

Detection of Citrus Tristeza Virus by RT-PCR and Status of CTV Infection among Citrus Trees in Cheju Island

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Citrus tristeza virus (CTV), an aphid-borne closterovirus, is one of the most destructive pathogens of citrus. It has caused rapid decline in growth, stem pitting and death in citrus trees. A reverse transcriptase polymerase chain reaction (RT-PCR) assay was developed for detection of CTV and investigation of the CTV infection status of citrus and its related cultivars in Cheju island. For RT-PCR based CTV detection, primers were designed to amplify 670bp of coat protein gene. A screening test for CTV in citrus cultivars was conducted from March to July in 1999. Seventy individual citrus trees representing 9 species of 3 genera were tested. The infection rates of CTV for leaves from ten years or older trees of late maturing citrus varieties such as Yuzu (*C. junos* Sieb. ex Tanaka), Navel orange (*C. sinensis* Osbeck), Kiyomi tanger (*C. unshiu* x *C. sinensis*), and Shiranuhi ((*C. unshiu* x *C. sinensis*) x *C. reticulata*) were 100%, 80%, 60%, and 60% respectively. The CTV infection rates in Early satsuma mandarins such as 'Miyagawa Early' Satsuma mandarins (*C. unshiu* Marc. var. Miyagawa) and 'Okitsu Early' Satsuma mandarins (*C. unshiu* Marc. var. Okitsu) were 100%, and 60%, respectively. CTV was not detected in Cheju native Danyooja (*C. grandis* Osbeck), Trifoliolate orange (*Poncirus trifoliata*) and Kumquat (*Fortunella margarita* Swingle). In conclusion, RT-PCR assay can be successfully applied to the detection of CTV in citrus trees.

Keywords : Citrus tristeza virus, CTV diagnosis, coat protein gene, RT-PCR.

Citrus tristeza virus (CTV), a member of the closterovirus group, is a positive sense, single-stranded RNA virus. Its host range is restricted to *Citrus* spp. and *Citrus* relatives in Rutaceae (Bar-Joseph et al., 1995; Coffin and Coutts, 1993; Dolja et al., 1994). CTV causes stem-pitting, shown as abnormalities in phloem and xylem development and

results in tree stunting, reduced sugar, and smaller fruit size, and yield loss in susceptible cultivars. It has been reported that it also causes rapid decline and death of trees grafted on sour orange (*C. aurantium* L.) rootstock, grapefruit (*Citroparadis* Macf.) and sweet orange (*C. sinensis* L., Osbeck) trees (Rocha-Pena et al., 1995; Yang et al., 1997)

CTV is transmitted by several species of aphids. Of them, *Toxoptera citricida* Kirk (brown citrus aphid) is the most efficient CTV vector (Gottwald et al., 1998; Rocha-Pena et al., 1995). The CTV and *T. citricida* resulted in the severe loss of citrus trees in South America and North America. Recently, *T. citricida* is spreading into all major citrus farms of Florida, California, Texas, and Arizona states in U.S.A. In case of Japan, Early Satsuma mandarins exhibit tolerance to CTV but late maturing mandarins such as Yuzu (*C. junos* Sieb. ex Tanaka), Kiyomi tangor (*C. unshiu* x *C. sinensis*), Iyo (*C. iyo* Hort ex Tanaka), Natsudaikai (*C. natsudaikai* Hayata) and Navel orange (*C. sinensis*) were more susceptible to CTV. Because CTV represents a serious problem to the citrus industry in the U.S.A. and other countries, development of resistant cultivars to a broad range of CTV strains is needed.

Most of citrus species are susceptible to CTV, so that severe symptoms can be induced when grafting on infected budwood. Since 1963, strategies aimed at minimizing the destructive effect of the tristeza disease include regulatory methods that are heavily dependent on appropriate diagnosis. Several million trees have been checked for CTV in California (Nikolaeva et al., 1995).

The most useful techniques for plant virus diagnosis are based on the use of serology with specific monoclonal antibodies. However, these techniques are not sensitive enough for detection of viral RNA targets in some woody plant tissue due to the low viral titers (Marinho et al., 1998; Mathews et al., 1997; Olmos, 1999; Rowhani et al., 1998). The enzyme linked immunosorbent assay (ELISA) testing is only reliable during certain months, when virus titer is high. Therefore, field collections are limited to these times. Recently, more sensitive techniques have been used to overcome the diagnosis problems. A more sensitive test for

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CTV has been developed: Sensitive molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) may be able to extend the time of testing throughout the year. RT-PCR assay can be designed to be more specific than serological methods in some cases. The RT-PCR has been used for specificity using specific primers and was able to detect woody plant viruses during dormant seasons (Singh et al., 1998). It was reported that RT-PCR was simple and required shorter time than other methods (Olmos, 1999). Recently many plant viruses were detected by RT-PCR (Barthe et al., 1998; James et al., 1999; Kinard et al., 1996).

Serological techniques for detection of CTV have been reported (Ahlawat, 1992; Nikolaeva, 1998; Rocha-pena, 1991). Recently, nucleotide sequences of the coat protein (CP) gene of CTV were reported and restriction analysis of the CTV coat protein gene amplified by the PCR based on CP gene sequences have been reported (Ahlawat et al., 1992; Gillings et al., 1993; Mathews, 1997; Nikolaeva et al., 1995; Nikolaeva et al., 1998; Sekiya et al., 1991). But in Korea CTV diagnosis and investigation of infection status by RT-PCR have not been reported. In this study, we developed an efficient and convenient CTV detection method using RT-PCR and investigated the infection status of citrus and related cultivars in Cheju island.

Materials and Methods

Plant materials. From March to July in 1999, young leaves of citrus trees from 4 to 20 year-old trees, were randomly collected from greenhouse in Cheju National University and citrus fields in Sogwipo-shi and Cheju-shi. These leaves were washed with diethyl pyrocarbonate (DEPC) treated water and stored at -70 °C (Table 1). Yuzu (*C. junos* Sieb. ex Tanaka), which shows typical CTV disease symptom such as stem-pitting, was used as a CTV positive control.

Isolation of total RNA from citrus leaves Total RNA was isolated by the methods of Chomzynski and Sacchi (1987). Citrus

leaf tissues (50 mg) were frozen in liquid nitrogen and ground to a powder in a prechilled mortar, and homogenized with 500 µl of Solution D [4 M guanidium isocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M β-Mercaptoethanol] and subsequently transferred to a Eppendorf tube. And then, 50 µl of 2 M sodium acetate (pH 4.0), 500 µl of water-saturated phenol, and 100 µl of chloroform were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 sec and cooled on ice for 15 min. Samples were centrifuged at 12,000 × g for 20 min at 4°C. After centrifugation, the aqueous phase was transferred to a fresh tube, mixed with the equal volume of isopropanol, and then placed at -20°C for 1hr to precipitate RNA. After centrifugation at 12,000 × g for 20 min the resulting RNA pellet was dissolved in 400 µl of Solution D, transferred into a Eppendorf tube, and precipitated with 1 vol of isopropanol at -20°C for 1 hr. After centrifugation in a microcentrifuge for 10 min at 4°C, the pellet was washed with 70% ethanol. The RNA pellet were vacuum dried and dissolved in 20 µl of DEPC treated water.

Detection of CTV by RT-PCR To develop CTV diagnosis by RT-PCR in citrus leaves infected with CTV, reverse transcription reaction (RT) was carried out. A pair of CTV specific primers, CTV-CP up primer (5'-CGGGATCCATGGACGACGAAAC-3') and CTV-CP down primer (5'-CGCGAGCTCTCAACGTGTGTTG-3'), was designed for the amplification of a 670bp based on CTV CP sequence analysis. One µg of total RNA was used as template for RT. First strand cDNA synthesis was performed using 1 µl of CTV CP specific down primer (20 pmol/µl), 2 µl of 10 × reverse transcription buffer, adding to deionized water to a final volume of 12 µl according to the instruction of the supplier of the M-MuLV-Reverse Transcriptase (MBI). The reaction mixture containing 2 µl of 5 mM dNTP, 2 µl of 0.1 M DTT, 2 µl of M-MuLV Reverse Transcriptase (MBI) was incubated at 37°C for 60 min, then heated to 70°C for 10 min and finally cooled to 4°C. After cDNA synthesis, PCR was performed using first stranded cDNA and CTV-CP specific primers. The cDNA was amplified by PCR in 50 µl reaction mixtures containing 50 mM Tris-HCl (pH 9.2), 2.25 mM MgCl₂, 2 µl of 1st-stranded cDNA, 50 pmol of dNTP, 50 pmol of each primer, 2.5 units of *Taq* DNA polymerase. The samples were subjected to 30 cycles of amplification with periods of 30 sec for melting at 94°C, 30 sec for primer annealing

Table 1. *Citrus* spp. and the related genera collected from fields in 1999

Citrus genotypes	Common name (local name)	Scientific name
<i>Citrus</i> LINN.	Yuzu	<i>Citrus junos</i> Sieb. ex Tanaka ^a
	Dangyooja	<i>Citrus grandis</i> Osbeck
	'Miyagawa Early' Satsuma mandarin	<i>Citrus unshiu</i> Marc. var. Miyagawa
	'Okitsu Early' Satsuma mandarin	<i>Citrus unshiu</i> Marc. var. Okitsu
	Kiyomi tanger	<i>Citrus unshiu</i> × <i>Citrus sinensis</i>
	Shranuhi	(<i>Citrus unshiu</i> × <i>Citrus sinensis</i>) × <i>Citrus reticulata</i>
	Navel orange	<i>Citrus sinensis</i> Osbeck
<i>Poncirus</i> RAF.	Trifoliolate orange	<i>Poncirus trifoliata</i> ^b
<i>Fortunella</i> SWINGLE	Kumquat	<i>Fortunella margarita</i> SWINGLE

^aPositive control with stem pitting symptoms

^bGenerally used in rootstock

at 55°C and 1 min for primer extension at 72°C. In the last cycle, primer extension was extended to 5 min and kept at 4°C prior to gel analysis. After RT-PCR, PCR reaction products (5 µl) were analysed by electrophoresis through a 1.2% agarose gel stained with ethidium bromide. The DNA bands on the gels were visualized on a UV transilluminator and photographed. Molecular size markers, 100 bp ladder (Takara), were loaded in each gel to estimate the size of the PCR products.

Results and Discussion

Detection of CTV by RT-PCR. The primer pair of CTV-CP up and CTV-CP down was used in RT-PCR to specifically detect CTV in citrus cultivars. Products of the expected size of 670bp of CTV-CP gene were observed in agarose gel in citrus cultivars (Fig. 1). And the nucleotide sequence of this product was determined (Choi, 1999). Citrus cultivars infected with CTV tested (Table 2) were successfully detected by RT-PCR using total RNA extracts. When used for detecting other citrus related genotypes, however, CTV were not detected in the leaves of Trifoliolate orange and Kumquat (Table 2), thus confirming specificity of this primer. The study of CTV has been hindered by the inherently low virus titers during dormant season. But, in this study CTV was detected by RT-PCR during virus dormant season. Mathews et al. (1997) reported that in the months of August and September sweet orange trees infected with CTV had a significant low level of virus titer. Therefore CTV was not reliably detected by ELISA. By contrast, RT-PCR methods gave definitive positive results for CTV detection in the samples collected during dormant seasons. In this study, we confirmed that RT-PCR is sensitive and effective for detecting CTV. So this RT-PCR assay will result in a significant saving of time, labor, and cost.

Status of CTV infection in Cheju Island. We estimated

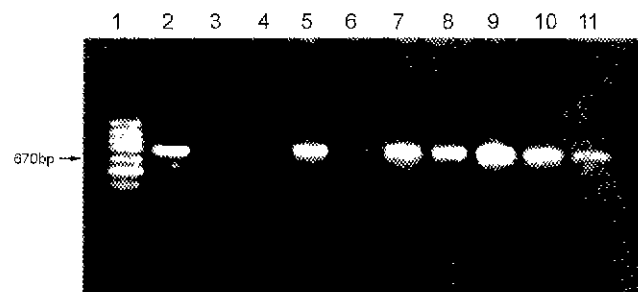


Fig. 1. RT-PCR analysis of CTV in citrus leaves Lane 1, 100 bp DNA ladder; Lane 2, *C. junos* Sieb. ex Tanaka with stem-pitting symptoms as a positive control (amplified fragment size: 670 bp); Lane 3, *P. trifoliata*; Lane 4, *Fortunella margarita* Swingle; Lane 5, *C. junos* Sieb. ex Tanaka; Lane 6, *C. grandis* Osbeck; Lane 7, *C. unshiu* Marc. var. Miyagawa; Lane 8, *C. unshiu* Marc. Var. Okitsu; Lane 9, *C. unshiu* x *C. sinensis*; Lane 10, *C. sinensis* Osbeck; Lane 11, (*C. unshiu* x *C. sinensis*)x *C. reticulata*.

Table 2. The present status of CTV infection in citrus trees in Cheju island as assayed by RT-PCR

Citrus species or related species	Age of tree ^d (year)	No of trees tested		Infectivity (%)
		tested	infected	
<i>C. junos</i> Sieb. ex Tanaka ^e	L 20(F)	5	5	100
<i>C. grandis</i> Osbeck ^e	L 20(F)	5	0	0
<i>C. unshiu</i> Marc. var. Miyagawa	E 4(G)	5	1	20
	20(F)	5	5	100
<i>C. unshiu</i> Marc. var. Okitsu	E 4(G)	5	3	60
	20(F)	5	3	60
<i>C. unshiu</i> x <i>C. sinensis</i>	E 4(G)	5	1	20
	10(G)	5	3	60
<i>C. sinensis</i> Osbeck	L 4(G)	5	2	40
	15(G)	5	4	80
(<i>C. unshiu</i> x <i>C. sinensis</i>) x <i>C. reticulata</i>	L 4(G)	5	2	40
	10(G)	5	3	60
<i>C. junos</i> Sieb. ex Tanaka ^a	20(F)	2	2	100
<i>Poncirus trifoliata</i> L ^{INN} ^b	1(F)	5	0	0
<i>Fortunella margarita</i> SWINGLE ^b	20(F)	5	0	0

^a Yuzu with stem-pitting symptoms as positive control.

^b Trifoliolate orange and Kumquat as negative control.

^c Cheju native species

^d F: Citrus trees in field, G: Citrus trees in green house, E: Early maturing, L: Late maturing

the infection rate of CTV by detection of virus specific PCR products. The incidence of virus infection ranged from 20% to 100% among citrus genotypes tested (Table 2). The incidence of CTV in Yuzu (*C. junos*) was 100% (Table 2), which ranked the first among the tested. This shows that Yuzu is the most susceptible to CTV. However CTV was not detected in the leaves of Cheju native citrus trees, Dangyooza (*C. grandis*). ‘Miyagawa Early’ Satsuma mandarins (*C. unshiu* Marc. var. Miyagawa) and ‘Okitsu Early’ Satsuma mandarins (*C. unshiu* Marc. var. Okitsu) occupy the major citrus cultivars in Cheju and the leaves of these two cultivars were positive in CTV detection. RT-PCR analysis represented 100% and 60% infectivity respectively in the leaves of 20-year old trees collected from field. Early satsuma mandarins as well as many other citrus cultivars has been reported to be tolerant to CTV and exhibited no obvious symptoms (Rocha-pena et al., 1995). In general, young citrus trees (4 years old) showed lower infection rates than old citrus trees (20 years old). Late maturing citrus varieties, Yuzu (*C. junos*), Kiyomi tanger (*C. unshiu* x *C. sinensis*), Shiranuhi ((*C. unshiu* x *C. sinensis*) x *C. reticulata*), Navel orange (*C. sinensis*) showed 100%, 60%, 60%, and 80% infection rates, respectively. These results show that the incidence of CTV in the late maturing citrus varieties is high and the late maturing citrus cultivars are more suscep-

tible to CTV. The infection in citrus varieties is thought to be caused by citrus pruning and insects feeding such as aphid.

CTV incidence of mandarin from Kamatakait area showed 50%, and therefore resistant rootstock to CTV are required (Ahlawat et al., 1992). Fang et al. (1998) reported that *P. trifoliata* has been an important citrus rootstock because of its tolerance to *Phytophthora* and resistance to CTV and nematodes. Many citrus species are known to be quite tolerant, either when grown on their own roots or when grafted on a tolerant rootstock. As mentioned earlier, sweet oranges, mandarins and grapefruits showed decline of growth when grafted onto the sour orange rootstock. Not all CTV isolates induced decline of growth. Many citrus trees infected with CTV for years showed no severe symptoms such as stem-pitting or growth decline in Florida and Israel. Therefore, it is important that citrus trees containing rootstock for grafting should be diagnosed for virus detection and prevented from the transmissible vector.

Early satsuma mandarins were known to be more tolerant to CTV than other citrus cultivars (Rocha-Pena et al., 1995). Recently the CTV infectivity of Shiranuhi, Navel orange, and Kiyomi tanger have been increased annually in Cheju and similar results also were reported in Japan, US, Israel, Caribbeau, Central and North America, Costa Rica and Dominican Republic and so on (Gottwald et al., 1998). These kinds of citrus cultivars may show severe virus disease symptoms. Therefore strategies such as eradication or cross protection require the procedures for detecting virus infection. The development of resistant citrus cultivars to CTV through genetic engineering is also desired. Therefore incorporation of CTV-resistant gene, for instance, from *P. trifoliata* into another citrus variety by genetic transformation may enable for us to develop a CTV-resistant cultivars (Fang et al., 1998). In conclusion, field evaluation of alternative rootstock is essential in all areas threatened by CTV, in addition to the development of immune scion varieties through genetic engineering or conventional breeding.

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