Restriction Fragment Length Polymorphism Analysis of Citrus Tristeza Virus Isolates in Japan and its Application to Cross-protection Experiments

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原著論文

Restriction Fragment Length Polymorphism Analysis of *Citrus Tristeza Virus* Isolates in Japan and its Application to Cross-protection Experiments †1

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Abstracts

Several primer pairs were designed between open reading frame 6 and the 3' untranslated region for amplification of *Citrus tristeza virus* (CTV) complementary DNA (cDNA). Reverse transcription polymerase chain reaction (RT-PCR) followed by restriction fragment length polymorphism (RFLP) analysis of the PCR products using an appropriate combination of primer pairs and restriction enzymes was shown to be useful for distinguishing diverse CTV isolates in Japan.

Three distinct experiments to investigate cross-protection against severe CTV infection were carried out using two mild isolates (M15A and M23A). The mild isolate M15A was previously reported to be a promising protective isolate. M15A, however, cannot be distinguished from severe isolates by means of existing monoclonal antibody techniques. Our work also demonstrates that RT-PCR-RFLP can be successfully applied to evaluation of the protective ability of mild isolates M15A and M23A against severe CTV isolates, since the results of RT-PCR-RFLP coincided with those of symptom observations in the cross-protection experiments.

Key words: *Citrus tristeza* virus, strain discrimination, diagnosis, cross-protection, RT-PCR-RFLP

Introduction

Citrus tristeza virus (CTV), a member of the genus *Closterovirus*, contains a single-stranded positive-sense RNA of about 20,000 nucleotides. The virus particles are flexuous, threadlike and approximately 2000 nm long by 10–12 nm in diameter. The disease caused by severe CTV strains is a major threat to citriculture (Lee and Bar-Joseph, 2000).

Most citrus trees in Japan are infected with severe strains of CTV. The aphid vectors *Toxoptera citricida* (Kirkaldy) and *Aphis gossypii* Glover are very common in citrus orchards. The protective ability of mild strains has therefore been tested for the control of diseases caused by severe strains. Discrimination of CTV strains is crucial to the development of control strategies, especially for cross-protection using a mild strain (Ieki et al., 1997; Ieki, 2003).

In Japan, CTV mild strains have proved effective in controlling diseases caused by severe strains (Ieki et al., 1997). The Japanese protective isolate M16A could be

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distinguished from severe CTV strains by means of the monoclonal antibodies MCA13 and 3DF1 (Kano et al., 1992b). Several mild Japanese isolates, such as M15A and M23A, however, cannot be distinguished from severe isolates using existing monoclonal antibody techniques (Kano et al., 1991, 1992a). We, therefore, wish to develop molecular diagnostic techniques that are able to distinguish such mild isolates from severe ones.

Kano et al. (1998) studied the molecular variability of Japanese CTV isolates using reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing. Nucleotide sequence analysis of PCR products including the 3'-end of the 27K protein gene, most of the 5'-end of the coat protein (CP) gene and the intercistronic region between the two genes enabled the 13 nucleotide sequences obtained from 12 Japanese isolates of CTV to be classified into three groups.

Pappu et al. (1993) reported that the amino acid residue at position 124 of the CTV coat protein was the most conserved and isolate-specific residue for mild and severe strains, always being phenylalanine for MCA13-reactive severe isolates and tyrosine for MCA13-nonreactive mild isolates. Of the Japanese CTV isolates examined, however, the two mild isolates M15A and M23A have phenylalanine at position 124, as do the severe isolates.

In this study, RT-PCR followed by restriction fragment length polymorphism (RFLP) analysis of the PCR products was shown to be useful for distinguishing diverse CTV isolates in Japan. Our work also demonstrates that RT-PCR-RFLP can be successfully applied to the evaluation of the protective ability of mild isolates M15A and M23A against severe CTV isolates, since the results of RT-PCR-RFLP coincided with those of symptom observations in the cross-protection experiments.

Materials and Methods

Virus isolates

The eleven CTV isolates used in this study (Table 2) have been previously described (Kano et al., 1992a, 1998; Suastika et al., 2001). The pathogenicity of CTV isolates has been classified roughly into mild, moderate or severe according to the degree that they induce stem pitting in Yuzu (*Citrus junos* Sieb. ex Tanaka) or Mexican lime (*Citrus aurantifolia* (Christm.) Swingle). Isolates 1595A-1 and S5agA are stem pitting strains, and the other severe isolates are seedling yellows strains. Infected plants were kept in aphid-free greenhouses at the National Institute of Fruit Tree Science, Tsukuba.

Reverse transcription polymerase chain reaction (RT-PCR)

The oligonucleotide primer pairs for RT-PCR were designed according to the determined sequences of the Japanese severe CTV isolates 1513A and KS3A2 and a Florida severe CTV isolate T36 (Table 1).

Table 1. Primer pairs used for amplification of CTV cDNA.

Primer Pair	Primer Code	Sequences (5' to 3')	Polarity	Binding site in CTV T-36 genome	Source of Sequences (isolate, accession number)
No. 1	FKS01	AAGGTTACGAGGAGGCAACC	+	16003---16022	KS3A2, AB011189
	RKS02	ACTCGAAGGGCGTTAGTACG	−	16548---16567	KS3A2, AB011189
No. 2	CTV52	CGAGGTATCATTCTTCGAGC	+	15957---15976	1513A, unpublished[z]
	CTV32	CGCCATAACTCAAGTTGCG	−	16617---16635	1513A, unpublished[z]
No. 3	CPN	ATGGACGACGAGACAAAGAAATTGA	+	16155---16179	1513A, AB011186
	CPC	TCAACGTGTGTTAAATTTCCCAAGC	−	16802---16826	1513A, unpublished[z]
No. 4	P4	CGGAGCTGGCTTGACTGATTTAGAA	+	16697---16721	1513A, unpublished[z]
	N6	ACTCAAAGGAATGATAACGATGCGT	−	17520---17544	T36, U16304
No. 5	P6	ACTTTCTACGCATCGTTATCATTCC	+	17513---17537	T36, U16304
	N7	TACACGCAAGATGGAGAGACTAAAT	−	18287---18311	T36, U16304
No. 6	P7	ATTTAGTCTCTCCATCTTGCGTGTA	+	18287---18311	T36, U16304
	N8	AGGAACTTATTCCGTCCACTTCAAT	−	19023---19047	T36, U16304

[z] Imanishi et al., unpublished

Total RNA from twig bark (100 mg) of citrus plants infected with CTV was extracted using the Isogen kit (Nippon Gene). RNA was denatured by boiling for 5 min to synthesize first-strand cDNA using a first-strand cDNA synthesis kit (Pharmacia) with a random primer. Synthesized cDNA was amplified in a 12 μl reaction volume (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 0.3 units of AmpliTaq Gold polymerase (Applied Biosystems), 1.5 mM $MgCl_2$, 0.5 μM each of sense and antisense primers and dNTPs (0.2 mM each of dATP, dCTP, dGTP and dTTP). PCR was performed in a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems or PC-800, ASTEC) under the following conditions: denaturation for 10 min at 95 °C and then 40 cycles of annealing for 1 min at 52°C, extension for 1 min at 72°C and denaturation for 1 min at 95 °C. The final extension was held at 72°C for 10 min. PCR products were analyzed by electrophoresis in 1.5 % agarose gels followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator. These were digested with restriction enzymes following the manufacturer's instructions and analyzed by electrophoresis in 2 % agarose gels.

Cross-protection experiments

Virus-free one-year-old Yuzu seedlings were graft-inoculated with two buds of mild isolates in the lower part of the seedling, approx. 10 cm from the soil surface. Pre-inoculated plants and virus-free ones were challenge-inoculated with two buds of severe isolates by grafting. The buds of the severe isolates were grafted immediately above the part where mild isolates had been inoculated. The challenge inocula were periodically removed (Table 3, Table 4). Plants were kept in temperature-controlled greenhouses (approx. 25°C) and observed for symptoms. Evaluation of cross-protection was carried out by symptom observation and RT-PCR-RFLP using primer pairs No. 1 and No. 4 (Table 1, Fig. 1).

Results and Discussion

Differentiation of Japanese CTV isolates by RT-PCR-RFLP analysis

Several primer pairs were used for amplification of CTV cDNA. The primer pairs were designed between the open reading frame (ORF) 6 and 3' untranslated region. Since this part of the genome was more conserved among isolates (Suastika et al., 2001), the primer pairs might be useful for constant detection of different isolates. Eleven Japanese isolates were analyzed by RT-PCR-RFLP using the five primer pairs (Table 1, Primer pair No. 2–No. 6). The obtained results showed that the isolates could be differentiated by using an appropriate combination of primer pairs and restriction enzymes (Table 2).

Confirmation of separation of an isolate from a mixed infection source

Kano et al. (1998) concluded, based on the sequence data, that one of the most severe CTV sources, 1595A, was a mixture of a severe isolate and a mild one. We therefore conducted aphid transmission experiments to separate each isolate from the mixed infection source (Fig. 2).

One to 30 viruliferous aphids (*T. citricida*) were used in the first transmission experiment from the donor plant (original source, 1595A) to receptor Yuzu seedlings. Thirty-

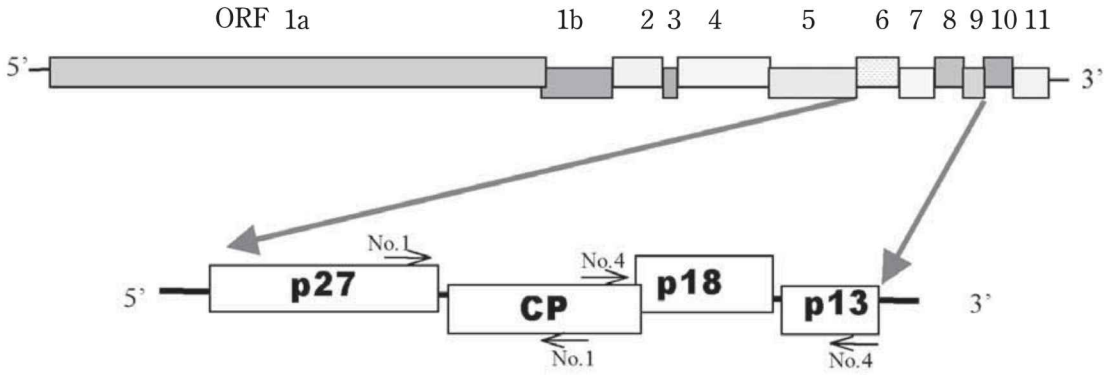

Fig. 1. Location of the primer pairs in the CTV genome.

Table 2. RFLP patterns of Japanese CTV isolates.

Isolates	Strain/Serotype [z]	Primer pair[y] / Restriction enzyme						
		No.1 *Dra* I	No.2 *Dra* I	No.3 *BstE* II	No.3 *Hae* III	No.4 *Hha* I	No.5 *Hae* III	No.6 *Hae* III
1215A	severe/ A	I[x]	I[w]	II[v]	II[u]	III[t]	I[s]	II[r]
1595A-1	severe/ A	--[q]	I	II	II	III	I	II
NUagA	severe/ A	I	I	II	II	III	I	II
KS3A2	severe/ A	I	I	II	I	III	I	II
S5agA	severe/ A	--	I	II	I	II	III	II
M16A	moderate/C	--	II	II	I	I	II	I
M15A	mild/ A	II	II	II	II	I	I	I
M12A	mild/ B	--	II	II	I	IV	I	I
M27A	mild/ C	--	II	II	I	I	I	I
M22A	mild/ A	--	III	I	II	III	I	I
M23A	mild/ A	III	III	I	II	I	I	II

[z] A: reactive to monoclonal antibodies 3DF1 and MCA13, B: reactive to 3DF1 but not to MCA13, C: non-reactive to both 3DF1 and MCA13.
[y] Refer to Table 1.
[x] The sizes (bp) of digested PCR products are approximately as follows;
I: 565 (not digested), II: 418, 147, III: 319, 246.
[w] I: 679, II: 485, 194, III: 366, 313.
[v] I: 672, II: 388, 284.
[u] I: 672, II: 508, 164.
[t] I: 848, II:740, 100, III: 591, 257, IV: 470, 260, 110.
[s] I: 799, II:515, 285, III: 436, 363.
[r] I: 761, II:526, 235.
[q] --: not tested

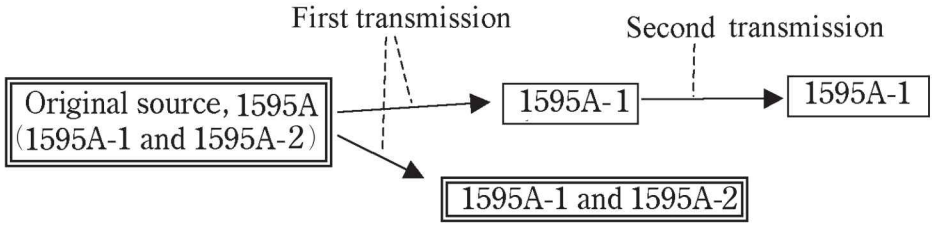

Fig. 2. Procedure of aphid transmission experiment.
: RFLP pattern "I" and "II" (primer pair No. 3, BstE II).
: RFLP pattern "II" (primer pair No. 3, BstE II).
For RFLP pattern, refer to Table 2.

two seedlings of 40 inoculated individuals gave positive results in PCR. In RT-PCR-RFLP using the primer pair No. 3 and the restriction enzyme *BstE* II, the donor plant (original source, 1595A) showed a mixed pattern of "I" and "II" (Fig 2, Table 2). Twenty-one seedlings showed pattern "II", but 11 seedlings showed a mixed pattern of "I" and "II". These results suggest that the seedling showing pattern "II" might be infected with a single isolate, i.e., 1595A-1. To confirm the result, a second experiment was carried out using one of the seedlings showing pattern "II" as a donor plant. One viruliferous aphid per receptor seedling was used for the transmission experiment. Six seedlings of 47 inoculated individuals were infected and these seedlings again showed pattern "II". Yuzu seedlings infected with 1595A-1 showed severe symptoms similar to those in the original source, 1595A.

In this experiment, we were able to confirm the separation of an isolate (1595A-1) from a mixed infection source 1595A containing both 1595A-1 and 1595A-2.

Cross-protection experiment analyzed by RT-PCR-RFLP

Three separate experiments were carried out using two mild isolates (M15A and M23A). The mild isolate M15A was previously reported to be a promising protective isolate

against severe CTV strains (Ieki et al., 1997). M15A, however, cannot be distinguished from severe strains with existing monoclonal antibodies (Kano et al., 1991, 1992a).

Experiments 1 and 2 were mainly planned to investigate whether RT-PCR-RFLP could detect mixed infections of different isolates and to find the appropriate duration of second (challenge) inoculation by grafting.

In Experiment 1 (Table 3, Figs. 3 and 4), plants pre-inoculated with M15A were challenge-graft-inoculated with severe isolates (seedling yellows strain) 1215A or KS3A2. The duration of the challenge inoculation was 7 days, 30 days, or "continuous". The results of RT-PCR-RFLP coincided with those of symptom observations. In Treatment 2 and 7, three pre-inoculated plants did not become infected with the severe isolates, even after 30 days of challenge-inoculations. In Experiment 2 (data not shown), plants pre-inoculated with M23A were challenge-graft-inoculated with severe isolates 1215A or KS3A2. The results of RT-PCR-

Table 3. Detection of a mild isolate (M15A) and severe isolates (1215A or KS3A2) by RT-PCR-RFLP in Experiment 1 [z].

Treatment	First-inoculation [y]	Second-inoculation	Duration of second inoculation [u]	RT-PCR-RFLP [t] (No. of plants)		Stem pitting [r] (No. of plants)	
1	M15A	1215A[w]	7 days	M[s]	(3)	-	(3)
2	M15A	1215A[w]	30 days	M	(2)	-	(2)
				M+S	(1)	S	(1)
3	M15A	1215A[v]	continuous	M+S	(3)	S	(3)
4	M15A	---		M	(3)	-	(3)
5	---[x]	1215A[v]	continuous	S	(3)	S	(3)
6	M15A	KS3A2[w]	7 days	M	(3)	-	(3)
7	M15A	KS3A2[w]	30 days	M	(1)	-	(1)
				M+S	(2)	S	(2)
8	M15A	KS3A2[v]	continuous	M+S	(3)	S	(3)
9	M15A	---		M	(3)	-	(3)
10	---	KS3A2[v]	continuous	S	(3)	S	(3)

[z] Three one-year-old Yuzu seedlings were used per treatment.
[y] Graft-inoculated with a mild isolate, M15A in August, 1996. Infection was confirmed by ELISA.
[x] '---' : no inoculation.
[w] Graft-inoculated with severe isolates, 1215A or KS3A2 in October, 1996.
[v] Graft-inoculated with severe isolates 1215A or KS3A2 in August, 1996.
[u] Inoculum sources were removed 7 or 30 days after inoculation by knives.
[t] PCR products amplified using the primer pair No. 1 were digested with *Dra*I. PCR products amplified using the primer pair No. 4 were digested with *Hha*I. RT-PCR-RFLP was carried out in December, 1998.
[s] M: RFLP pattern of a mild isolate, M15A. S: RFLP pattern of severe isolates, 1215A or KS3A2
M+S: mixed pattern of M and S.
[r] Degree of stem pitting observed in December, 1998 . -: no symptom, S: severe stem pitting.

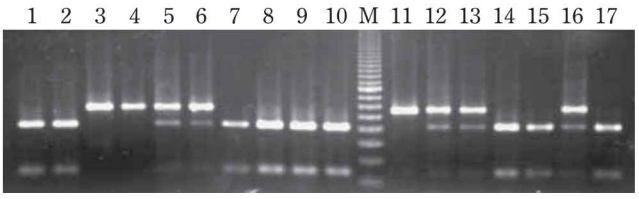

Fig. 3. RFLP analysis of RT-PCR products using primer pair No. 1 and *Dra*I (Experiment 1, *cf*. Table 3).
Lanes 1, 2, 7-10, 14, 15, 17: plants infected with M15A only.
Lanes 5, 6: plants infected with M15A and 1215A.
Lanes 12, 13, 16: plants infected with M15A and KS3A2.
Lanes 3, 4: plants infected with 1215A only.
Lane 11: plants infected with KS3A2 only.
Lane M: marker DNA (100bp ladder, Pharmacia).

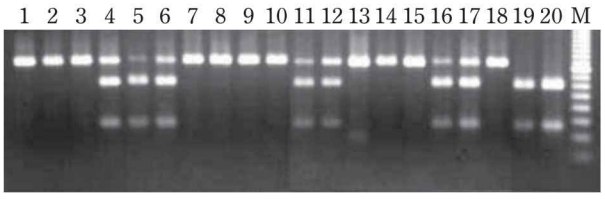

Fig. 4. RFLP analysis of RT-PCR products using primer pair No. 4 and *Hha*I (Experiment 1, *cf*. Table 3).
Lanes 1, 2, 3, 7-10, 13-15, 18: plants infected with M15A only.
Lanes 4-6: plants infected with M15A and 1215A.
Lanes 11, 12, 16, 17: plants infected with M15A and KS3A2.
Lanes 19, 20: plants infected with KS3A2 only.
Lane M: marker DNA (100bp ladder, Pharmacia).

RFLP similarly coincided with those of the symptom observations. The above results indicate that RT-PCR-RFLP is applicable to the detection of mixed infections and that about 30 days is an appropriate duration for second inoculation by grafting.

In Experiment 3 (Table 4, Fig 5), pre-inoculated plants with M15A were challenge-graft-inoculated with the severe isolate (seedling yellows strain) NUagA. The duration of challenge inoculation was 7 days, 14 days, 28 days, or "continuous". Super-infection with a severe isolate occurred after 14 days of challenge-inoculation. Eight months after challenge-inoculation, 15 plants in Treatment 6 and 8 showed the RFLP pattern of mixed infection of M15A and NUagA. Severe stem pitting symptoms did not appear on 6 plants; however, eleven months after challenge-inoculation, severe symptoms appeared on those same plants. The results of RT-PCR-RFLP eight months after challenge-inoculation eventually coincided with the symptom

Table 4. Cross-protection between a mild isolate (M15A) and a severe isolate (NUagA) in Experiment 3[z].

Treatment	First-inoculation[y]	Second-inoculation[x]	Duration of second inoculation[w]	RT-PCR-RFLP [8 mo after second-inoculation][v] (No. of plants)		Stem pitting [8 mo after second inoculation] (No. of plants)		Stem pitting [11 mo after second inoculation] (No. of plants)	
1	---	NUagA	7 days	-[u]	(4)	-[t]	(4)	-	(4)
2	M15A	NUagA	7 days	M	(8)	-	(8)	-	(8)
3	---	NUagA	14 days	-	(3)	-	(3)	-	(3)
				S	(1)	S	(1)	S	(1)
4	M15A	NUagA	14 days	M	(8)	-	(8)	-	(8)
5	---	NUagA	28 days	S	(4)	-	(1)	S	(4)
						S	(3)		
6	M15A	NUagA	28 days	M	(2)	-	(2)	-	(2)
				M+S	(6)	-	(1)	S	(6)
						M	(1)		
						S	(4)		
7	---	NUagA	continuous	S	(4)	S	(4)	S	(4)
8	M15A	NUagA	continuous	M+S	(9)	M	(4)	S	(9)
						S	(5)		
9	---	---		-	(4)	-	(4)	-	(4)
10	M15A	---		M	(6)	-	(6)	-	(6)

[z] Four, six, eight or nine one-year-old Yuzu seedlings were used per treatment.

[y] Graft-inoculated with a mild isolate, M15A in October, 1998. Infection was confirmed by ELISA. '---' : no inoculation.

[x] Graft-inoculated with a severe isolate, NUagA in January, 1999.

[w] Inoculum sources were removed 7, 14 or 28 days after inoculation by knives.

[v] PCR products amplified using the primer pair No. 1 were digested with *Dra*I.

[u] M: RFLP pattern of a mild isolate, M15A. S: RFLP pattern of a severe isolate, NUagA. M+S: mixed pattern of M and S. -:not detected

[t] Degree of stem pitting. -: no symptom. M: mild stem pitting, S: severe stem pitting.

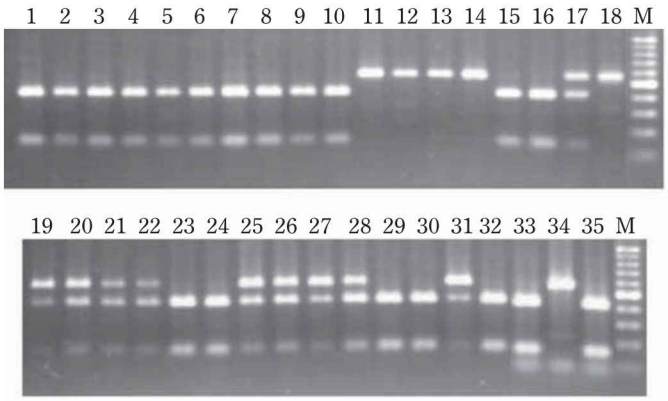

Fig. 5. RFLP analysis of RT-PCR products using primer pair No. 1 and *Dra*I (Experiment 3, *cf.* Table 4).
Lanes 23, 24, 30, 33: inoculated with M15A only.
Lanes 1-10, 15, 16, 29, 32, 35: plants infected with M15A were challenge -inoculated with NUagA but not infected with NUagA.
Lanes 17, 19-22, 25-28, 31: plants infected with M15A were challenge-inoculated with NUagA and infected with NUagA.
Lanes 11-14, 18, 34: plants inoculated with NUagA only.
Lane M: marker DNA (100bp ladder, TOYOBO).

observations eleven months after challenge-inoculation. Furthermore, eight months after challenge-inoculation, in Treatment 6, two pre-inoculated plants were not infected with severe isolates, probably due to cross-protection. These two plants did not show any symptoms eleven months after challenge-inoculation. Thus, RT-PCR-RFLP was able to confirm the super-infection of a severe isolate prior to symptom development.

The results obtained here show that RT-PCR-RFLP is a potentially useful tool for cross-protection experiments, as Gillings et al. (1996) previously reported in field experiments in Australia. This technique may be valuable in evaluating mild isolates like M15A and M23A which cannot be distinguished from severe isolates using existing monoclonal antibodies.

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日本産カンキツトリステザウイルス分離株のRT-PCR-RFLP分析と弱毒株の干渉効果評価への適用

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摘　要

カンキツトリステザウイルス(CTV)のopen reading frame 6 と 3'末端非翻訳領域の間にCTV相補DNAを増幅するためのいくつかのプライマーペアを設計した。これらのプライマーペアと制限酵素を組み合わせてRT-PCR-RFLP分析することにより、日本産の異なるCTV分離株を識別することができた。

本手法がCTV弱毒株の強毒株に対する干渉効果の評価に有効であるか調べるため、弱毒株(M15AまたはM23A)を用いた干渉効果試験に本手法を適用した。M15Aは干渉効果能が高いことが報告されているが、既存のモノクローナル抗体で強毒株と識別できない分離株である。これらの試験において、RT-PCR-RFLPの結果は病徴観察結果と一致し、また、評価期間短縮の可能性が示唆された。