Comparative Study on Stem-Pitting Strains of CTV in the Asisn Countries

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ABSTRACT. A comparative study was made of three distinct CTV strains which cause severe stem-pitting (SP) and stunting, respectively, in pummelo, sweet orange and mandarin trees, and an ordinary seedling-yellow (SY) strain. The ordinary SY strain caused symptomless infections in sweet orange and mandarin trees, but did not persist in pummelo trees which are grown widely in Asian countries. The pummelo SP strain (Pum/SP), called dwarf strain of CTV (CTV-D), has become common in pummelo trees in recent years. This strain was also found in southern China recently. The Pum/SP replicated well and caused severe dwarfing and SP in pummelo, grapefruit, and lime trees. It replicated poorly in mandarin, and caused no seedling yellows symptoms in Eureka lemon. The sweet orange SP strain (SwO/SP) found in Indonesia and southern China, caused severe SP and moderate stunting in sweet orange trees and SY symptoms in Eureka lemon, but did not cause any symptoms in mandarin and pummelo trees. It persisted in mandarin, but not in pummelo. The mandarin SP strain (Mand/SP) found in Malaysia and Thailand was similar to the sweet orange SP strain but also caused severe SP in mandarin. Three monoclonal antibodies (MAB) were prepared for differentiating CTV strains. The MAB 3E10 recognized an epitope common to all isolates, while 10E3 did not react with the mild strain and 4G12 did not react with SY and mandarin SP strains.

Index words. pummelo dwarf strain of CTV, stem-pitting strain, ELISA, monoclonal antibody.

Citrus tristeza closterovirus (CTV) is present worldwide and consists of a complex of virus strains. Strain variation is very complicated and ranges from mild isolates to several severe and destructive isolates (6). In Taiwan, all citrus cultivars are propagated on CTV-tolerant rootstocks, and infected trees are normally symptomless (7). Most of these CTV isolates show typical seedling yellows symptoms in Eureka lemon. The destructive isolates causing dwarfing and severe stem-pitting in Wentan pummelo trees were first reported from Taiwan in 1981 and were designated as CTV-D strain (7). The CTV-D strain has been spread over the whole island, mainly by movement of infected plants during the last 10 yr. The isolate (IN-1) collected from sweet orange in Indonesia is a sweet orange stem-pitting strain as described by Muharam *et al.* (4). It also caused severe stem-pitting when transmitted to Luchen sweet orange in Taiwan. The isolate MY-2, collected from Langkat mandarin in Malaysia, caused severe stem-pitting symptoms in Ponkan mandarin.

In this paper we compare three destructive stem-pitting strains with an ordinary SY strain with regard to symptom expression, virus replication, and persistence in major citrus cultivars. We also show partial differentiation of these strains by panel assays with three monoclonal antibodies.

MATERIALS AND METHODS

Virus isolates and their characterizations. The Taiwanese isolates of CTV-D (designated Pum/SP) and ordinary SY strains were obtained through aphid (*Toxoptera citricidus*, 20 aphids per plant) transmission from field-infected materials. The sweet orange stem-pitting isolate IN-1 (designated SwO/SP) and mandarin stem-pitting isolate MY-2 (designated Mand/SP) were collected from Indonesia and Malaysia, respectively. All of these isolates were maintained in an isolated double-walled greenhouse and characterized by symptom expression on such differential indicators as Eureka lemon, Mexican lime grafted on sweet orange, Luchen sweet orange, Ponkan mandarin, and Peiyu pummelo. Each indicator with two repeats was grafted with two CTV strain-infected buds and kept in greenhouse at about 20 to 32 C. The isolates were also indexed with Rusk citrange and Etrog S-1 citron for tatter leaf virus and exocortis viroid in-

fection. Symptom expression was observed at two-month intervals for two years, and virus concentration in leaf tissue was examined by DAS ELISA at three-month intervals.

Production and characterization of monoclonal antibodies specific for CTV strains. The method for production and characterization of MABs followed the process previously described (8). The immunogens used were purified CTV from CTV-D-inoculated Peiyu pummelo seedlings and ordinary SY strain-inoculated rough lemon seedlings. Freeze-dried extracts of Florida CTV mild isolate T-30 were used as the source of CTV-M for ELISA tests.

ELISA test. The double antibody sandwich (DAS) method was used for detecting virus replication and virus persistence in the host. The purified polyclonal antibody 1052 antisera prepared against Florida CTV isolate T-36 was used as the coating antibody and for preparation of the conjugate. The indirect double antibody sandwich (I-DAS) method was used for epitope diversity analysis of MABs, as described by Garnsey *et al.* and Permar *et al.* (1,5).

RESULTS

The Wentan pummelo trees in the field infected with Pum/SP showed severe symptoms including bunchy twigs and curly leaves, stem-pitting on twigs, trunk, and roots, stunting, and fruit deformation. The pummelo dwarf has become epidemic in Taiwan. A survey showed a primary infection of 28% in field samples. The Pum/SP strain was also detected from grapefruit and lime trees showing dwarfing, stem-pitting, severe bunchy twigs, leaf curling, and vein-clearing in the field. The lime may have had a mixed infection because Pum/SP does not cause severe symptoms in Mexican lime.

Comparative characterization of Pum/SP, other stem-pitting strains, and an ordinary SY strain from Asian countries in major citrus cultivars is shown in Table 1. The pummelo stem-pitting strain (Pum/SP) did not induce seedling yellows symptoms in Eureka lemon, produced very mild or no symptoms in Mexican lime, no symptoms in Luchen sweet orange and Ponkan mandarin, but caused severe SP in Peiyu pummelo. The SwO/SP, Man/SP, and ordinary SY caused severe seedling yellows in Eureka lemon, severe vein-clearing and stem-pitting in Mexican lime, and stem-pitting of variable severity in Luchen sweet orange. These did not cause symptoms in Peiyu pummelo. The SwO/SP, Mand/SP, and ordinary SY strains induced different

TABLE 1
COMPARATIVE DIFFERENTIATION OF STEM-PITTING STRAINS OF CITRUS TRISTEZA VIRUS (CTV) FROM AN ORDINARY SEEDLING-YELLOWS STRAIN BY SYMPTOM EXPRESSION IN CITRUS INDICATORS

	CTV strain				
Citrus indicator	Pum/SP[z] (F3-7)	SwO/SP (IN-1)	Mand/SP (MY-2)	SY-S (PW-11)	CTV-M (T-30)
Eureka lemon	SY : -[y]	SY : +++	SY : +++	SY : +++	SY : -
Mexican lime	VC : +/-	VC : +++	VC : +++	VC : +++	VC : -
	SP : -	SP : +++	SP : +++	SP : ++	SP : -
Luchen sweet orange	SP : -	SP : +++	SP : ++	SP : +	SP : -
	ST : -	ST : ++	ST : -	ST : -	ST : -
Ponkan mandarin	SP : -	SP : -	SP : ++	SP : -	SP : -
	ST : -	ST : -	ST : +	ST : -	ST : -
Pieyu pummelo	SP : +++	SP : -	SP : -	SP : -	SP : -

[z]Pum/SP = pummelo stem-pitting strain; SwO/SP = sweet orange stem-pitting strain; Mand/SP = mandarin stem-pitting strain; SY-S = seedling yellows severe strain; CTV-M = non-seedling yellows mild strain; virus isolate number in parenthesis.
[y]SY = seedling yellows; VC = vein clearing; ST = stunting; Symptom intensity: +++ = severe; ++ = intermediate; + = mild; - = no symptoms.

symptoms in Luchen sweet orange and Ponkan mandarin. The SwO/SP strain induced severe stem-pitting and stunting in Luchen sweet orange but no symptoms in Ponkan mandarin. However, the Mand/SP strain caused intermediate stem-pitting but no stunting in Luchen sweet orange and intermediate stem-pitting and stunting in Ponkan mandarin. The ordinary SY strain caused mild stem-pitting in Luchen sweet orange and no symptoms in Ponkan mandarin.

All four strains could replicate very well in Eureka lemon, Mexican lime, and Luchen sweet orange, and persistent infections were maintained (Table 2). The Pum/SP strain did not replicate or persist in Ponkan mandarin but replicated well in Peiyu pummelo. The SwO/SP, Mand/SP, and ordinary SY strains did not replicate well or persist in pummelo.

Two new monoclonal antibodies (MABs), 3E10 and 4G12, were recently obtained to the Pum/SP strain and were used in a panel combined with the MAB 10E3 for differentiating Pum/SP, SwO/SP, Mand/SP, ordinary SY, and CTV-M. The MAB 3E10 recognized all of those strains (Table 3). The MAB 10E3 reacted with those SP strains and ordinary SY but did not react with CTV-M as shown before (3). The 4G12 did not react with ordinary SY and Mand/SP strains but reacted with Pum/SP, SwO/SP, and CTV-M strains.

DISCUSSION

The destructive Pum/SP strain has spread over Taiwan since it was first reported in 1981, and has became a threat to pummelo production. The Pum/SP strain is quite distinct from the other stem-pitting strains and ordinary SY strains as shown in Tables 1 and 2. This Pum/SP strain is more like mild strain CTV-M in its reaction in Eureka lemon, Mexican lime, Luchen sweet orange, and Ponkan mandarin. The main difference is that it causes severe symptoms in pummelo. The CTV-M strain T-30 caused the same symptom expression as Pum/SP strain but did not replicate in pummelo. However, CTV-M isolates such as B-29 (Beltsville collection, 2) did persist in pummelo. The Pum/SP strain may have evolved from a mild, non-SY strain. If the standard host range (SHR) is used to identify Pum/SP, the symptoms will appear as a mild strain like CTV-M as shown in Table 1. This will cause a great problem if a new citrus cultivar containing Pum/SP virus is introduced into pummelo growing areas, since pummelo and grapefruit will be threatened. This Pum/SP strain has been found in Taiwan and in southern mainland China so far.

TABLE 2
ELISA ASSAY FOR REPLICATION AND PERSISTENCE OF STEM-PITTING STRAINS AND AN ORDINARY SEEDLING-YELLOWS STRAIN OF CITRUS TRISTEZA VIRUS (CTV) IN CITRUS INDICATORS

	CTV strain				
Citrus indicator	Pum/SP[z] (F3-7)	SwO/SP (IN-1)	Mand/SP (MY-2)	SY-S (PW-11)	CTV-M (T-30)
Eureka lemon	++[y]	++	++	++	+++
Mexican lime	+++	+++	+++	+++	+++
Luchen sweet orange	++	++	+++	+++	+++
Ponkan mandarin	-	+++	++	+++	++
Pieyu pummelo	+++	-	-	-	-

[z]Pum/SP = pummelo stem-pitting strain; SwO/SP = sweet orange stem-pitting strain; Mand/SP = mandarin stem-pitting strain; SY-S = seedling yellows severe strain; CTV-M = non-seedling yellows mild strain; virus isolate number in parentheses.
[y]Average of ELISA values (OD^{405}) measured four times at three-month intervals by DAS ELISA test with polyclonal antibody. ELISA value index: - = <0.1; + = 0.1-0; ++ = 0.05-1.0; +++ = 1.0-1.5.

TABLE 3
ANTIGENIC SPECIFICITY OF MONOCLONAL ANTIBODIES (MABs) AGAINST STEM-PITTING AND SY STRAINS OF CITRUS TRISTEZA VIRUS (CTV) IN DOUBLE ANTIBODY SANDWICH INDIRECT (I-DAS) ELISA

MAB no.	CTV strain				
	Pum/SP[z] (F3-7)	SwO/SP (IN-1)	Mand/SP (MY-2)	SY-S (PW-11)	CTV-M (T-30)
3E10	+[y]	+	+	+	+
10E3	+	+	+	+	-
4G12	+	+	+/-	+/-	+

[z]Pum/SP = pummelo stem-pitting strain; SwO/SP = sweet orange stem-pitting strain; Mand/SP = mandarin stem-pitting strain; SY-S = seedling yellows severe strain; CTV-M = non-seedling yellows mild strain; virus isolate number in parentheses.
[y]I-DAS ELISA test results from three separate assays, + = positive reaction; - = negative reaction.

The antigenic specificity test of MABs, showed that 3E10 recognized all strains tested as a polyclonal antibody, presumably because all have a common epitope. MAB 10E3 could differentiate severe strains and mild strains as shown previously (8). The new MAB 4G12 did not react to the ordinary SY strain and the Mand/SP strain showing SY symptoms in Eureka lemon; however, it reacted with the SwO/SP strain which shows SY in Eureka lemon. MAB 4G12, used in combination with polyclonals or another MAB which recognizes a common epitope, is useful for differentiating the ordinary SY strain from Pum/SP and CTV-M. The MAB array of 3E10, 10E3, and 4G12 could distinguish Pum/SP strain, the CTV-M strain, and part of the SY strains.

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