モノクローナル抗体を用いたELISAによる‘森田ネーブル'におけるカンキツトリステザウイスル分離株M16Aの干渉効果の検定

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Cross-Protection by Protective Strain M16A of Citrus Tristeza Virus (CTV) in 'Morita Navel' Orange Monitored by Enzyme-Linked Immunosorbent Assay (ELISA) Using Two Monoclonal Antibodies†1

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Synopsis

Cross-protection by a protective strain M16A of CTV in 'Morita navel' orange trees was monitored by enzyme-linked immunosorbent assay (ELISA). A unique strain of citrus tristeza virus (CTV), M16A, which does not react to the monoclonal antibodies (MAbs) MCA13 and 3DF1 but reacts to polyclonal antibodies (PAb) was used for pre-inoculation. Pre-inoculated and virus-free trees were challenged with a severe CTV strain (S5) two to seven months after pre-inoculation. Sixteen months after challenge inoculation, all the trees were exposed to natural infection by aphids. Six years after pre-inoculation, the trees in the field were checked by ELISA and by biological indexing. Five pre-inoculated trees were superinfected with CTV serotypes other than M16A and at least three of the five trees were infected with a severe CTV strain. Nine years after pre-inoculation, three trees remained free of superinfection, and eight pre-inoculated trees were superinfected with other serotypes. Four of eight superinfected trees were infected with a severe strain of CTV, based on visual inspection and biological indexing. Four control trees which were originally virus-free and subsequently challenged by aphids showed severe symptoms, and tested positively for the two MAbs and PAb. Even seven

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years after exposure to natural infection by aphids, three pre-inoculated trees provided a cross-protection against the severe strain of CTV. This suggests that the MAbs 3DF1 and MCA13 can be used to monitor the cross-protection ability of the protective strain, M16A, prior to symptom development by super-infection of severe strains.

Key words : citrus tristeza virus, cross-protection, enzyme-linked immunosorbent assay, monoclonal antibody, navel orange

Introduction

A cross-protection experiment with protective strains of citrus tristeza virus (CTV) was carried out in the field at Okitsu Branch, Fruit Tree Research Station, to control CTV stem pitting disease of 'Morita navel' orange (*Citrus sinensis* Osbeck var. *brasiliensis* Tanaka). M15A and M16A are promising protective strains of CTV (Ieki and Yamaguchi 1988, Koizumi *et al.* 1991). M16A is a unique CTV strain which does not react to the monoclonal antibodies (MAbs) 3DF1 and MCA13, but reacts to the polyclonal antibodies (PAbs) (Kano and Koizumi 1991). M16A can be easily distinguished from other serotypes which react to 3DF1 and/or MCA13. Thus, challenge infection with strains which react to either 3DF1 or MCA13 should be distinguished from that with the protective strain M16A.

In this paper, we monitored the protective ability of M16A by ELISA using two MAbs. Symptom observation and biological indexing were also carried out for comparative purposes.

Materials and Methods

Virus-free trees

A 'Morita navel' orange tree infected with a severe strain of seedling yellows of CTV (S5) was heat-treated to inactivate CTV (Ieki and Yamada 1984). Virus-free scions obtained were grafted to trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstocks.

Pre-inoculation and challenge inoculation

CTV-free 'Morita navel' orange trees on trifoliate orange rootstocks were graft-inoculated with M16A in October 1982 or February 1983. Infection of M16A was confirmed by ELISA with a polyclonal antiserum to CTV. Pre-inoculated and virus-free trees were challenged with a severe CTV strain (S5) using viruliferous citrus brown aphid (*Toxoptera citricidus* Kirk.) in April or May 1983. The inoculation feeding period was two days. All the trees were maintained in vector-free screenhouses until September 1984, and were then put outside and exposed to natural infection by aphids. The trees were transplanted to the field at Okitsu Branch, Fruit Tree Research Station, in April 1987.

Biological indexing

Seedlings of Mexican lime (*C. aurantifolia* (Christm.) Swingle) were used to identify CTV strains in 'Morita navel' orange trees. Inoculated plants were kept in a greenhouse maintained at 15 to 30℃ minimum/maximum temperatures.

Enzyme-linked immunosorbent assay (ELISA)

Double antibody sandwich (DAS) ELISA was performed with a polyclonal antibody (PAb). The PAb was produced in our laboratory using CTV purified from Hassaku dwarf-infected fruit (Koizumi and Kuhara 1985). Double antibody sandwich indirect (DAS-I) ELISA tests with MAbs, 3DF1 (Vela *et al.* 1986) and MCA13 (Permar *et al.* 1990) were performed as described by Kano *et al.* (1991).

Reaction of each sample with MCA13, 3DF1 and PAb is shown in that order in each table. For example, (− − +) indicates that the sample have a negative reaction to MCA13 and 3DF1, and positive reaction to PAb. In Table 2, '#' indicates that the positive reaction of the test sample (OD_{405}) was more intense than that of the positive standard sample (uniform, freeze-dried extracts from citrus tissues infected with a stem pitting strain of CTV, 1595) in the same plate.

Evaluation of tree growth

'Morita navel' orange trees in the field were examined for stem pitting, tree vigor and trunk girth.

Results

Indexing and Symptoms

Results of indexing showed that all the control trees without pre-inoculation and one pre-inoculated trees (K-9) had been infected with the severe CTV strain by October 1985 (Table 1).

Pre-inoculated trees K-1 and K-3 were found to be infected with the severe CTV strain in December 1988 by biological indexing. The presence of stem pitting also showed that K-1, K-9 and four control trees (K-12, 13, 14, 15) were infected with the severe CTV strain. The pre-inoculated tree K-7 apparently became infected with the severe CTV strain, recently, based on the degree of stem pitting and the trunk girth. The canopy foliage of the trees infected with the severe strains was visually thinner than that of the other trees, although the trunk girth of K-9 and K-13 had not reflected that effect even in 1991.

ELISA in 1988

The results showed that trees K-1, 3, 6, 9, 10 and 14 were infected with CTV serotypes other than M16A (Table 2). Based on this indexing and the degree of stem pitting, it was shown that K-1, 3, 9 and 14 were infected with the severe CTV strain. Mexican lime seedlings graft-inoculated with the source from the field trees in 1988 were assayed by DAS-I-ELISA and DAS-ELISA (Table 1). Most of the inoculated lime seedlings showed the same serological reaction as that of the field trees. The only exception was K-6 whose field source reacted well to the MAbs (Table 2). The Mexican lime seedlings graft-inoculated with the field source (K-6) did not react to the MAbs.

ELISA in 1991

Three control trees (K-12, 13 and 15) which were originally healthy and subsequently showed severe symptoms were not assayed by ELISA in 1988, but were tested in 1991 and they reacted to both MAbs and PAb. The pre-inoculated trees K-2, 4 and 8 reacted to a PAb, but not to MAbs, which indicated that no superinfection with other serotypes had occurred in those three trees (Table 2). Trees K-5, 7 and 11 which did not react to the MAbs in 1988, did react in 1991, indicating that serotypes other than M16A had infected K-5, 7, 11 between December 1988 and October 1991. Trees K-6, 7 and 10 showed a variable reaction to the MAbs in October 1991, and were retested in December 1991. Most samples collected from trees K-6, 7 and 10 in December 1991 reacted to both MAbs, although one sample from K-7 did not react to MCA13 (Table 2). These results showed that K-6, 7 and 10 were superinfected with other serotypes. K-4 and K-8 were again negative.

The tissue samples of the trees (K-1, 3, 9, 12, 13, 14 and 15) infected with the severe CTV strain reacted more strongly to the antibodies ('#' in Table 2) than the others tested in October 1991.

Table 1. Biological indexing results and symptom observation for 'Morita navel' orange trees pre-inoculated with a protective strain M16A of CTV and exposed to challenge infection.

Tree code	CTV[z]		Indexing[y]		Degree of SP[x]			Trunk girth (mm)[w]	
	Pro-tecting	Chal-lenge	'85	'88	Dec. '85	Jan. '91	Dec. '91	Jan. '91	Dec. '91
K−1	M16A	S5	M	S (+ + +)	7	53	34	151	175
K−2	M16A	S5	M	M (− − +)	10	0	3	177	202
K−3	M16A	S5	M	S (+ + +)	0	13	6	141	162
K−4	M16A	S5	M	M (− − +)	0	0	3	187	210
K−5	M16A	S5	M	M (− − +)	5	0	14	205	213
K−6	M16A	S5	M	M (− − +)	0	7	6	164	190
K−7	M16A	S5	M	M (− − +)	0	13	34	140	166
K−8	M16A	S5	M	M (− − +)	0	13	6	159	185
K−9	M16A	S5	S	S (+ + +)	73	67	46	176	207
K−10	M16A	−	M	M (+ + +)	0	0	17	172	195
K−11	M16A	−	M	M (− − +)	0	0	14	179	225
K−12	−	S5	S		84	73	43	155	180
K−13	−	S5	S		53	33	66	180	210
K−14	−	S5	S		73	73	26	155	170
K−15	−	S5	S		76	47	32	155	178
K−16	M16A			M (− − +)					

[z] M16A : Pre-inoculation with M16A by grafting in October 1982 or February 1983.
S5 : Challenge inoculation with a seedling yellows strain of CTV (S5) by aphids in April or May 1983.
K−10 and K−11 : Challenge-inoculated naturally in the field.
K−12 to K−15 : No pre-inoculation.
K−16 : Original source of M16A maintained in a greenhouse.

[y] Inoculated to Mexican lime seedlings in October 1985 and in December 1988.
M : mild, S : severe. Mexican lime seedlings inoculated in 1988 were checked by DAS-I-ELISA and DAS-ELISA. Reaction to antibodies was described in parentheses. See Table 2.

[x] Figures represent the degree of stem pitting (SP) observed on 3 to 5 twigs per tree. 0=no SP, 100=very severe SP. (Yamada *et al.* 1981)

[w] Trunk girth was measured 5cm above from the graft-union.

Table 2. Serological assays for 'Morita navel' orange trees in a cross-protection experiment to monoclonal antibodies (MAbs) MCA13 and 3DF1 and to a polyclonal antibody (PAb)

Tree code	CTV[z]		Reaction to MCA13, 3DF1 and PAb[y x]		
	Protecting	Challenge	December 1988	October 1991	December 1991
K−1	M16A	S5	(− − +) (+ + +) (+ + +) (+ + +)		
			(+ + +)	(# + #) (+ + +)	
K−2	M16A	S5	(− − +) (− − +) (− − +) (− − +)		
			(− − +)	(− − +) (− − +)	
K−3	M16A	S5	(+ + +) (+ + +) (+ + +) (+ ± +)		
			(+ + +)	(# + #) (# + #)	
K−4	M16A	S5	(− − +) (− − +) (− − +) (− − +) (− − +) (− − +) (− − +)		
			(− − +)	(− − +) (− − +) (− − +) (− − +)	
K−5	M16A	S5	(− − +) (− − +) (+ − +) (+ − +)		
			(− − +)	(+ − +) (+ ± +)	
K−6	M16A	S5	(+ + +) (+ + +) (− − +) (± − +) (+ + #) (+ + #) (+ + #)		
			(+ + +)	(± − +) (+ ± +) (+ + #) (+ + #)	
K−7	M16A	S5	(− − +) (− − +) (+ + +) (− − +) (+ + #) (+ + #) (# + #)		
			(− − +)	(+ + +) (− + +) (+ + #) (− + #)	
K−8	M16A	S5	(− − +) (− − +) (− − +) (− − +) (− − +) (− − #) (− − #)		
			(− − +)	(− − +) (− − +) (− − +) (− − +)	
K−9	M16A	S5	(+ + +) (+ + +) (+ + #) (+ + +) (# + #) (# + #) (+ + #)		
			(+ + +)	(+ + #) (+ + #) (+ + #) (# + #)	
K−10	M16A	−	(± ± +) (+ + +) (± − +) (− − +) (+ + #) (+ + #) (+ + #)		
			(+ + +)	(+ + +) (+ − +) (+ + #) (+ + #)	
K−11	M16A	−	(− − +) (− − +) (+ ± +) (+ ± +)		
			(− − +)	(+ ± +) (+ + +)	
K−12	−	S5		(+ + #) (+ + #) (# + #) (# + #) (# + #)	
				(# + #) (# + #) (# + #) (# + #)	
K−13	−	S5		(+ + #) (+ ± +)	
				(+ + #) (# + #)	
K−14	−	S5	(+ + +) (+ + +) (+ + #) (+ + +)		
			(+ + +)	(+ + #) (# + #)	
K−15	−	S5		(+ + #) (+ + #)	
				(# + #) (# + #)	
K−16	M16A	−	(− − +) (− − +) (− − +) (− − +)		
			(− − +)	(− − +) (− − +)	

[z] See Table 1.

[y] Samples for ELISA were collected from the three or four different twigs of a 'Morita navel' orange tree in December 1988 or October 1991, respectively. Results are shown for each separate sample. Reaction of each sample to MCA13, 3DF1 and PAb is indicated in parentheses in that order. For example, (− − +) means negative to MCA13 and 3DF1, positive to a PAb. − : negative, ± : questionable, + : positive, # : positive and its absorbance value at 405 nm was higher than that of the positive standard sample in the same plate. The same source of standard sample was used in all the plates in October 1991. Details of ELISA procedures and rating system of reactions were described by Kano *et al.* (1991)

[x] Samples for ELISA were collected from five different twigs of a tree in December 1991. The same source of standard sample was used in all the plates in December 1991.

Discussion

Superinfection with other CTV serotypes in pre-inoculated 'Morita navel' orange trees was examined by serological methods, biological indexing and symptom observation.

Three of the 11 pre-inoculated trees (K-1, 3 and 9) were infected with the severe CTV strain based on biological indexing and ELISA. Another five trees (K-5, 6, 7, 10 and 11) were also found to be superinfected with DAS-I-ELISA. One tree, K-7, showed severe symptoms, while superinfection in the other trees was detected before symptom expression. Three trees (K-2, 4, and 8) remained free of superinfection, even seven years after exposure to natural infection by aphids. Samples were collected from three to five different twigs for ELISA. In some cases, the reaction to the MAbs fluctuated among samples from a single pre-inoculated tree. This variable reaction was attributed to the low concentration or uneven distribution of the 'challenging' strain within the tree. In 1988, a pre-inoculated tree (K-6) in the field and Mexican lime seedlings graft-inoculated with the same field source showed different reactions to the MAbs, which also indicated that the inoculum source for indexing may have lacked the serotypes reactive to the MAbs.

Koizumi and Kuhara(1985) who had monitored the cross protection by ELISA using a PAb, reported that the relationship between the CTV concentration and tree symptoms was not conspicuous. In our study, the trees infected with the severe CTV strain showed higher absorbance values (OD_{405}) than those containing only the protecting strain. These results suggest that there may be a relationship between the virus concentration and the pathogenicity of the CTV strain in 'Morita navel' orange trees. It will be further necessary to compare the virus concentration in trees with different combinations of protective and severe strains.

Several citrus virologists in the United States have reported the successful application of MCA13 MAb to monitor the cross-protection ability of protective strains (Rocha-Peña *et al.* 1991, Yokomi *et al.* 1991, Powell *et al.* 1992). Yokomi *et al.* showed that ELISA with MCA13 may contribute to the prediction of decline in trees which do not show symptoms. In our experiment, likewise, the superinfection with the CTV strain was detected in a field tree by ELISA prior to symptom development. Even seven years after exposure to natural infection by aphids, three pre-inoculated trees provided a cross-protection against the severe strain of CTV. Our results here also indicate that the 3DF1 MAb can be used to monitor the protective ability of M16A. Several other Japanese protective strains like M15A, M23A (Koizumi *et al.* 1991) cannot be distinguished from severe strains with the existing MAbs (Kano *et al.* 1992). We plan to develop some cDNA probes to distinguish such protective strains from severe strains, since genetic differences between the strains may be present outside of the coat protein gene.

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モノクローナル抗体を用いたELISAによる'森田ネーブル'におけるカンキツトリステザウイルス分離株M16Aの干渉効果の検定

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摘　要

カンキツトリステザウイルス（CTV）のprotective strainを利用したステムピッティング病防除のための干渉効果試験を実施中の圃場栽植'森田ネーブル'において，干渉効果成立の有無をモノクローナル抗体（MAb）を用いたELISAにより調べた．2種のMAbと反応せずポリクローナル抗体とのみ反応するCTV分離株M16Aを11樹に前接種し，ウイルスフリーの4樹を対照に用いた．強毒系統S5の二次接種を2～7か月後に行い，さらにその1年4か月後から自然感染下に暴露した．前接種6年後にELISA，病徴観察，生物検定を実施したところ，前接種樹のうち5樹でM16A以外の血清型を示すCTVによる二次感染が認められ，そのうち3樹は強毒系統に感染していた．前接種9年後でも，前接種した3樹では二次感染が認められなかった．ELISAで二次感染が明らかになった8樹のうち4樹は強毒系統に感染していた．実験開始時にウイルスフリーであった対照の4樹は強毒系統に感染し，いずれの抗体とも反応した．以上のように，自然感染下に暴露後7年後においても3樹（27.2%）で，干渉効果が保持されていた．また，MAbを用いたELISAにより，病徴発現の有無にかかわらず二次感染したCTVを検出できることがわかった．

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