カンキツトリステザウイルスアブラムシ伝染株の生物学的性質

誌名	果樹試験場報告 = Bulletin of the Fruit Tree Research Station
ISSN	09165851
著者名	加納,健 小泉,銘冊 家城,洋之
発行元	農林水産省果樹試験場
巻/号	23号
巻号補足	
掲載ページ	p. 145-154
発行年月	1992年9月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

AgriKnowledge

果樹試報 Bull. Fruit Tree Res. Stn. 23 : 145～154, 1992

Biological Properties of Aphid-Transmitted Subcultures of Citrus Tristeza Virus in Japan.[†1]

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Synopsis

Aphid-transmitted subcultures (ATSs) of Japanese citrus tristeza virus (CTV) were obtained, and biological properties of the ATSs were tested. The biological properties of most of the ATSs were similar to those of their parent field sources (FSs). However, two ATSs obtained from a FS of a seedling yellows strain showed a different pathogenicity in sour orange seedlings. The results obtained here and in previous studies on serotype separation by aphid vectors indicate that selective transmission of CTV by aphids occurs in the field.

Japanese mild sources reactive to two monoclonal antibodies (MAbs) (MCA13, 3DF1) and a polyclonal antibody (PAb) induced mild or no symptoms in both Yuzu seedlings and grafted combination of sweet orange on sour orange rootstocks, confirming the existence of several mild sources reactive to MCA13.

Yuzu seedlings were successfully used for the indexing of biologically distinct CTV isolates. As it is much easier to collect seeds of Yuzu than those of Mexican lime, it is considered that Yuzu could be a useful substitute for Mexican lime in evaluating the severity of CTV isolates in the induction of stem pitting disease in Japan.

Key Words : citrus tristeza virus, aphid-transmitted subculture, biological indexing, serotype, *Citrus junos* Sieb. ex Tanaka

†1 Received for publication June 8, 1992. Contribution No. B-187 of the Fruit Tree Research Station

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Introduction

The disease caused by citrus tristeza virus (CTV) is one of the major threats to citriculture. Several severe and destructive strains of CTV have been reported worldwide (Roistacher and Moreno 1991). Mild or protective strains of CTV have also been reported. The protective ability of such strains has been used for the control of diseases caused by severe strains. Identification of CTV strains, based on their pathogenicity, is very important for developing control strategies in different citrus-growing areas.

Several studies on the pathogenicity of CTV strains have been conducted in Japan (Sasaki 1974, Miyakawa 1977, Yamada *et al.* 1981, Koizumi and Kuhara 1985, Ieki and Yamaguchi 1986, Miyakawa 1987, Koizumi 1991). Different strains were recognized in the field trees, including severe (stem pitting, seedling yellows, tristeza, natsudaidai) and mild strains. Most of these studies, however, were carried out with field sources (FSs) of CTV.

In areas where CTV is endemic, propagation of infected trees is common. However, since CTV is an aphid-borne virus, natural transmission to citrus plants in the field also occurs. Severe strains with a high aphid-transmissibility contribute to the CTV epidemics in the field. A FS of CTV may exist as a complex or mixture of different strains (McClean 1974), which may vary in their aphid-transmissibility. Aphid transmission is the principal method to separate or isolate the individual components of a complex at present, because there are no local lesion hosts. Characterization of aphid-transmitted subcultures (ATSs) of CTV obtained from FS is important to analyze complexes or mixtures of FSs and to study the epidemiology of CTV in the field.

In this paper, we compared the reaction of different CTV ATSs in some indicator plants and discussed the relationship between ATSs and their parent FSs. We also compared the serological and biological characteristics of Japanese CTV isolates, and indicated that Yuzu seedlings could be used as an indicator plant for CTV.

Materials and Methods

CTV sources

HM55 and HS34 were kindly provided by Dr. A. Sasaki (Sasaki 1974). Other sources were isolated in our laboratory, as follows : 0001, 0137, 145R, 1513, BOUQ, M2, M26, M27 (Ieki and Yamaguchi 1986, Kano *et al.* 1991, Koizumi 1991), and 1215A, 1417A, 1513A, 1595A, BOUQA, KS3A, KSA, M12A, M15A, M16A, M22A, M23A, M27A, PM8A, PM25A, S5A2, SIYA (Ieki and Yamaguchi 1988, Koizumi *et al.* 1989, Koizumi *et al.* 1991, Kano and Koizumi 1991). The name of a virus source was altered, *e. g.* S5A to S5A2, to indicate subculturing by aphid transmission. For example, KS3A and KS3A2 were different ATSs obtained from the same FS, KS3. The donor source of PM8A was first named M8 (Ieki and Yamaguchi 1988) and renamed PM8 (Kano *et al.* 1991).

Transmission by the aphid, Toxoptera citricidus Kirk.

Several additional ATSs (0001A, 0137A, 1672A, KS3A2, M2A, M16A2, M26A, M27A5) were obtained in this study by aphid transmission methods described previously (Kano and Koizumi 1991).

Transmission by the aphid, Aphis gossypii Glover

ATS, NUagA was transmitted by *Aphis gossypii* from the donor satsuma mandarin (*Citrus unshiu* Marc.) infected with a seedling yellows strain (SY) of CTV (Ieki 1986). S5agA was similarly obtained from the donor 'Morita navel' orange (*Citrus sinensis* Osbeck var. *brasiliensis* Tanaka) infected with CTV-SY (S5).

Aphids were reared on the donor tree, and viruliferous aphids were transferred to the receptor Yuzu (*Citrus junos* Sieb. ex Tanaka) seedlings. The inoculation feeding period was two or three days.

Enzyme-linked immunosorbent assay (ELISA)

Double antibody sandwich (DAS) ELISA with a polyclonal antibody (PAb) was used. The PAb was produced in our laboratory using CTV purified from Hassaku dwarf-infected fruit. Double antibody sandwich indirect (DAS-I) ELISA with monoclonal antibodies (MAbs), 3DF1 (Vela *et al.* 1986) and MCA13 (Permar *et al.* 1990) was also used (Kano *et al.* 1991).

Biological indexing

CTV sources were graft-inoculated to three Mexican lime (*Citrus aurantifolia* (Christm.) Swingle) seedlings, three Yuzu seedlings, three sour orange (*Citrus aurantium* L.) seedlings, and two grafted combinations of 'Madam vinous' sweet orange (*Citrus sinensis* Osbeck) on sour orange rootstock. Inoculated plants were kept in a greenhouse maintained at 15 to 30 ℃ minimum /maximum temperatures. Pests were carefully controlled with chemicals. All the plants used were grown using modified UC soil and plastic long pots (Koizumi and Kano 1991).

Results

Reaction in Mexican lime and Yuzu seedlings

CTV isolates which induced a severe reaction in Mexican lime generally induced a severe symptoms in Yuzu (Table 1). However, the symptom severity induced by a few isolates differed somewhat between the two indicator plants. That is, ATS 1215A induced a severe reaction in Mexican lime and a moderate one in Yuzu. Conversely, 1417A, 1513A and PM8A induced moderate reactions in Mexican lime and severe ones in Yuzu.

Vein-clearing symptoms appeared on young expanding leaves in Yuzu seedlings. A symptom we call 'vein necrosis' developed in the center of cleared veins on young leaves and also on old expanded leaves (Table 1).

Table 1. Reaction of Mexican lime and Yuzu seedling to aphid-transmitted subcultures of citrus tristeza virus in Japan.

Name[z] of isolate	FS[y] or ATS	Sero-[x] type	Reaction of indicator plants[w]	
			Mexican lime	Yuzu
0001	FS	(+ ± +)	mVC, mSP	no
0001A	ATS	(+ + +)	−	no
0137	FS	(− + +)	mVC, mSP	mSP
0137A	ATS	(− + +)	−	no
1215A	ATS	(+ + +)	VC, sSP, St	VN, SP
1417A	ATS	(+ + +)	VC,SP	mVN, sSP
145R	FS	(+ + +)	no	no
1513	FS	(+ + +)	VC, sSP, St	mVN, sSP
1513A	ATS	(+ + +)	VC,SP	mVN, sSP, St
1595A	ATS	(+ + +)	VC, sSP, St	LC, VN, sSP, St
1672A	ATS	(+ + +)	VC, sSP, St	mVN, sSP
BOUQ	FS	(+ + +)	mVC, mSP	mSP
BOUQA	ATS	(− + +)	mVC, mSP	mSP
HM55	FS	(+ + +)	mVC	no
HS34	FS	(+ + +)	VC,SP	VN, sSP
KS3A	ATS	(+ + +)	VC, sSP, St	VN, sSP, St
KS3A2	ATS	(+ + +)	VC, sSP, St	VN, sSP, St
KSA	ATS	(+ + +)	VC, sSP, St	LC, VN, sSP, St
M2A	ATS	(+ + +)	−	no
M12A	ATS	(− + +)	−	no
M15A	ATS	(+ + +)	mVC	no
M16A	ATS	(− − +)	mVC, SP	VN, SP
M16A2	ATS	(− − +)	mVC, SP	mVN, SP
M22A	ATS	(+ + +)	mVC, mSP	no
M23A	ATS	(+ + +)	no	no
M26A	ATS	(+ + +)	−	no
M27	FS	(+ + +)	mVC,SP	mVN, SP
M27A	ATS	(− − +)	mVC,SP	mVN, mSP
M27A5	ATS	(+ + +)	−	mVN, SP
NUagA	ATS	(+ + +)	VC, sSP, St	VN, sSP, St
PM8A	ATS	(+ + +)	VC,SP	mVN, sSP
PM25A	ATS	(+ + +)	mVC,mSP	no
S5A2	ATS	(+ + +)	VC, sSP, St	mVN, sSP
S5agA	ATS	(+ + +)	VC,mSP-sSP, St	mVN, sSP
SIYA	ATS	(+ + +)	VC,mSP-sSP, St	mVN, sSP

[z] See text.

[y] FS : field source, ATS : aphid-transmitted subculture.

[x] Reaction of each sample to MCA13, 3DF1, PAb is shown in parentheses in that order. For example, (− − +) means negative to MCA13 and 3DF1, positive to a PAb. − : negative, ± : questionable, + : positive. Details of ELISA procedurdes and rating system of reactinos were described by Kano *et al.* (1991).

[w] no : no symptom, mSP : mild stem pitting, SP : moderate stem pitting, sSP : severe stem pitting, St : stunting, mVC : mild vein-clearing, VC : vein-clearing, LC : leaf curl, mVN : mild vein necrosis, VN : vein necrosis, − : not tested.

Reaction in sour orange seedlings

Several CTV isolates caused stem pitting in sour orange seedlings (Table 2). However, the property of such isolates was not significantly correlated with the ability of causing seedling yellows reaction in sour orange or severe symptoms in other citrus species. ATS S5A2 caused seedling yellows and stem pitting in sour orange, just like its parent FS S5, although ATS S5agA caused only mild stem pitting.

Table 2. Reaction of sour orange seedlings and 'Madam vinous' sweet orange grafted on sour orange rootstocks (SW/SO) to aphid-transmitted subcultures of citrus tristeza virus in Japan.

Name[z] of isolate	FS[y] or ATS	Sero-[x] type	Reaction of indicator plants[w]	
			Sour orage	SW/SO
0001	FS	(+ ± +)	no	no
0001A	ATS	(+ + +)	no	−
0137	FS	(− + +)	mSP	no
0137A	ATS	(− + +)	no	no
1215A	ATS	(+ + +)	Y, St	−
1417A	ATS	(+ + +)	no	−
145R	FS	(+ + +)	no	no
1513	FS	(+ + +)	Y, St	D
1513A	ATS	(+ + +)	Y, St	−
1595A	ATS	(+ + +)	no-Y	sSP
1672A	ATS	(+ + +)	Y	−
BOUQ	FS	(+ + +)	no	no
BOUQA	ATS	(− + +)	mSP	no
HM55	FS	(+ + +)	no	no
HS34	FS	(+ + +)	mSP	no
KS3A	ATS	(+ + +)	Y	−
KS3A2	ATS	(+ + +)	Y	−
KSA	ATS	(+ + +)	Y, St	−
M2A	ATS	(+ + +)	no	no
M12A	ATS	(− + +)	no	no
M15A	ATS	(+ + +)	no	no
M16A	ATS	(− − +)	no	no
M16A2	ATS	(− − +)	no	no
M22A	ATS	(+ + +)	no	no
M23A	ATS	(+ + +)	no	no
M26A	ATS	(+ + +)	no	no
M27	FS	(+ + +)	no	no
M27A	ATS	(− − +)	no	no
M27A5	ATS	(+ + +)	no	no
NUagA	ATS	(+ + +)	Y, St	−
PM8A	ATS	(+ + +)	Y, St	no
PM25A	ATS	(+ + +)	no	no
S5A2	ATS	(+ + +)	Y, SP,St	−
S5agA	ATS	(+ + +)	mSP	−
SIYA	ATS	(+ + +)	no-Y, SP, St	−

[z] See text.

[y, x] See Table 1.

[w] no : no symptom, Y : leaf yellowing, St : stunting, mSP : mild stem pitting, SP : moderate stem pitting, sSP : severe stem pitting, D : decline, − : not tested.

Reaction in combination trees of sweet orange on sour orange

CTV which induced mild reactions in Mexican lime and sour orange seedlings (Kano *et al.* 1991), was graft-inoculated to combination trees of sweet orange on sour orange rootstock. Such mild sources did not induce any symptoms (Table 2). Only severe CTV isolates, 1513 and 1595A, caused disease in the grafted-combinations.

Discussion

Biological properties of ATSs and some FSs were tested using several citrus indicator species. The data obtained here were compared with the reactions of their parent FSs (Ieki and Yamaguchi 1986, Kano *et al.* 1991, Koizumi and Kuhara 1985, Koizumi 1991). Most of the ATSs shared the same biological properties as their parent FSs (Tables 1, 2). However, two ATSs, S5A2 and S5agA, obtained from a FS of a seedling yellows strain, S5, differed in their pathogenicity to sour orange seedlings, reflecting strain segregation by the vector aphids. Similar segregation had been reported by Martinez and Wallace (1964), and Yokomi *et al.* (1989). Segregation or separation of serotypes through aphid transmission was also observed in our previous experiments (Kano and Koizumi, 1991). These results suggest that selective transmission of strains by vector aphids occurs in the field.

Based on the reaction of Mexican lime and Yuzu, the sources 1595A, KS3A, KS3A2, KSA and NUagA were the most severe among the ATSs tested. The original field source of 1595A severely affected sweet orange (Koizumi 1991). The others were obtained from satsuma mandarin. Since ATS NUagA was obtained by transmission using *A. gossypii* (Ieki 1986), the dominant aphid species in citrus orchards, in early summer (Komazaki *et al.* 1985), we should pay more attention to this species even though its transmission efficiency was very low (Ieki 1986).

In Japan, CTV has been classified roughly into mild or severe strains for the induction of stem pitting. However, several CTV sources tested here (M16A, M16A2 and M27) showed moderate symptoms in indicator plants. These should be classified as moderate strains.

Vein enation symptoms were also observed on the leaves of Yuzu or rough lemon (data not shown) inoculated with 0137, 0137A, HM55, M15A, M16A, M26A and S5A2. The causal agent of citrus vein enation disease apparently does not affect symptom development by CTV, when a citrus tree is infected with both pathogens simultaneously.

Permar *et al.* (1990) reported that mild CTV isolates in the U. S. A. did not react to a MAb, MCA13. It was reported previously (Kano and Koizumi 1991, Kano *et al.* 1991) that several isolates, such as 145R, BOUQ, HM55, M15A, M22A, M23A, PM25A reacted to MCA13 but were classified as mild based on the reactions of Mexican lime and sour orange seedlings. In this study, Japanese mild sources reactive to MCA13, 3DF1 and PAb induced mild or no symptoms in both Yuzu seedlings and combination trees of sweet orange on sour orange rootstocks (Tables 1, 2). Thus, we confirmed the existence of several mild sources reactive to MCA13 in Japan.

Miyakawa (1976) indicated that Yuzu was highly susceptible to severe CTV strains. Kurata *et al.* (1986) also investigated the occurrence of stem pitting disease of Yuzu in the field. Miyakawa and Sasaki (1977) recommended Yuzu as an alternative indicator for CTV. In this paper, we successfully used Yuzu seedlings for the indexing of CTV in Japan. Yuzu is a polyembryonic species (ca. 20 seeds/fruit) tolerant to freezing. In contrast, seed sources of Mexican lime must be grown inside greenhouses due to their high susceptibility to CTV and cold. Moreover since seed production per Mexican lime fruit is low, we conclude that Yuzu is a useful substitute for Mexican lime for the evaluation of the severity of CTV isolates in causing stem pitting disease in Japan, although the relative severity of the symptoms in Yuzu may not be correlated exactly with that in Mexican lime.

Recently, Garnsey *et al.* (1987, 1991) have developed a worldwide collection of CTV isolates in Beltsville, U. S. A. and they are currently characterizing some properties of the collected CTV isolates, including three Japanese isolates 1513, HS34 and HM55. These isolates which were also used as standard isolates in this study, were effective. It is suggested that the Japanese CTV isolates can be compared with other foreign isolates through the three standards.

Acknowledgements

The authors wish to thank Dr. S. M. Garnsey, USDA, Orlando, FL for the critical review of this manuscript, and for providing monoclonal antibodies. The assistance of Dr. T. A. Permar, USDA, Orlando, FL, Dr. C. Vela, Ingenasa, Madrid, Spain and Dr. M. Cambra, I. V. I. A., Mon cada, Valencia, Spain, for providing monoclonal antibodies is gratefully acknowledged. We thank Mrs. Chizuko Suzuki and Mrs. Chieko Azeyanagi, Okitsu Branch, Fruit Tree Research Station, Japan and also Miss Hiromi Takeda, Division of Plant Protection, Fruit Tree Research Station, Japan for their technical assistance.

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カンキツトリステザウイルスアブラムシ伝染株の生物学的性質

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摘　要

日本産カンキツトリステザウイルス（CTV）のアブラムシ伝染株を分離し，その病原性を各種木本検定植物を用いて調べた．大部分のアブラムシ伝染株は，元株である圃場分離株と同じ病原性を示した．しかし，同一の圃場分離株から異なる病原性を持つアブラムシ伝染株が得られる事例があった．この結果及びアブラムシによる血清型の異なるCTVの分離事例（Kano *et al.* 1991）は，圃場においてアブラムシによるCTV系統の選択的伝染が起こっていることを示唆している．メキシカンライムとサワーオレンジの反応から弱毒系統と判定され，2種のモノクローナル抗体（MCA 13と3 DF1）及びポリクローナル抗体と反応したCTVは，ユズ及びサワーオレンジ台スイートオレンジ上でも弱毒系統の反応を示した．これにより，モノクローナル抗体MCA 13と反応する弱毒系統の存在の確証が得られた．病原性の異なる多くのCTV分離株をユズに接種し，その反応を調べたところメキシカンライムの反応と概ね一致し，ユズがわが国におけるカンキツステムピッティング病の検定植物として優れていることがわかった．ユズは1果あたりの種子も多く，耐寒性もあり，メキシカンライムより採種，育苗が容易であった．

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