Studies on Mild Strain Cross Protection Against Stem-pitting *Citrus tristeza virus*

C. Y. Zhou, D. L. Hailstones, P. Broadbent, R. Connor, and J. Bowyer

ABSTRACT. Glasshouse trials were conducted to investigate the capacity of a mild strain of *Citrus tristeza virus* (PB61) to protect seedlings against super-infection with a severe grapefruit stem-pitting isolate (PB219) or two orange stem-pitting (OSP) isolates (PB155 or PB235). Symptoms were monitored, and the presence of each isolate followed using isolate-specific restriction fragment length polymorphism (RFLP) analysis of amplicons generated by reverse transcription and polymerase chain reaction (RT-PCR), and multiplex RT-PCR. Pre-immunization with PB61 gave partial protection against super-infection using aphid-inoculation, and delayed super-infection when challenge was by grafting. Pre-immunization with PB61 did not ameliorate the expression of OSP symptoms once super-infection with OSP-inducing isolates was observed, nor prevent movement of the challenge virus. Pre-immunization with a severe OSP isolate (PB155) did not delay super-infection by PB61 when introduced via grafting.

Preimmunization with PB61 protected plants more effectively against OSP isolate PB235 than against OSP isolate PB155. This is significant because PB235 has closer nucleotide sequence homology to PB61 than PB155. A model based on post-transcriptional gene silencing (PTGS) is presented, that may explain mild-strain cross-protection against CTV, and would be consistent with these results.

Index words. CTV, MSCP, Preimmunization, mechanism, PTGS.

Mild strain cross-protection (MSCP) has been used extensively to control losses caused by stem-pitting isolates of *Citrus tristeza virus* (CTV) in Australia (5), Brazil (19), South Africa (26), and Japan (24). Field trials at Somersby and Dareton in NSW, Australia, have shown that some mild isolates of CTV from apparently healthy Marsh and Thompson grapefruit trees in orchards declining with CTV stem-pitting, when grafted into virus-free Marsh grapefruit, protect against stem-pitting isolates transmitted by the brown citrus aphid, *Toxoptera citricida* (7, 10). The degree of protection varied with the CTV isolate and climatic conditions at the trial site (3). Pre-immunization with a mild CTV isolate (PB61) has protected Marsh grapefruit trees in the Australian Citrus Budwood Scheme against stem-pitting for 35 yr. However, a gradual increase in CTV strain severity occurred in preimmunized trees used as budwood sources, commencing 17 yr after planting. Trees nevertheless remained vigorous and productive, with only 6 of 64 trees showing mild trunk-pitting. Breakdown in mild-strain protection has been more evident in sub-propagations from trees with mixed infections (5). In 1990, severe stem-pitting symptoms were found in Washington navel orange and Ortanique tangor trees in the Central Burnett area of Queensland (4, 6, 20). Losses are continuing in the absence of an effective preimmunizing isolate for use in sweet orange.

Several problems still limit the use of MSCP: a) screening for protective isolates is empirical (18), b) the most effective protecting isolate for a given citrus species usually comes from the same species, indicating host specificity (18), c) breakdown may occur over time, d) difficulties have been experienced in pre-immunizing red grapefruits in Australia (5) and South Africa (9).

Understanding the mechanism of MSCP against CTV may improve our ability to solve these problems and to recognize or engineer more effective pre-immunizing isolates. MSCP against CTV might involve failure of the challenge virus to infect, to replicate, or to move, or to

provoke symptoms. This paper examines these possibilities.

MATERIALS AND METHODS

Hosts. Marsh grapefruit (MGF) buds grafted onto 1.5 yr-old Symons sweet orange (SSwO) seedlings were used for trials 1 and 2, and 1-yr-old SSwO seedlings were used for trials 3 to 6. Plants were grown in glasshouses at 24-28°C. The virus-negative Marsh grapefruit budwood was from a virus-free mother tree in the screenhouse of Citrus Foundation Repository at Elizabeth Macarthur Agricultural Institute (EMAI), NSW Agriculture. Pre-immunized Marsh grapefruit budwood was from a mother tree graft-inoculated with mild isolate PB61, grown in the screenhouse of Fruit Variety Foundation Repository at EMAI.

CTV isolates. The following isolates were used:

1) PB61, the pre-immunizing isolate used commercially to protect grapefruits for over 30 yr in Australia (4). Molecular and biological characterization of PB61 and 10 subisolates derived from it via single-aphid transmissions, suggest that PB61 consists of a stable and homogenous viral population (Zhou et al., unpublished);
2) PB155 and PB235 induce OSP symptoms (12) and were derived by single-aphid transmissions from field isolates (4), and are therefore referred to as subisolates;
3) PB219 is a grapefruit stem-pitting (GFSP) isolate. Restriction fragment length polymorphism (RFLP) analysis of cDNAs to the coat protein (CP) gene, amplified by RT-PCR and digested with *Hin*f I, indicates this isolate contains a mixture of variants (12).

Inoculation methods. Plants were challenge-inoculated by feeding with viruliferous brown citrus aphids (*Toxoptera citricida*) or by grafting with two pieces per plant of CTV-infected SSwO bark, as indicated in Table 1. The CTV status of all virus-negative control plants and all young shoots of pre-immunized plants was confirmed by direct tissue blot immunoassay (DTBIA) prior to challenge-inoculation.

TABLE 1
SUMMARY OF *CITRUS TRISTEZA VIRUS* MILD STRAIN CROSS-PROTECTION TRIALS 1 TO 6

Trial	Host[a]	Challenge method	Challenge isolate/ subisolate	DPP/I[b]	P/I[c]	No. of plants positive for the challenge isolate (or subisolate)/total no. of plants[d]				
						7 dpi	15 dpi	30 dpi	60 dpi	90 dpi
1	MGF/ SSwO	100 aphids	PB219	55	none		0/8	2/8	7/8	7/8
					PB61		0/8	0/8	0/8	0/8
2	MGF/ SSwO	grafting	PB219	57	none	0/7		7/7		
					PB61	0/7		2/7	7/7	
3	SSwO	grafting	PB61	56	none	0/8		6/8	8/8	
					PB155	0/8		6/8	8/8	
4	SSwO	50 aphids	PB235	85	none			14/15	14/15	14/15
					PB61			0/15	0/15	1/15
5	SSwO (large)	50 aphids	PB155	70	none			20/20		
					PB61			8/20	8/20	8/20
6	SswO (small)	50 aphids	PB155	29	none			10/10		
								9/10	10/10	

[a]MGF/SSwO = Marsh grapefruit (MGF) on Symons sweet orange (SSwO) rootstock. [b]DPP/I = days post pre-immunization when challenge-inoculated. [c]P/I = preimmunizing isolate; none = plants were not preimmunized but inoculated with the challenge isolate/subisolate only. [d]blank = either that monitoring was not conducted at that time point or that all plants were confirmed positive for the challenge isolate/subisolate by 30 or 60 dpi and monitoring was discontinued.

Aphid transmissions were based on the method of Broadbent et al. (4). Half the plants used in each experiment served as non-pre-immunized controls, challenged in the same manner and at the same time as the pre-immunized plants. Each trial also included a "mock-inoculated" virus-negative control plant and a "mock-inoculated" preimmunized control plant, which were subjected to aphid feeding by virus-negative aphids or by grafting with two pieces of virus-negative SSwO bark.

Monitoring the superinfection of preimmunized plants. After challenge inoculation, plants were monitored at 7, 15, 30, 60 and 90 days post-inoculation (dpi). The pre-immunizing and challenge isolates/subisolates were discriminated within the same plant (in young bark and rootlets) using either RFLP analysis of the CP gene and/or multiplex RT-PCR of the p23 gene (Connor et al., unpublished).

RFLP analysis of the CP gene. Total nucleic acid used for RT-PCR was extracted from ca. 10 mg of CTV-infected tissue using a rapid micro-extraction method (14, 28). cDNAs to the CP gene were amplified by RT-PCR and digested with *Hin*f I (13).

Multiplex RT-PCR. Primer pairs were designed on the p23 genes to selectively amplify isolate PB61, subisolate PB155 and isolate PB219 (Table 2). The size of the amplified product was different with each primer pair so the three isolates/subisolates could be identified within a single extract, even if all three were present. Reverse-transcription was conducted using reagents from Promega Corporation and 2.5 µM random primer, the PCR mix contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.75 mM $MgCl_2$, 0.1 mg/ml BSA, 0.6 µM of each primer; 2.5 U *Taq* DNA polymerase. cDNA was amplified using the following temperature program: 95°C for 2 min; 95°C for 30 s, 60°C for 30 s touching down in 0.9°C decrement to 43°C (one cycle at each temperature), 72°C for 1 min (length of cycle increased by 3 s per cycle), 40 cycles; finally 72°C for 5 min.

OSP symptom expression. After challenge inoculation, plants were grown at about 26°C in glasshouses. Plants were cut back at 15 cm above the soil, periodically after challenge inoculation, and stem-/root-pitting were recorded. Both PB155 and PB235 cause stem- and root-pitting in SSwO within 3-5 mo post-challenge, whereas PB61 and PB219 do not induce such symptoms in SSwO.

RESULTS

The results of MSCP trials are summarized in Table 1.

Trial 1. The presence of preimmunizing isolate PB61 effectively protected MGF/SSwO against superinfection by severe isolate PB219 inoculated using 100 aphids per plant (none of eight preimmunized

TABLE 2
SEQUENCE OF OLIGONUCLEOTIDE PRIMERS USED IN MULTIPLEX RT-PCR

Primer	Sequence (5' to 3')*	Specific for	Amplicon size (bp)
61F2	^{18422}ACTAGAGTTGAAAACGTAAAATCG^{18445}	PB61	468
61R	^{18889}GTTGAGTTCCGGTAACATCGCTG^{18867}	PB61	
155F	^{18543}GAATAATAGGAGTGTGCGTA^{18562}	PB155	378
155R2	^{18920}AAGTGTCTTCGTTATCACCAACGA^{18897}	PB155	
219F	^{18422}ACTRAAGTYGAAAMCGTAAATTCG^{18445}	PB219	115
219R	^{18536}GAAAGCGAGCRCCCTGATAAG^{18516}	PB219	

*Nucleotide (nt) numbers of the selective primers refer to nt position in the p23 gene of CTV isolate T30 (1), M = A or C, R = A or G, Y = C or T.

plants infected compared to seven of eight non-preimmunized controls).

Trial 2. The presence of PB61 delayed super-infection by challenge isolate PB219, introduced by grafting, in five of seven plants by about 30 days compared to non-preimmunized control plants. All grafted plants contained the mixture of CTV genotypes present in PB219, as checked by RFLP profiling (not shown).

Trial 3. When SSwO seedlings were preimmunized with PB155, and challenged with the mild isolate PB61 by grafting, no cross-protection against PB61 was observed (Table 1). Results were confirmed by multiplex RT-PCR and RFLP analyses of the CP gene (not shown). PB155 alone caused moderate OSP symptom in the mock-inoculated control SSwO seedling, whereas PB155 and PB61 together caused moderate to severe OSP symptoms.

Trial 4. Only 1 of 15 SSwO preimmunized with PB61 and challenged with PB235 by aphid-inoculation was super-infected by 90 dpi (Fig. 1, Table 1), compared to 14 of 15 non-preimmunized control plants.

Trial 5. Eight of 20 PB61-preimmunized SSwO seedlings were super-infected with PB155 via aphid-inoculation by 30 dpi (Table 1). In contrast, all non-pre-immunized control plants were infected by this time. These results show that PB61 effectively protected large SSwO plants (2.5-3.5 mm stem diameter at 10 cm above soil) against super-infection by PB155.

Trial 6. Multiplex RT-PCR (Fig. 2) indicated that 9 of 10 of the PB61-preimmunized small plants (1-1.5 mm stem 10 cm above soil) were super-infected with PB155 by 30 days after aphid-inoculation, and all 10 by 60 dpi. The protection afforded to large plants by preimmunization with PB61 in Trial 5 contrasts with the results in the small plants in Trial 6 (Table 1). This suggests that host physiology may affect MSCP.

Stem-pitting symptoms were observed in all SSwO plants in which the challenge (OSP) subisolate was detected, and plants negative for the OSP subisolate did not display stem-pitting symptoms.

DISCUSSION

In these studies the efficacy of preimmunization against super-infection was influenced by the inoculation method, the challenge isolate involved, and the host and its physiological status.

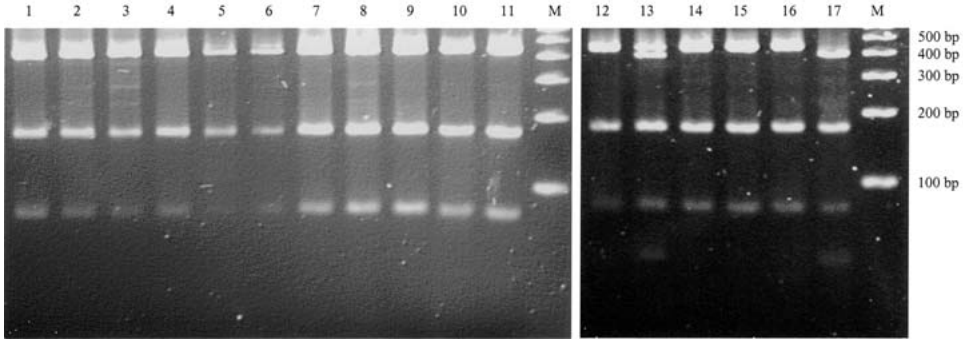

Fig. 1. Detection by CP/*Hinf* I RFLP profiles of the challenge CTV severe OSP subisolate PB235 and the preimmunizing isolate PB61 in Symons sweet orange (SSwO) seedlings at 90 days post-challenge (Trial 4). 1-15 = SSwO seedlings preimmunized with PB61 and challenged with severe OSP subisolate PB235; 16 = a preimmunized SSwO seedling subsequently "mock-inoculated" with 50 vitus-free aphids; 17 = a SSwO seedling inoculated with PB235; M = 100 bp ladder DNA marker. Note: only plant no. 13 was superinfected with PB235 by 90 dpi.

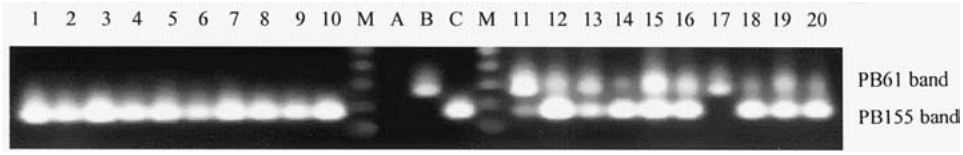

Fig. 2. Detection of the severe OSP subisolate PB155 and the preimmunizing mild isolate PB61 in small Symons sweet orange (SSwO) seedlings by multiplex RT-PCR, at 30 days post inoculation (trial 6). 1-10: non-pre-immunized control SSwO seedlings inoculated with PB155 using 50 aphids per plant. M: DNA marker (100 bp ladder, the bottom band is 300 bp). A: water control in RT-PCR mix. B: mock control plant pre-immunized with PB61 and exposed to 50 virus-free aphids. C: mock control plant infected with PB155. 11-20: small SSwO seedlings pre-immunized with PB61 and challenged with PB155 using 50 aphids per plant.

When challenge was by grafting, pre-immunization with PB61 only delayed detection of the severe isolate by about one month in some plants, and the challenge virus could be detected within 2 to 3 mo in all plants (Trial 2). Exclusion of the challenge isolate from pre-immunized plants was not observed under the high inoculum pressure provided by bark inoculation. This is consistent with some studies based on symptom expression (7). However other studies have indicated that MSCP against CTV can be effective when challenge was by grafting (22, 23), although this may reflect the host species used as inoculum source in these experiments (22). In the present study, RFLP analysis indicated that the three CP/Hinf I variants present in isolate PB219 were all transmitted by grafting, whereas we have previously observed segregation of these variants after aphid transmission (data not shown).

In all cases, plants super-infected with OSP subisolates PB155 and PB235 showed symptoms within 3 to 5 mo once infection was confirmed (Trials 3 and 4). This is consistent with the results of Moreno et al. (16) that whenever the dsRNA profile of a severe isolate was detected, the plants showed symptoms characteristic of that isolate.

Our results provide some evidence that MSCP against CTV at an early stage involves prevention of super-infection, but if this occurs, symptom expression is not prevented. This could explain certain field observations. Why does MSCP not work against quick-decline CTV in sweet orange or mandarin on sour orange? Probably because symptoms develop so quickly, due to the hypersensitivity of these combinations to quick decline, and MSCP does not prevent symptom development. In contrast, grapefruit on sour orange is less hypersensitive to quick decline CTV, and this combination could therefore persist in the field for some years with less disease pressure as reported by Stubbs in Australia (25) and by Powell et al. (21) in Florida. Stem-pitting develops more slowly in grapefruits than in sweet oranges in Australia, so in general MSCP could be expected to protect the former for longer in areas where severe GFSP and OSP isolates are endemic.

The results also suggest that MSCP did not prevent movement of the challenge virus, because once super-infection occurred, the challenge virus was detected both in shoots and feeder roots, indicating systemic movement.

PB61 protected more effectively against OSP isolate PB235 (Trial 4) than PB155 (Trial 5). The levels of nucleotide sequence identity between PB61 and PB235 are much higher than between PB61 and PB155 (97.6% vs 91.2% identical over the coat protein, p18 and p23 genes) (results not shown); the better protective capacity of PB61 against

PB235 may therefore be a direct function of the sequence homology between them. Effective MSCP against CTV may thus require a high level of homology between preimmunizing and challenge isolates.

For CTV, a model of RNA-mediated defence (2) can be envisaged whereby infection with the preimmunizing isolate triggers the host to produce a dsRNA specific-nuclease, which targets the viral RNA for degradation to low levels. This may result in the appearance of small nucleotide fragments such as those observed during PTGS (15, 17). Once PTGS is established, other transcripts homologous, or nearly so, to the silenced gene are also subsequently degraded if they infect the plant (8, 27). If citrus plants resist infection by CTV using PTGS, effective protection would depend on a low inoculum pressure and presumably would be most successful where there is close sequence identity between the preimmunizing and the challenge isolates.

ACKNOWLEDGMENTS

Zhou Changyong was funded by a John Allwright Fellowship from the Australian Center for International Agricultural Research (ACIAR) to undertake the Ph.D. degree in the University of Sydney with experimental work conducted at EMAI. Many thanks to Mrs. Carol Dephoff for technical assistance, and to Dr. Mui-keng Tan for technical advice.

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