Serological Diversity of Field Sources of Citrus Tristeza Virus (CTV) in Japan

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ABSTRACT. Reaction of field sources (FS) of citrus tristeza virus (CTV) collected in Japan was tested with the Spanish CTV monoclonal antibody (MAb) 3DF1, the Florida MAb MCA13, and polyclonal antisera to CTV produced in Florida and Japan. Four FSs (three mild and one intermediate) reacted strongly to 3DF1 and not to MCA13. One mild FS, M16, did not react to either 3DF1 or MCA13. Other FSs, including mild sources, reacted strongly to both MAbs. All Japanese CTV isolates reacted to polyclonal antisera from Florida and Japan. The results show a greater serological diversity among Japanese CTV sources than that found previously in the USA and Spain. The 3DF1 MAb was previously believed reactive to all CTV isolates, but since it does not react to all Japanese sources, it should not be considered a universal probe for CTV. The MCA13 MAb, reported nonreactive to mild CTV isolates from Florida and Spain, reacted to several CTV sources in Japan which are considered mild.
Index words. ELISA, monoclonal antibodies, epitopes.

The diseases caused by citrus tristeza virus (CTV) are important threats to citriculture. Various indexing procedures have been developed by citrus virologists worldwide. These include use of indicator plants (18), serology (1, 3, 7, 9, 12, 15, 17), light and electron microscopy (4), and nucleic acid analysis (13). Serological techniques used for CTV detection include SDS-immunodiffusion (3), fluorescence antibody technique (15), immunoelectron microscopy (4), radioimmunoassay (9), and enzyme-linked immunosorbent assay (ELISA) (1, 7, 12). ELISA has been the most useful, because the procedure is rapid and can be applied to large-scale indexing. Although CTV isolates vary greatly in biological effects, they have been considered to be serologically uniform, based on reaction to polyclonal antibodies (PAb) and to a monoclonal antibody (MAb) produced to a Spanish isolate (17). Results with the latter indicated presence of several epitopes in the CTV coat protein (16). Recently, Permar *et al.* (12) produced MAbs against the Florida CTV isolate T-36 which causes decline in sweet orange grafted on sour orange and seedling yellows (SY). One MAb selected, MCA13, reacted well to decline and SY isolates not to mild isolates which caused symptoms only in Mexican lime (7, 11, 12). In this paper, we describe the reaction of different Japanese CTV field sources (FS) to MCA13 and to the Spanish MAb 3DF1.

MATERIALS AND METHODS

Virus sources. CTV sources were collected mainly in the field at the Okitsu Branch, Fruit Tree Research Station (FTRS), and the Kuchinotsu Branch, FTRS. A mild CTV, HM55, was kindly provided by Dr. A. Sasaki, Hiroshima Prefectural Fruit Tree Experiment Station. After collection, these CTV sources were kept in vector-free screenhouses. Biological characters of these sources have been described by Koizumi (8), Ieki and Yamaguchi (5, 6) and Sasaki (14). Seedlings of Mexican lime, sour orange, or Eureka lemon, and sweet orange grafted on sour orange rootstock (SW/SO) trees were used for the indexing of CTV. Inoculated indicator plants and the virus sources for ELISA were kept in a greenhouse maintained at 15 to 27 C minimum/maximum. Tissues collected for ELISA (bark, petiole or

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midrib) were stored at -70 C until tested.

Antibodies. Japanese PAb produced against a severe CTV (stem pitting) strain was used in double antibody sandwich (DAS) ELISA. Florida PAb (1052) produced against the Florida CTV isolate T-36 was used as the coating antibody in double antibody sandwich indirect (DAS-I) ELISA. The MAbs MCA13 and 3DF1 were used as intermediate antibodies in DAS-I ELISA.

ELISA. DAS-ELISA was conducted essentially as described by Clark and Adams (2).

Monoclonal antibodies were tested using DAS-I-ELISA. Vinyl chloride plates (Sumitomo Bakelite, Japan) were coated with PAb (1052, 1 μg/ml) for 4 hr at 30 C and washed. Plant extracts were prepared by macerating tissue in Tris buffer (0.05 M, pH 7.8) with a mortar and pestle. The ratio of tissue to buffer was 1/10 (w/v). Extracts were added to plates and incubated at 4 C overnight. After antigen incubation and washing MAbs were added. MCA13 was used at 1:15,000 dilution of ascites fluid in PBS. 3DF1 was used at a 0.125 μg/ml in PBS. Incubation of the MAbs was for 90 min at 37 C. Next, a goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma, USA) was added at a 1:1500 dilution in PBS and incubated for 90 min at 37 C. After washing, p-nitrophenyl phosphate substrate (1 mg/ml) in 10% diethanolamine (pH 9.8) was added and incubated at room temperature (ca. 25 C). The absorbance at 415 nm was measured at 10-minute intervals on a Corona ELISA reader MTA-32 (Corona Electric, Japan), until the absorbance of the positive control reached 1.000 (A_{415}).

Evaluation of data. As a positive control, extracts of tissue infected with T-36, or with Japanese stem-pitting isolates 1595 or G28 were added to each plate. Those standard sources were lyophilized extracts or tissue aliquots frozen at -70 C. The reaction rate was calculated by reading the plates at two different times and measuring the change in OD_{415} per minute. Three general ratings were made. If the reaction rate of a test sample was less than 1/10 the reaction rate of the positive control in the plate, the sample was considered negative (–). If the reaction rate was 1/10 to 1/5 the rate for the positive control, the sample was considered questionable (?). If the reaction rate was more than 1/5 the rate of the positive control, the test sample was considered positive (+).

DAS-ELISA was used to check the total antigen content. Samples weakly reactive to PAb were not evaluated for MAb reaction unless a definite reaction to the MAb was noted.

RESULTS AND DISCUSSION

Pathogenicity of FS tested is described in Table 1. Some information is from previous reports (6, 7, 8). According to the reaction of indicator plants, symptomatology was classified into SY (seedling yellows), T (tristeza decline), SP (stem pitting), INT (intermediate), M (mild), and ND (not yet determined). SY, T, and SP were considered severe isolates. INT isolates showed intermediate virulence between SP and M and did not cause seedling yellows. These criteria are similar to those described by McClean (10). Mild sources have been selected as causing mild symptoms (vein clearing) in Mexican lime, and no SY in sour orange or Eureka lemon. Most of them have not been indexed on SW/SO for the T reaction.

The total ELISA data for all FS is shown in Table 1. ELISA tests for each source were repeated at least three times. Three patterns of reaction of the CTV sources to the different antibodies were observed. Although the identity of FS 0001 is still pending, Group 1 reacts to 3DF1, MCA13, and PAb and includes 8 SY, 3 SP, one T, 2 INT, 8 M and 2ND sources. Group II reacts only to 3DF1 and PAb and includes one INT and three M sources. Group III reacts only to PAb and includes one M source.

The FS 0001 showed an unstable reaction (Table 2). FS 0001 was col-

TABLE 1
REACTION OF JAPANESE CITRUS TRISTEZA VIRUS (CTV) FIELD SOURCES TO THE CTV MONOCLONAL ANTIBODIES MCA13 AND 3DF1 AND TO CTV POLYCLONAL ANTIBODIES (PAb)

CTV sources	Code of tree	Variety[z]	Cumulative evaluation[y]			Symptomatology
			MCA13	3DF1	PAb	
0001	0701	MT-H	+[w]	?	+	ND
0137	0702	MT-H	–	+	+	INT
1215	0703	RL	+	+	+	SY
1417	0707	RL	+	+	+	T
145R	0703	MT-H	+	+	+	M
1513	0709	RL	+	+	+	SY
1522	0713	RL	+	+	+	SY
1595	0714	RL	+	+	+	SP
1672	0721	RL	+	+	+	SY
1722	0723	RL	+	+	+	SY
1990	0724	RL	+	+	+	SP
2192	0729	RL	+	+	+	SP
BOUQ	0935	RL	+	+	+	?[v]
HM55	0785	ML	+	+	+	M
KS3	0770	RL	+	+	+	SY[v]
M2	0778	ML	+	+	+	M[v]
M4	0780	ML	–	+	+	M[v]
M12	0787	ML	–	+	+	M[v]
M16	0791	ML	–	–	+	M[v]
M19	0794	ML	+	+	+	M[v]
M20	0795	ML	–	+	+	M[v]
M22	0796	ML	+	+	+	M[v]
M23	0797	ML	+	+	+	M[v]
M26	0801	ML	+	+	+	M[v]
M27	0802	ML	+	+	+	M[v]
PM8	0783	ML	+	+	+	INT[v]
PM25	0800	ML	+	+	+	INT[v]
S5	0807	RL	+	+	+	SY[v]
SIY	0810	MI	+	+	?	SY[v]

[z]MT-H = Matoh buntan X Hassaku, RL = rough lemon, ML = Mexican lime, MI = Miyauchi iyokan.
[y]At least 3 different samples were tested to make a total evaluation.
[x]SY = seedling yellows, SP = stem pitting, T = tristeza decline INT = intermediate, M = mild, ND = not yet determined.
[w]+, ?, –: see text for rating system.
[v]Not yet indexed in sweet orange grafted on sour orange rootstock.

lected from a pummelo hybrid in the field of Kuchinotsu Branch, FTRS, by Koizumi (8). This FS has shown mild vein clearing on Mexican lime and mild yellowing on Eureka lemon seedlings, but pathogenicity is being further tested. Among 14 samples of FS 0001, the reaction to MCA13 was constantly strong, but the reaction to 3DF1 was sometimes weak or negative. This suggests the FS 0001 might have two components, one reactive to both MCA13 and 3DF1, and the other reactive only to MCA13. The relative proportion of those components might vary in different parts of the tree or with environmental conditions. If the latter component is isolated, it will expand the range of serological diversity in CTV. Until now, only the tissues of the original variety and graft-inoculated Mexican lime have been used for ELISA. Tests of other varieties infected with 0001 and of aphid-transmitted subcultures have been untaken to obtain more information on the serological character of this source.

FS SIY showed strong reaction to MAbs, though its reaction to PAb was low in two of three tests (Table 1). In other tests, Madam Vinous sweet orage graft-inoculated with SIY showed strong reaction to both PAb and MAbs (data not shown). Some

TABLE 2
REACTION OF THE JAPANESE FIELD SOURCE (FS) 0001 OF CITRUS TRISTEZA VIRUS (CTV) TO CTV MONOCLONAL AND POLYCLONAL ANTIBODIES IN ELISA

Sample No.[z]	Antibody[y]		
	MCA13	3DF1	PAb
1	+	–	+
2	+	–	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	?	+
10	+	?	+
11	+	?	+
12	+	?	+
13	+	?	+
14	+	?	+

[z]No. 1-11 were collected from the pummelo hybrid plant (Tree code 0701) which was the original source of 0001, and No. 12-14 were collected from a Mexican lime (0794) graft inoculated with 0001.
[y]+, ?, –: see text for rating system.

other sources whose virus content was estimated to be low by DAS-ELISA often reacted weakly to MAbs. Therefore, checking the virus content with DAS-ELISA using PAb is very important for accurately analyzing the data of the MAb reaction. Only samples showing good reaction to PAb should be used for further evaluation.

The results in this paper showed a greater serological diversity among Japanese CTV sources than that found in the USA and Spain. The 3DF1 monclonal was previously believed to be reactive to all CTV isolates, but since it does not react to certain Japanese sources, it cannot be considered a universal probe for CTV.

The MCA13 monoclonal which has been reported nonreactive to mild CTV isolates in the USA and Spain reacted to several CTV sources in Japan which are considered to be mild CTV. It will be necessary to further evaluate the reaction of these mild sources in SW/SO trees. Further comparative evaluation of mild isolates from different countries may be needed.

Isolates, like M16, which are nonreactive to both 3DF1 and MCA13, have never been found in other areas where CTV is endemic.

Monclonal antibodies may be effective for research work or routine indexing for certain types of isolates. For example, as M16 can be distinguished from other CTV sources by DAS-I assays using 3DF1 and MCA13, challenge inoculation to M16-infected plants can be tested by MAb to clarify the mechanism of cross-protection in citrus trees.

In Florida, Irey *et al.* (7) showed that application of ELISA with MCA13 was successful to verify infection by CTV decline isolates in young field plantings. In Japan, trifoliate orange is the predominant rootstock, and the stem-pitting disease of scion varieties is the major CTV problem. Therefore, protective strains which cause few stem-pitting symptoms on scion varieties and protect against more virulent strains could be used for cross-protection in the fields, even if the protective strains cause decline symptoms in SW/SO trees. No MAb can currently discriminate such protective strains in Japan from more virulent ones.

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