Characterization of Citrus Tristeza Virus Isolates Infecting Pummelo and Sweet Orange in Sichuan Province, China

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ABSTRACT. In recent years, citrus tristeza virus has become more severe in pummelo cultivars, Fenghuang-you and Taibei-you, and in sweet orange cultivar, Jinchen-Beibei 447, in Sichuan Province of China. Nine CTV isolates were compared using indicator plants, ELISA, and double stranded RNA (dsRNA) analysis. A wide variety of symptoms were induced on Mexican lime (ML), Duncan Grapefruit (GF), Eureka lemon (EL) and sour orange (SO), ranging from mild vein clearing in ML with no symptoms in other indicators to severe stem pitting or seedling yellows response in GF and SO. All the isolates reacted in ELISA with polyclonal antibody, and all but one reacted also with the MCA13 monoclonal antibody. Several dsRNA patterns were observed among the nine isolates, but no relation was observed between these patterns and pathogenic characteristics.

Citrus tristeza virus (CTV) is present worldwide and its strains vary from mild to highly destructive (11). In China, although CTV is widely distributed, decline is not apparent since the rootstocks commonly used are tolerant or immune (2, 7). Recently, CTV has become a problem in Sichuan province with the introduction of pummelo and some sweet orange varieties. Pummelo cultivars, Fenghuang-you in Dachuan Prefecture and Taibei-you in Shantai County, show symptoms that include dwarfing with short internodes, leaf curl, severe stem pitting, small fruits and poor yield. Symptoms found in a superior line of sweet orange, Jinchen-Beibei 447, two years after being top-worked to a local sweet orange on trifoliate orange rootstock included dwarfing, yellowing, severe stem pitting, and brittle twigs near limb junctures. Many died of this problem in Beibei, Chongqing Municipality. CTV isolates collected from these diseased trees and six other isolates were compared using indicator plants, ELISA, and double stranded RNA (dsRNA) analysis and results are reported here.

MATERIALS AND METHODS

Virus isolates. The following nine CTV isolates were used: CT1, obtained from a Fenghuang-you pummelo with severe symptoms of dwarfing, bunchy twigs, leaf curl and stem-pitting; CT2 from Fenghuang-you pummelo (Dachuan prefecture) with yellowing and bunchy twigs; CT3 from Taibei-you pummelo (Shantai county) with severe symptoms of dwarfing, bunchy twigs, leaf curl, stem-pitting on twigs and roots, and fruit deformation; CT4 from Jinchen-Beibei 447 sweet orange top-worked onto sweet orange on trifoliate orange rootstock, showing severe dwarfing, stem-pitting and brittle twigs; CT5 from Okitsu satsuma; CT6 from Owari satsuma (Beibei, Chongqing Municipality); CT7 from a recovered grapefruit which first showed the seedling yellows response after graft-inoculation with CTV from a shoot-tip-grafted budline of Anliu-chen sweet orange; CT8, an isolate causing stem-pitting in Eureka lemon; and TR-L514, an isolate described previously (13). All isolates were kept in Xinhui-chen (sweet orange) seedlings in the screenhouse.

Biological indexing. Indexing was done with four indicators: Mexican lime (ML) grafted on Goutouchen, and seedlings of Duncan grapefruit (GF), Eureka lemon (EL) and sour orange (SO). Indicator plants were grown and maintained

in a screenhouse. Four plants of each indicator were graft-inoculated with two pieces of infected bark in September 1993. Symptom expression was observed over a 2-yr-period in spring and autumn.

ELISA test. All isolates were graft-inoculated in seedlings of Anliu-chen sweet orange in September 1993 and kept in the screenhouse. In July 1995, bark extracts (1:10 w/v) from three twigs/plant were prepared in 0.02 M PBS-Tween, pH 7.4, containing 0.05% TGA and 2% PVP buffer, and assayed by double antibody sandwich ELISA (DAS-ELISA) using polyclonal antibody (Sanofi Sante Animale, Libourne, France) and monoclonal antibody MCA13 (3, 10, 13).

DsRNA analysis. Young bark from shoots of CTV-infected Anliu-chen sweet orange seedlings were collected in spring and frozen at -20°C, ground to a powder consistency in liquid nitrogen, and dsRNAs were purified by CF-11 cellulose column chromatography and separated by polyacrylamide gel electrophoresis according to the method described by Dodds et al.(4). *Lambda* DNA digested with Hind III (Promega, U.S.A.) was used as size markers. The dsRNA bands were observed by silver staining (1).

RESULTS

Biological indexing. Results for indexing of nine isolates are summarized in Table 1. CT1 and CT8 caused no seedling yellows (SY) response on GF, EL and SO, but moderate to severe stem-pitting (SP) on ML and GF, and CT-8 even caused moderate SP on EL. Both were classified as severe SP isolates. CT2 and CT5 only caused mild vein clearing (VC) on ML, and no symptom on GF, EL and SO. Both were classified as mild tristeza isolates. CT3 and CT7 caused no SY on GF, EL and SO and only mild Sp on GF. CT3 also induced moderate SP on ML. These two isolates were classified as mild to moderate SP isolates. CT4 caused severe SP on ML and GF, and moderate SY on GF and SO. It was classified as a complex of severe SP and moderate SY. CT6 induced severe Sp on ML, mild Sp on GF, and severe SY on GF, EL and SO. It was classified as a complex of moderate SP and severe SY. TR-L514 caused moderate SP on ML and GF, and mild SY on GF and EL. It was classified as a complex of moderate SP and mild SY.

ELISA. All nine isolates reacted positively by ELISA using polyclonal antibody. The OD_{405} values of the infected plant extracts varied from 0.71 to 1.92, except for CT3 that yielded an OD value of 0.18 (Table 1). The OD value of the uninoculated control was 0.05. All the isolates but CT3, reacted with MCA13, the OD values ranging between 0.15 and 0.96 (Table 1). The OD values yielded by CT3 and the uninoculated control were 0.03.

DsRNA analysis. The dsRNA patterns of the nine isolates are drawn in Fig. 1. All the isolates had three readily detected dsRNA bands: the full length replicative form of the genome (M_r 13.3 $\times$ 10^6); and two lower intensity bands at 2.0 $\times$ 10^6 and 0.8 $\times$ 10^6. These results are similar to those reported by Dodds et al. (5). All isolates except CT3 (Fig. 1, Lane 5) had a 1.7 $\times$ 10^6 band. Only CT2 (Fig. 1, Lane 9) and CT6 (Fig. 1, Lane 10) had a small 0.5 $\times$ 10^6 band.

DISCUSSION

Many differences between the nine field CTV isolates were observed by biological indexing. Variation ranged from mild tristeza with no SP or SY on any of the indicators tested, to SP or SY isolates, sometimes combined with SY or SP components, respectively.

As shown in Table 1, Fenghuang-you pummelo with field symptoms of

TABLE 1
INDEXING RESULTS OF NINE CTV ISOLATES MAINLY COLLECTED FROM SICHUAN PROVINCE

Results of indexing		CTV isolate[z]								
Host	Symptom[z]	CT1	CT2	CT3	CT4	CT5	CT6	CT7	CT8	TR-L-514
Mexican lime on Goutou	ST	++	-	+	+++	-	+++	-	++	+
	VC	+++	+	++	+++	+	+++	+	+++	++
	SP	++	-	++	+++	-	+++	-	++	++
	LC	++	-	-	++	+	++	+	+	+
Grapefruit	ST	++	-	+	+++	-	+++	+	++	+
	SY	-	-	-	++	-	+++	-	-	+
	SP	+++	-	-,+	+++	-	-,+	+	+++	++
Eureka lemon	ST		-	-		-	+++	-	+	+
	SY		-	-		-	+++	-	-	+
	SP		-	-		-	-	-	++	-
Sour orange	ST	-	-	-	++	-	+++	-	-	-
	SY	-	-	-	++	-	+++	-	-	-
ELISA[y]	PAb	1.92	1.86	0.18	1.90	1.83	1.29	1.92	0.71	1.89
	MCA13	0.44	0.55	0.03	0.50	0.31	0.26	0.96	0.15	0.35
$1.7x10^6$ M_r dsRNA band[x]		+	+	-	+	+	+	+	+	+
Estimated strain of CTV[w]		SP-S	M	SP-M	SY- M+ SP-S	M	SY-S SP-M+	SP-M	SP-S	SY-M SP-M

[z]ST = stunting; VC = vein clearing; SP = stem-pitting; LC = leaf-curl; SY = seedling yellows; +++ = severe; ++ = moderate; + = mild; - = no symptom.
[y]Mean OD_{405} values obtained by DAS-ELISA using two replicates with polyclonal antibody (PAb) or four replicates with monoclonal antibody MCA13. Zero was adjusted with buffer. OD values with healthy plant extracts were 0.05 and 0.03 with PAb and MCA13, respectively.
[x]The dsRNA band of $1.7x10^6$ (see Fig. 1) was observed (+) or undetected (-).
[w]Isolate classified: SP-S = severe stem-pitting; SP-M+ = moderate stem pitting; SP-M = mild stem pitting; SY-S = severe seedling yellows (SY); SY-M+ = moderate seedling yellows; SY-M = mild seedling yellows; M = mild tristeza.

dwarfing, short internodes, leaf curl, stem-pitting and small fruit (CT1) was infected with a severe Sp isolate while the trees of Taibei-you pummelo with similar field symptoms (CT3) was infected with a moderate SP isolate, and the trees of Jinchen-Beibei 447 sweet orange showing symptoms of dwarfing, yellowing, severe stem-pitting and brittle twigs (CT4) contained a complex of severe SP and moderate SY.

The sweet orange trees on trifoliate orange with severe CTV symptoms were located in the cool mountain area of Chongqing. Pummelo trees severely damaged by severe or moderate SP isolates were found in other areas in Sichuan province. This leads us to predict that the tristeza problem will become more severe as new plantings of pummelo and certain sweet orange varieties occur in Sichuan province as well as in other parts of China. Screening of effective mild CTV strains for cross protection should be immediately undertaken.

Differences in dsRNA pattern were also detected among CTV isolates, but these differences could not be associated with biological characteristics. For example, CT1, CT4 and CT8 showed dramatic differences in symptom expression, but no difference in their dsRNA profile. Only CT2 and CT6 had the 0.5×10^6 band which was suggested to be associ-

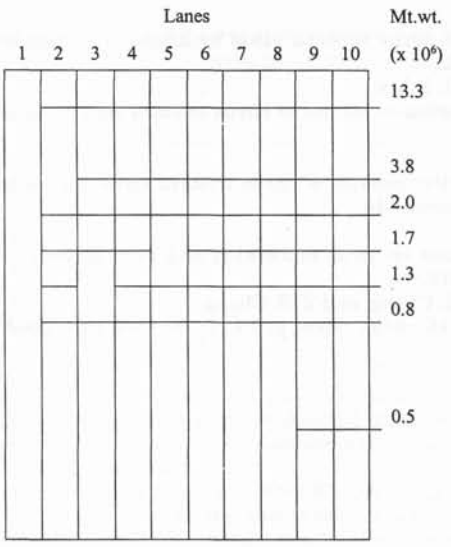

Fig. 1. Comparison of dsRNA profiles of nine isolates of CTV. Lane 1 is negative control; Lane 2 is CT5; Lane 3 is CT7; Lane 4 is TR-L514; Lane 5 is CT3; Lane 6 is CT8; Lane 7 is CT7; Lane 8 is CT1; Lane 9 is CT2; Lane 10 is CT6.

ated with isolates causing SY or severe SP in grapefruit seedlings (5, 6). In this study, CT6 was a severe SY isolate while CT2 was a mild isolate which only induced the vein-clearing on ML/GT. This result confirms previous reports that pathogenicity and dsRNA profile are not necessarily related (9). Recently, Mawassi et al. (8) showed the presence of defective RNAs in some CTV isolates. Some of the subgenomic dsRNAs observed in this work might be replicative forms of defective RNAs and these might affect symptom expression (12).

It was interesting to note that CT-3, the only isolate that lacks the 1.7×10^6 band had a very weak reaction with polyclonal antibody and no reaction with MCA13. This might indicate poor replication of this isolate in Anliu-chen sweet orange.

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