Restriction Analysis of Amplified CTV Coat Protein cDNA is a Sensitive and Rapid Method for Monitoring and Controlling CTV Infections

Michael Gillings, Patricia Broadbent, and James Indsto

ABSTRACT. Strategies to manage citrus tristeza closterovirus (CTV) diseases, such as eradication of severe strains or the use of mild strain cross protection, require procedures to accurately and reliably identify individual virus strains. Restriction analysis of the CTV coat protein gene (CPG) amplified using the polymerase chain reaction (PCR) places strains into groups with similar properties. Although there are some variations in CTV strains not revealed by this technique, it has considerable advantages in that it can identify strain mixtures in complex field isolates using a single reaction from samples taken directly from field trees. In this paper we show some of the practical applications of CPG restriction analysis. These include: (1) its ability to distinguish the mild cross-protecting strain used in Australia to control grapefruit stem pitting from all other Australian strains; (2) prediction of cross protection breakdown as the result of appearance of new RFLP groups in cross-protected trees by introgression of severe strains; (3) identifying CTV isolates transmitted by single aphid transfers (SAT). In the latter case, SAT and restriction analysis of CPG can be used to obtain and document that an isolate is a pure strain (as defined by the presence of a single RFLP group in the SAT).

Index words. Mild strain cross-protection, aphid transmission, *Toxoptera citricida*, stem pitting, polymerase chain reaction, PCR, RFLP.

Citrus tristeza closterovirus (CTV) is the most important viral pathogen of citrus. It is spread by propagation of infected budwood, and is semi-persistently transmitted by various aphid species in the genera *Aphis* and *Toxoptera*, the most efficient vector being *T. citricida* (Kirkaldy) (30). Field isolates of CTV may be composed of a mixture of virus strains, as first suggested by Grant and Higgins (14). In the past, evidence of strain diversity has been found in the number of strains with distinct biological properties that can be recovered from field isolates, particularly during serial grafting or aphid transmission experiments (1, 19, 31, 37). Recent developments in molecular and serological techniques have furnished more direct evidence of strain diversity. Monoclonal antibodies prepared to single strains (17) can exhibit reactivity with only a subset of the strains in CTV collections (19, 28, 29), demonstrating the variability in the coat protein sequence of different CTV isolates as reflected by a diversity of epitopes (8). This protein sequence variation has also been demonstrated using electrophoretic analysis of partial digests of the coat protein (15, 21, 22). Sequence variation at the RNA level has been demonstrated using differential cDNA probes in hybridization assays (32, 36), and by comparison of the coat protein gene (CPG) sequence from various isolates (27, 35, Gillings et al. unpublished). There is also considerable variation in the electrophoretic profiles of CTV specific double stranded RNA (dsRNA) (9, 16, 20, 24).

Each of the methods for demonstrating variation in CTV strains has also been used as a means of detecting and identifying individual strains, but all have limitations. Electrophoretic analysis of partial digests of the CTV coat protein can distinguish some isolates, but the method is impractical for routine identifications. Immunological and hybridization assays both have limitations in that multiple tests with a large panel of discriminatory probes or antibodies are required to distin-

guish between the large number of distinct CTV strains. In some cases monoclonal antibodies cannot distinguish between strains that have quite different biological properties (Broadbent unpublished). DsRNA profiles have not been associated with specific symptoms, and may vary according to the indicator host and time of year (18, 25). DsRNA analysis is also complicated by the presence of strain mixtures in field isolates (26).

To date, glasshouse indexing is the only method available to reliably determine the biological properties of a given CTV field isolate. However, glasshouse indexing is time consuming and labor intensive. There may also be strains within the isolate that are 'hidden' through cross-protection or interference mechanisms (4, 25). Furthermore, differences in environment, indicator hosts, and terminology have created uncertainty as to the relative severity of CTV isolates from different citrus producing areas. To overcome some of these problems, a biocharacterization index has been proposed to standardize results from different laboratories (11).

Clearly, a more rapid means of identifying individual CTV strains and predicting their biological properties is needed to screen budwood source trees, for epidemiological studies, and to evaluate cross-protection trials. This need is even more pressing when highly destructive strains are introduced to a country such as the orange stem pitting strains recently introduced to South Africa (23) and Australia (6). A method we have used with success is the rapid analysis of restriction digests of the CTV CPG (13) amplified using the polymerase chain reaction (PCR) (33). This method has advantages of speed, sensitivity, and the ability to detect many different CTV coat protein genotypes in a single reaction. The method can be used directly on field trees, and although it does not discriminate all the strains of CTV known to exist on biological indexing criteria, it does provide a rapid analysis of CTV strain mixtures (13). In this paper, we evaluate the use of this method for tracking the introgression of severe CTV strains into pre- immunized grapefruit trees and, thereby, predict the breakdown of mild strain cross protection. We also demonstrate that restriction analysis can be used to rapidly screen the products of aphid transmission experiments and identify the different strains separated by aphid transmission from CTV field isolates.

MATERIALS AND METHODS

Virus isolates and indexing. Detailed information on all field isolates and subsequent bud or aphid transmissions is presented in Tables 1 and 2. Propagating material for the experiment on the breakdown of mild strain cross protection was collected from Marsh grapefruit mother trees. These trees were maintained in budwood source blocks at the Agricultural Research and Advisory Station (ARAS), NSW Agriculture, Dareton (5). Isolates capable of causing stem pitting on sweet oranges were collected from commercial blocks of Washington navel orange and Ortanique tangor in the Central Burnett region of Queensland (6). Reference isolates were from the international collection of CTV strains held at the Beltsville Agricultural Research Center in Maryland, USA (12). To establish the biological properties of the CTV isolates, and to estimate their severity, a range of seedlings was bud-inoculated with the source material. Seedlings used as indicators included West Indian lime (WIL, for the vein clearing reaction), Eureka lemon and bitter sweet Seville orange (for the seedling yellows reaction) and sweet orange (for orange stem pitting). Indicator plants were held at

TABLE 1
LIST OF FIELD TREES INOCULATED WITH MILD CROSS PROTECTING STRAIN(S) SUBSEQUENTLY TESTED FOR THE INTROGRESSION OF SEVERE STRAINS

Identification[z]	Row/tree/no.[y]	Tissue[x]	Biological indexing[w]	RFLP[v]
Marsh grapefruit 3970	R28 T1	WIL	+++	5, 3, (1)
	R28 T2	FT	++	5, 1
	R28 T6	WIL	++	5, 1
	R28 T9	WIL	+++	5, 1
	R28 T19	WIL	+++	5, 3, 1
	R28 T20	FT	+++	5, 3, 1
	R28 T22, T24	FT	+	5
	R28 T30	WIL	+	5
	R28 T27	FT	+	5
	R28 T32, T33	WIL	+++	5, 3, 1
Marsh grapefruit 3962	R13 T6, T7	WIL	+	5
	R13 T7	FT	+	5
	R28 T59*	FT	+++	5, 3, (1)
	R28 T63*	FT	+++	3, (5)
	R28 T64	FT	++	5, 3
Marsh grapefruit 3970	R9 T19	FT	++	5, 1
	R9 T20	FT	++	5, 3, 1
	R9 T21	FT	+	5, 1
	R9 T22	FT	+	5, 3, (1)
	R9 T23,T24,T25	FT	+	5
	R9 T7	WIL	+	5
	R9 T7	DG	+	5
	R9 T26,T27,T28	FT	+	5
	R9 T30,T31,T32	FT	+	5
	R9 T34,T35	FT	+	5

[z]Scion variety and accession number.
[y]Row and tree numbers from budwood blocks planted at Dareton, NSW. Row 28 is in mother tree block 1. Individual trees from this block were sampled 25 to 27 years after planting. Row 13 is in the foundation repository at Dareton. Row 9 is in budwood block 2, and trees 19-35 were propagated in 1985 from R28 T2. Individual trees from this block were sampled 8 to 10 years after planting.
[x]Source of dsRNA used in the amplification reaction. WIL = West Indian lime, FT = Field tree, in this case Marsh grapefruit leaves, DG = Duncan grapefruit; w: Biological indexing on WIL. Symptom severity: +++ (severe), ++ (moderate), + (mild) and - (none).
[v]CTV isolates were tested using restriction endonuclease analysis of amplified coat protein genes. *Hinf* 1 RFLP groups present in the field tree or indicator plant, defined by Gillings *et al.* (1993). RFLP groups in brackets are present as minor components of the strain complex. Since introgression of CTV strains is a dynamic process, the RFLP analysis and biological indexing were performed using tissue collected during the same time period.
*T 59 and 63 exhibit stem pitting in the field.

ambient temperature (15-35°C) in an insect proof glasshouse and symptoms were scored at 2-month intervals.

Aphid transmissions. Virus-free apterous *T. citricida* were fed on semi expanded leaves of inoculated sweet orange and then single or multiple aphids were transferred to receptor plants. Details of the method used have been previously published (38). Receptor sweet orange plants were tested for CTV at 8 weeks after inoculation using ELISA (2). Grafting wood of CTV-positive plants was used as inoculum for transmission of CTV to the indicator plants, previously listed, to assess strain severity. In each case, there were five graft inoculated

TABLE 2
LIST OF CTV ISOLATES OBTAINED BY BUD TRANSMISSION FROM FIELD TREES, OR BY SINGLE APHID OR MULTIPLE APHID TRANSMISSIONS

Isolate no.	Transmission[z]	Biological indexing[y]			RFLP groups[x]
		SSO	WIL	EUR	
Ortanique tangor					
PB 72	BT	OSP	+++	+++	5, 1, 3, 8, 9, 10
PB 92	1°SAT ex PB 72	-	+++	+++	5, 1, 3, 8, (10)
PB 95	1°SAT ex PB 72	-	+++	+	5, 1, 8
PB 94	1°SAT ex PB 72	-	+++	+++	1, (3), (10)
PB 93	1°SAT ex PB 72	-	+++	+	5,(3), (8), 10
PB 91	1°SAT ex PB 72	-	+++	+++	3, 10
PB 96	1°SAT ex PB 72	OSP	+++	+++	3, 10
PB 155	2°SAT ex PB 74	OSP	+++	+++	3
Benyenda navel					
PB 224	BT	OSP	+++	+++	5, 1, 3, 10, 11
PB 114	MAT ex PB 113	OSP	+++	+++	1, 3, 10
PB 135	MAT ex PB 113	nd	nd	nd	1, 3, 10
PB 131	MAT ex PB 113	OSP	+++	+++	1, 3, 10, 11
PB75	BT	OSP	+++	+++	5, 1, 3, 10, 11
PB 123	MAT ex PB 117	nd	+++	nd	1, 3, 10, 11
PB 118	MAT ex PB 117	OSP	+++	+++	1
PB 83	1°SAT ex PB 75	OSP	+++	-	1
PB 80	1°SAT ex PB 75	OSP	+++	+	1 (+)
PB 164	2°SAT ex PB 80	-	+++	+++	1
PB 167	2°SAT ex PB 80	-	+++	+	1 (+)
PB 192	2°SAT ex PB 86	OSP	+++	+++	1
PB 235	4°SAT ex PB 228	OSP	+++	-	1

[z]BT = Bud transmission; SAT = Single aphid transmission; MAT = Multiple aphid transmission
[y]Biological indicators used were seedlings of Symons sweet orange (SSO), West Indian lime (WIL) and Eureka lemon (EUR). OSP = severe orange stem pitting symptoms. The severity of the vein clearing and stunting reaction on WIL and the seedling yellows reaction on EUR are denoted by +.; nd = not determined.
[x]Isolates were analyzed using amplification of the CTV coat protein gene and digestion with *Hinf* 1. RFLP groups as defined in Table 1, (+) = other very faint components present in the restriction digest.

seedlings per pot, with one seedling left as a negative control.

Amplification and analysis of the CPG. DsRNA was extracted from field trees or indicator plants (listed in Table 1) using a CF 11 cellulose chromatography procedure (13). DsRNA was used as a template for synthesis of CPG cDNA using the primer CTV-CP3 (5' TCA ACG TGT GTT GAA TTT 3', corresponding to nucleotides 654 to 672 of the CPG of CTV strain T36, (35)). After reverse transcription, the forward primer CTV- CP1 (5' ATG GAC GAC GAA ACA AAG 3', corresponding to nucleotides 1 to 18 of the published sequence of CTV strain T36, (35)) was added and the cDNA was amplified in a standard PCR. The cDNA synthesis and amplification of the CTV CPG was performed using the Perkin Elmer Cetus Geneamp RNA PCR kit. Details of the reaction and thermal cycling conditions are given in Gillings et al. (13). The success of the amplification was monitored by analyzing an aliquot on 1.2% agarose gels cast and run in TBE buffer, pH 8.3 (34). Gels were run at 100 volts for 90 min and stained with ethidium bromide.

PCR products were separated from the overlaid paraffin oil by

extraction with chloroform and precipitated from the supernatant with PEG 6000 (13). Amplified CPGs were digested with *Hinf* 1 according to the manufacturer's instructions. Digests were analyzed by electrophoresis on 4% NuSieve 3:1 agarose gels (FMC) cast and run in TBE buffer (34). Gels were stained with ethidium bromide and photographed using transmitted UV light with an orange filter and Polaroid film.

Monitoring orange stem pitting isolates. In March 1990, orange stem pitting (OSP) symptoms were found in a block of 5 yr-old Washington navel orange on sweet orange stock in the Central Burnett area of Queensland. Shortly thereafter, similar symptoms were also found in Ortanique tangor (6). Before eradication could be attempted, means of identifying the causal strain needed to be developed. This in turn required isolation of the CTV strain(s) responsible for OSP from within the mixture of endemic CTV strains in the field trees. To isolate individual CTV strains from the field trees we used single aphid transmissions (SATs), and to assess the efficiency of the SATs we used analysis of the CPG in the receptor plants. Isolates which had been SAT were also indexed biologically.

RESULTS

Amplification of the CTV CPG. After reverse transcription and amplification (RT PCR), samples prepared from CTV infected plants produced a DNA fragment of approximately 670 base pairs (bp), indistinguishable from the known CPG size of 672 bp (35). Amplifications were successfully performed on dsRNA prepared both from all common indicator plants and directly from field trees. No amplification products were detected using samples prepared from uninfected seedlings or from reaction mixes to which no RNA was added. When amplified CPGs from field or bud-transmitted isolates were digested with the restriction enzyme *Hinf* 1, individual bands larger than 170 base pairs (bp) were found to be derived from a distinct CTV genotype (genotypic "strain") (13). Although there were other polymorphic bands within the patterns, those above 170 bp were the easiest to resolve, and simpler to analyze. The presence of multiple bands above 170 bp indicated there were multiple CTV strains in the original sample. The ratios and presence of these bands remained constant regardless of whether the analysis was performed on samples from the original field tree or from common indicator plants inoculated by bud transmission (Table 1) (13).

The pre-immunizing CTV strain used in Australia to cross protect Marsh grapefruit conforms to RFLP 5 (13), characterized by a single band of 428 bp above 170 bp (Fig. 1, tracks B and C). All apparently healthy grapefruits which index as carrying mild CTV isolates contain only the RFLP 5 strain. Isolates derived from aphid transmissions from apparently healthy, pre-immunized Marsh grapefruit all conform to RFLP 5 (13) and have identical biological and serological properties (4). All RFLP 5 strains which have been analyzed have very similar CPG sequences (Gillings et al. unpublished). The evidence from the CP sequences suggests that the pre-immunizing RFLP 5 strain is a single well-defined genotype of CTV. There may, of course, be diversity among the RFLP 5 strains that is only reflected in other genes within the CTV genome.

Other RFLP groups exhibit different band sizes in the 170 to 450 bp range. RFLP 1, a characteristically severe strain group, has a 386 bp fragment (Fig. 1, track J), while RFLP 3, another group of severe strains, has a 300 bp fragment (Fig. 1, track L). A group of mild strains

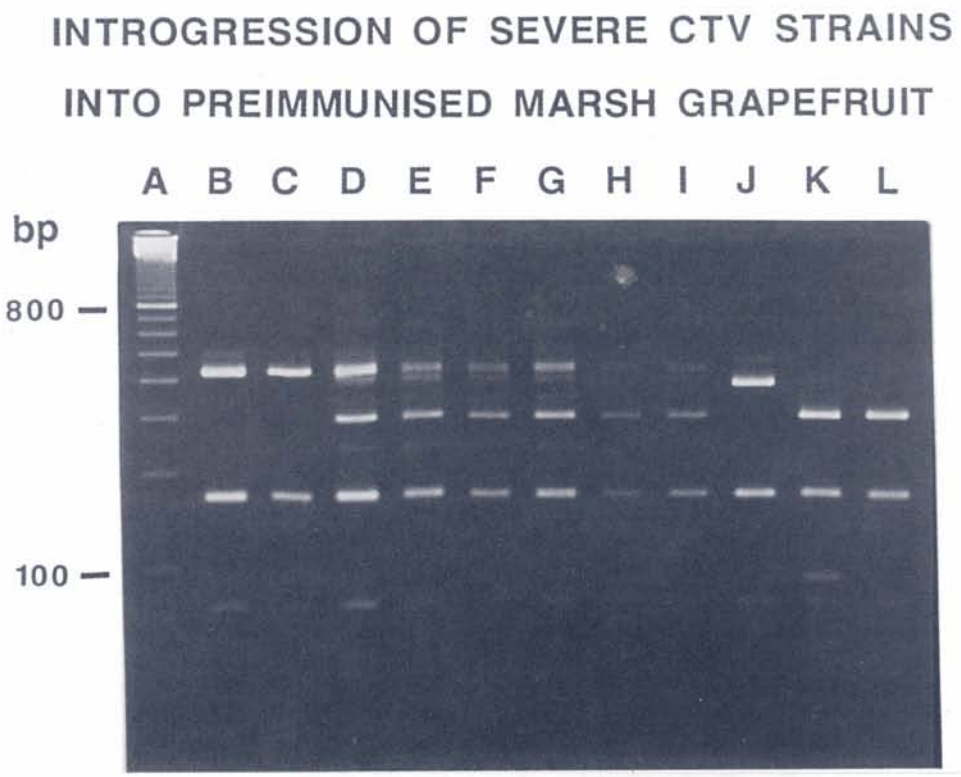

Fig. 1. Analysis of the introgression of CTV strains into Marsh grapefruit trees pre-immunized with CTV strain PB 61, the mild strain used for cross protection of grapefruit in Australia. PB 61 conforms to RFLP group 5. The CTV coat protein gene was reverse transcribed and amplified from field trees or indicator plants, digested with *Hinf* 1 and separated by electrophoresis on a 4% NuSieve 3:1 agarose gel. Samples were generated from the following trees: (B) Row 9 Tree 7 Budwood Block 2 (RFLP 5 only), (C) to (I) Trees from Row 28, Mother Tree Block 1; trees 22, 24, 20, 27, 63, 59, and 64 respectively. All trees exhibit additional RFLP groups except R28 T22 which still contains only RFLP 5. Tracks (A) and (J) to (L) are reference tracks; (A) 100 base pair ladder, (J) T66, severe Florida isolate; CTV-cp RFLP group 1; (K) T30, mild Florida isolate; CTV-cp RFLP group 4; (L) B31, severe grapefruit stem pitting, CTV-cp RFLP group 3.

found in Florida (RFLP 4) also contains a 300 bp band but also have an additional 91 bp band (Fig. 1, track K) (see 13).

Separation of restriction digests of the CPG can therefore be used to quickly identify multiple components of a CTV infection. To test the efficacy of this technique, we used it to trace the introgression of severe strains of CTV into blocks of Marsh grapefruit that had been pre-immunized with an RFLP 5 strain 28 years ago.

Monitoring the breakdown of mild strain cross protection. A mild CTV strain, whose CPG is now known to conform to RFLP 5, was used to pre-immunize grapefruit to provide cross-protection against severe strains (10, 7, 3). A budwood source block of 64 Marsh grapefruit trees (Acc. Nos 3970 and 3962, Row 28), pre-immunized with this protective isolate, is maintained at ARAS, Dareton and has been routinely indexed on WIL since 1986. No increase in strain severity, as assessed by biological indexing, was observed until 17 years after planting. At present, six trees show mild trunk pitting, and the majority of

trees exhibit moderate to severe vein clearing on West Indian lime indicators. However, there has, as yet, been no decline in tree vigor, size, and yield or changes to fruit morphology (5).

CTV isolates from this Marsh grapefruit block were processed for amplification and analysis of the CPG. At the same time, biological indexing was carried out on WIL. Apparently healthy trees which gave mild reactions on WIL indicators carried only RFLP 5 strains (Fig. 1, tracks B and C), while trees that indexed with severe or moderate reactions on WIL carried other RFLP groups, in particular RFLP groups 1 and 3 (Fig. 1, tracks D to I, Table 1). These RFLP groups are associated with severe symptoms on indicators and in the field (13). In two cases, trees containing multiple RFLP groups indexed mild on WIL. It is not known whether this was due to cross protection within the indicator plant, but it highlights the need for a combined approach (biological and molecular) to indexing of CTV, since either method alone may give an incomplete picture.

There were some other poorly characterized RFLP groups present in the Marsh field trees, as evidenced by faint bands at 250 and 320 bp (Fig. 1). There was also some evidence of proximity effects in the introgression of severe strains, for instance, a group of trees in the center of Row 28 (T22 to 30) still index mild, while both ends of the row carry moderate to severe strains (Table 1). In two cases (T59 and T63), RFLP 3 has come to dominate the profile of RFLP groups present in the field tree, and in one of these cases (T63) it is difficult to detect RFLP 5 at all (Table 1). Both T59 and T63 exhibit stem pitting in the field, suggesting that the RFLP 3 strain might be the cause of the stem pitting symptoms. One of the reference isolates, B31 (Fig. 1, track L), is known to cause grapefruit stem pitting and is also RFLP 3.

In 1985 sub-propagations of Marsh grapefruit 3970 were made from R28 T2 which, at that time, carried only the mild pre-immunizing strain. The sub-propagations now constitute budwood block 2, Row 9. The trees in this block were also tested at 8 to 9 years after planting. The majority of trees in this block still indexed mild and carried only RFLP 5 (Table 1, Fig. 1 track B). Four of the subpropagations tested exhibit additional RFLP groups. Again these additional groups conform to RFLP 1 and/or 3. Two of the four trees now index moderate on West Indian lime (Table 1). There may be some local foci of infection in this block, since trees carrying the introgressing RFLP groups 1 and 3 are clustered together (T19-T22, Table 1). The other trees in Row 9 carried only RFLP 5, and indexed mild on WIL (Table 1).

Monitoring aphid transmission of orange stem pitting isolates. Analysis of bud transmissions from an Ortanique tangor affected by the OSP isolate showed the presence of RFLP groups 5, 3 and 1 (bands of 428, 386 and 300 bp respectively) as well as a number of other potential RFLP groups generating bands of 450, 250 and 270 bp (Fig. 2, track B). For the purposes of this section, we have called the strains generating these bands RFLP groups 8, 9 and 10, although the strict proof that they are separate entities has not been made. SATs from the original strain mixture transmitted subsets of the strains present in the original bud inoculated indicator. None of the primary SATs transmitted only a single strain, as assessed by the recovery of a single RFLP group, although in each case fewer strains were present in the transmissions than in the donor indicator (Fig. 2 tracks C to H, Table 2). Many of the initial aphid transmissions did not elicit OSP symptoms on indicators. Those

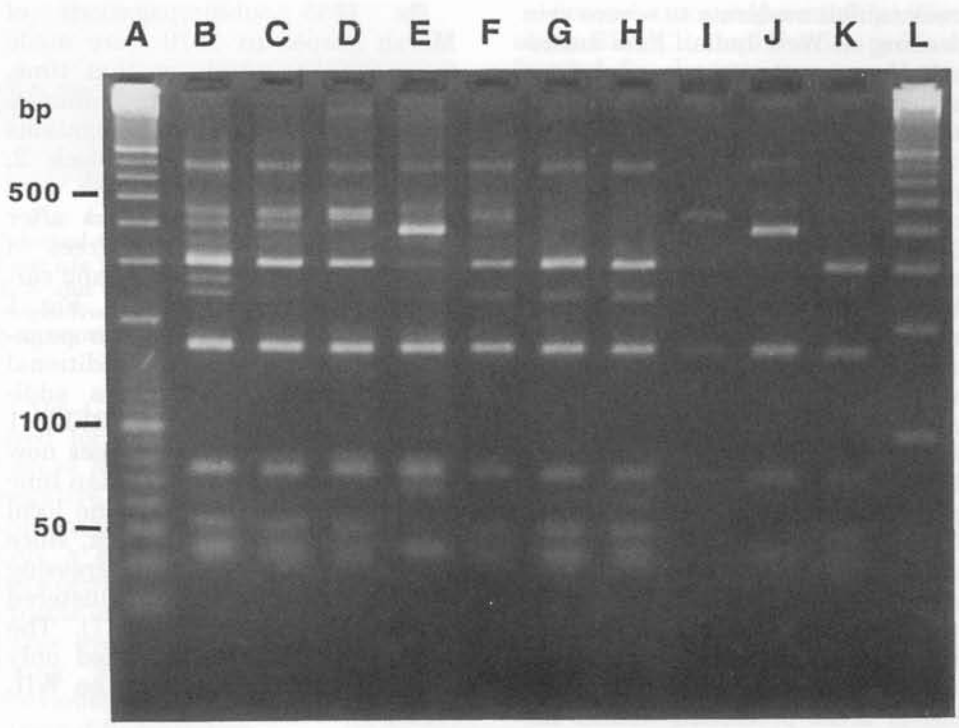

Fig. 2. Analysis of the effect of single aphid transmissions (SATs) on the composition of CTV strains in recipient plants. SATs were made from a CTV field isolate (Ortanique/Cleopatra mandarin). The CTV coat protein gene was reverse transcribed and amplified from the recipient plants, digested with *Hinf* 1 and separated by electrophoresis on a 4% NuSieve 3:1 agarose gel. Tracks are as follows: (B) PB 72, primary bud transmission from Ortanique/Cleo, and representative of the CTV strains present in the field tree, (C) to (H) PB 92, 95, 94, 93, 91 and 96 respectively, primary SATs from PB 72. Tracks (A) and (I) to (L) are reference tracks; (A) Mixture of 100 and 10 base pair ladders, (I) CTV-cp RFLP group 5: Marsh 3970 mild strain, (J) CTV-cp RFLP group 1: T66 severe strain, (K) CTV-cp RFLP group 3: B31 severe grapefruit stem pitting, (L) 100 base pair ladder.

transmissions that did elicit OSP symptoms all had high concentrations of RFLP 3, suggesting that the RFLP 3 group might be associated with the syndrome. The analysis was complicated by the fact that some transmissions did carry RFLP 3 strains, yet did not elicit OSP symptoms. It is possible that there are several distinct CTV genotypes that have CPGs conforming to RFLP 3, yet have critical differences in other genes associated with pathogenic effects. Nevertheless, a secondary SAT containing only RFLP 3 (PB155) induced OSP symptoms on indicators (Table 2). Virions purified from PB155 induced OSP when inoculated onto sweet orange, confirming the association of RFLP 3 with OSP in the Ortanique tangor.

Analysis of the RFLP groups present in Benyenda Washington navel orange trees exhibiting OSP symptoms also showed complex mixtures of strains were common in field trees. Bud transmissions from the single field tree (PB 224) exhibited bands characteristic of RFLP groups 5, 1, 3, 10 and a further presumptive RFLP group, 11, characterized by a band of 225 bp. Transmissions from another tree in the same block had similar RFLP groups, although RFLPs 5 and 11 were not present in some transmis-

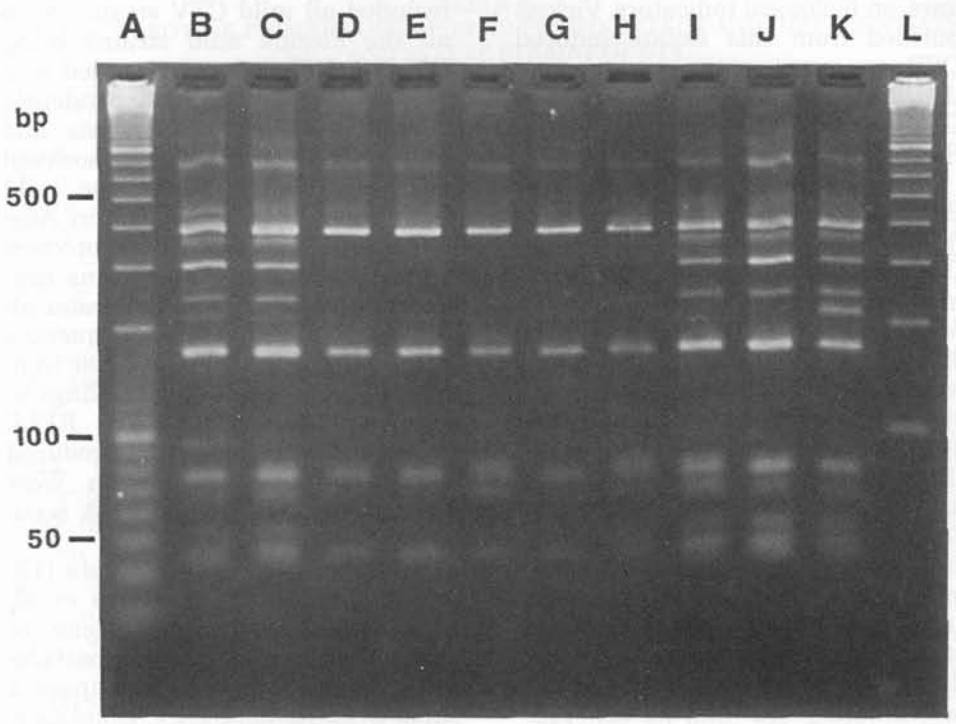

Fig. 3. Analysis of the effect of bud and aphid transmissions on the composition of CTV strains in recipient plants. Transmissions were made from CTV field isolates of Benyenda Washington navel. The CTV coat protein gene was reverse transcribed and amplified from the recipient plants, digested with *Hinf* 1 and separated by electrophoresis on a 4% NuSieve 3:1 agarose gel. Tracks are as follows: (B) PB 75, primary bud transmission from Benyenda navel, and representative of the CTV strains present in the field tree, (C) and (D) PB 123 and PB 118 respectively, are multiple aphid transmissions; (E) and (F) PB 83 and PB 80 respectively, are primary single aphid transmissions from PB 75; (G) and (H) PB 164 and PB 167 respectively, secondary aphid transmissions from PB 80; (I) to (K) PB 144, PB 135, and PB 131 respectively, multiple aphid transmissions from bud transmissions of PB 113. Tracks (A) and (L) are reference tracks; (A) Mixture of 100 and 10 base pair ladders, (L) 100 base pair ladder.

sions (Fig. 3, Table 2). The SAT transmissions from the original bud transmission of Benyenda navel all conformed to a single RFLP group, RFLP 1 (Fig. 3 tracks E to H). Even one of the multiple aphid transmission isolates exhibited only RFLP 1 (Fig. 3 track D, Table 2). Thus, in the transmissions from Benyenda navel one RFLP group dominated. All the transmissions in this experiment carried RFLP 1, and most induced OSP symptoms on sweet orange indicators (Table 2). Two SATs (PB 164 and 167) did not induce OSP. The RFLP 1 SATs also exhibited varying seedling yellows reactions when indexed on Eureka lemon (Table 2). This suggested that although all the SAT isolates conformed to RFLP 1, and were presumed to have similar CPGs, there was still genetic variability present, most probably in other genes within the CTV genome. However, the strong association of RFLP 1 with OSP symptoms suggested that an RFLP 1 strain was responsible for OSP in the original Benyenda navel. To further investigate this possibility, serial SATs were conducted. After four such transmissions, the resulting RFLP 1 isolate (PB 235) induced OSP, but not seedling yel-

lows, on biological indicators. Virions purified from this isolate induced OSP on sweet orange, supporting the view that an RFLP 1 strain of CTV was the cause of OSP in the Benyenda navel.

The symptoms elicited by the Benyenda OSP isolate were different from those elicited by the Ortanique OSP isolate, both in the field and in indicators. Admittedly, differences in field symptoms might be caused by differences in the host, but there were characteristic differences in the symptoms elicited by the two isolates in the sweet orange indicators. Ortanique isolates tended to exhibit fine honeycombed pits, while the Benyenda isolates had deep longitudinal pits. Clearly, the biological and molecular evidence show that there are at least two distinct CTV strains capable of causing OSP in Queensland, a result that could not have been predicted.

DISCUSSION

Citrus tristeza virus exists as a large number of distinct strains. Field isolates may be mixtures of strains, some of which may not express symptoms in either field trees or when graft inoculated to biological indicators (4). To fulfill the need for a rapid means of discriminating between strains, we have developed a method based on restriction analysis of the CTV CPG amplified using RT-PCR (13). Using this protocol, we amplified the CPG from representatives of the known biological variants of CTV held in the world wide collection of isolates at Beltsville (12, 13).

Analysis of the amplified CPG with restriction digests showed considerable polymorphism is present between different strains, and seven RFLP groups have been defined on this basis. Each of these RFLP groups contained CTV strains with similar biological properties (13). For instance RFLP groups 4 and 5 included all mild CTV strains, with all the Florida mild strains being RFLP 4. RFLP 4 corresponded to a group of strains independently defined by sequence analysis and failure to react with the monoclonal antibody MCA 13 (27). The mild strains found in grapefruit in Australia and used for cross protection were all RFLP 5 (13). Strains conforming to RFLP 5 in Australia all have very similar CPG sequences regardless of the time or area from which they are collected (Gillings et al. unpublished). Other RFLP groups contain strains that produced either severe symptoms on West Indian lime, with or without seedling yellows.

Although the biological data (13) and sequence data (Gillings et al. unpublished) show that some of these severe RFLP groups (particularly RFLPs 1 and 3) encompass a number of distinct CTV genotypes, the use of RFLP analysis is still a useful tool for investigating the strain composition in CTV field isolates.

The RFLP 5 strain used for cross-protection of grapefruit in Australia can be distinguished from all other Australian strains (13). We found field Marsh grapefruit trees that had been pre-immunized with an RFLP 5 strain up to 27 years ago in which little changes in strain severity had occurred at 17 years. However, by 25 years, some trees were indexing moderate or mild on West Indian lime, and two trees were showing mild stem pitting of the trunk (5). There was a strong correlation between increased strain severity and the presence of RFLP groups additional to RFLP 5. Field trees where RFLP 3 was a major component of the profile were those that exhibited stem pitting. However, sub-propagations made from a field tree in the Marsh block are now 8 years old and the majority of the trees still carry only the RFLP 5 strain, and index mild on WIL.

The RFLP analysis has allowed us to follow the introgression of severe strains into pre-immunized grapefruit trees. However, even under constant disease pressure from *T. citricida*, a number of the pre-immunized trees have remained free of severe strains for 27 years. Even where severe strains have established within field trees, symptoms of grapefruit stem pitting are slow to establish when RFLP 5 is present within the tree. The restriction analysis can identify trees containing severe strains long before symptoms might appear, and with greater speed and efficiency than biological indexing, and, hence, the health status of proposed budwood sources can be readily checked coincident with distribution.

Strain transmission using SATs depended on the field isolate used. In one case (Ortanique tangor), SATs did not generate pure strains, but, rather, transmitted a subset of the strains present in the original parental tree. A series of sequential aphid transmissions was required to generate "pure" strains as assessed by the presence of a single RFLP group. This observation has important consequences for efforts to produce serological or molecular markers for particular CTV strains. The first step in any such effort must be to obtain a pure (genotypically homogenous) strain. Aphid transmissions have been used in the past, but our results show that this approach may not always be successful.

In transmission studies using a second field isolate (Benyenda navel), a different problem was encountered. The majority of SATs from this isolate consisted of a single RFLP group, RFLP 1, even though many other distinct RFLP groups were present in the field tree. In a scheme where aphid transmissions are indexed in an attempt to understand the biology of a field syndrome, the presence of other CTV genotypes, poorly transmitted by aphids, may remain undetected. It may be these genotypes that are the cause for concern in the field.

CPGs are a logical first choice for RT-PCR analysis, because they are well described, and the subgenomic dsRNAs coding for the coat protein are likely to be at high concentration. However, in a number of cases, the biological data clearly show that there is genetic variation within CTV groups defined purely on the basis of RFLP analysis of the coat protein. The coat protein itself is probably not the cause of symptoms within the host, and RFLP analyses based on genes associated with pathogenicity may give better separation of symptom types. The sensitivity of such assays will be dependent on the degree of divergence between these genes. If large changes in pathogenic characters are caused by point mutations, development of diagnostic tests may be more difficult. Certainly, in preliminary tests conducted on two genes adjacent to the CPG (p18 and p27), less polymorphism was detected than that exhibited by the CPG (Gillings and Lee, unpublished).

At the present time, the coat protein method has potential for immediate application to a number of problems. These include monitoring the introgression of severe strains into new geographic regions or into trees that have been pre-immunized with cross-protective mild strains (3). The interaction between strains within individual trees could be analyzed directly, allowing more critical experiments on the mechanism(s) of mild strain cross protection to be performed.

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