Characterization of Citrus Tristeza Virus Isolates by Single-Strand Conformation Polymorphism Analysis of DNA Complementary to Their RNA Population

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ABSTRACT. Citrus tristeza virus (CTV) isolates, like other RNA viruses, contain a population of genomic variants, the composition of which may affect their biological characteristics. Analysis of single-strand conformation polymorphism (SSCP) is a simple technique that allows detection of minor variations in the nucleotide sequence of DNA fragments without sequencing. To assess if SSCP could be used to characterize the population of sequence variants present in CTV isolates, dsRNA from two isolates, differing by their SSCP profile of the gene p20, was combined at various ratios and the mixtures used as templates to synthesize cDNA by RT-PCR. The SSCP profile obtained from each dsRNA mix was a composite of the individual profiles of the two isolates, and the intensities of the cDNA bands reflected the relative proportion of each isolate in the dsRNA mix. The SSCP pattern of each component could be observed in the composite pattern when its dsRNA was in a ratio of at least 10%. Graft or slash-inoculation of CTV isolate T317 from citron to sweet orange, or from citrus to lime and then to sweet orange, caused changes in its SSCP profile, indicating host selection of the predominant sequence variants in the population. In some cases, this change was reversed when the isolate was back inoculated into the first host species, whereas in others the change was permanent. Variations in the viral population after change of host may help to explain biological diversity observed among CTV isolates.

Citrus tristeza virus (CTV), an aphid-transmitted closterovirus, is the causal agent of one of the most economically important diseases of citrus. CTV isolates differ widely in the range of symptoms induced in different citrus varieties (2, 4, 18) and the possibilities to control disease damage are highly dependent on the type of isolates predominant in each citrus area. Therefore, biological and molecular characterization of CTV isolates is of outmost importance for disease control, and recently various procedures have been developed for this purpose (10).

CTV virions are filamentous particles, ca. 2000 nm long and 11 nm diameter. Its genome is a single-stranded, positive-sense RNA molecule of 19,226 to 19,296 nucleotides (7, 9, 20). The RNA is encapsidated by two proteins of 25- and 27-kDa, coating about 95% and 5% of the particle length, respectively (6).

The nucleotide and the deduced amino acid sequences of various genes have been used to compare CTV isolates and cluster them in dendrograms (15, 17), but most times a unique or very few clones were sequenced from each isolate. CTV-infected citrus, like plants infected with other RNA viruses, do not contain a unique genomic sequence; rather, they have a population of variants usually clustered around one or more consensus sequences (1, 8). The composition of this population may determine, in part, the pathogenic characteristics of CTV isolates and, therefore, rapid characterization of genomic populations may provide some clues for identification of specific groups of isolates.

Sequencing multiple variants of one or more genes could be an accurate procedure to characterize the CTV populations, but it is too costly and time consuming for routine identification of isolates. Single-strand conformation polymorphism (SSCP) analysis is a quick and simple technique that allows discriminating between clones of the coat protein gene differing by a single nucleotide (19). Here, we evaluated

the sensitivity of SSCP analysis to detect minor sequence variants in an RNA population and changes occurred in genomic populations after host change.

MATERIALS AND METHODS

CTV isolates. The CTV isolates used in this study belong to the IVIA (Moncada, Spain) collection. Infected citrus plants were grown in an artificial potting mix (50% sand and 50% peat moss) in 25 l pots in a screenhouse.

T388 is a very severe isolate obtained from an early satsuma illegally imported from Japan (3). T21 and T317 are subisolates obtained from the mild isolate T385 when a Mexican lime, which had been recently aphid-inoculated with this isolate, was used as inoculum source to graft inoculate multiple citron plants (13). These isolates differed by their double-stranded RNA (dsRNA) profile, but both induced mild symptoms in Mexican lime. Isolate T317 was always maintained in Etrog citron. T318 was obtained by graft-transmission of T317 to sweet orange soon after obtaining T317, and T305 by graft transmission of T318 to Mexican lime and then to sweet orange. Contrasting with T317, these two isolates caused very severe symptoms in Mexican lime and citron, seedling yellows in sour orange, grapefruit or lemon, and stem pitting in sweet orange, rough lemon and other hosts. The dsRNA pattern of T305 differed that of T317 and T318 (11, 12). T317L was obtained by graft transmission of T317 to Mexican lime and then to sweet orange. Its dsRNA pattern was similar to that of T305, but T317L was asymptomatic in sweet orange or grapefruit and only caused mild symptoms in Mexican lime (12). Finally, isolates T317D and T317M were obtained various years later by graft and mechanical transmission, respectively, of T317 to sweet orange. Both of these isolates were asymptomatic in sweet orange and citron.

Complementary DNA preparation. Complementary DNA (cDNA) of the gene p20 was prepared by reverse transcription and amplification by the polymerase chain reaction (RT-PCR) using CTV dsRNA as template and the primers 5'ACAATATGCGAGCTTACTTTA3' (nucleotides 17,759 to 17,780 in the genomic RNA of T36 (16)) and 5'AACCTAACAGCAAGATGGA3' (nucleotides 18,316 to 18,297 in the same genomic RNA).

DsRNA was purified from infected sweet orange bark following a protocol previously established (14). The dsRNA equivalent to ca. 0.25 g fresh tissue was heat denatured at 95°C for 10 min, chilled on ice and immediately used for RT-PCR. This was performed in a single step using a 20 µl reaction volume containing 20 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 nM dNTPs, 1 µM of each primer, 0.2 U of AMV reverse transcriptase and 0.5 U of *Taq* DNA polymerase (Promega Corp.). RT was performed at 42°C for 45 min, and PCR included 30 cycles of 30 s at 94°C, 30 s at 36°C and 40 s at 72°C, and a final extension at 72°C for 2 min.

SSCP analysis. For SSCP analysis, 1 µl of the RT-PCR product was added to 9 µl of the denaturing mixture (95% formamide and 0.05% bromophenol blue), heated for 10 min at 95°C, chilled on ice, and quickly loaded on a non-denaturing polyacrylamide minigel (8% acrylamide) (19). Electrophoresis was carried out at 4°C, using 300 volts for 1.5 h or 200 V for 3 h. Gels were silver stained following the protocol of Beidler et al. (5).

RESULTS

RT-PCR using dsRNA of the different CTV isolates in all cases yielded a single DNA band of 558 bp. A critical question in character-

izing RNA populations by SSCP analysis of the cDNA, is how faithfully the DNA population reflects the natural RNA population. To determine if the main RNA variants had not been excluded by nucleotide mismatching of the primers during RT-PCR, probes were prepared by digoxigenin-labeling the RT-PCR product or the most frequent cDNA sequence variant (detected by SSCP analysis of at least 30 clones). When these probes were hybridized in dot blot with the homologous dsRNA, a strong hybridization signal was always observed.

To assess if SSCP analysis would allow an estimate of the relative concentration of different sequence variants in the RNA population of a CTV isolate, we used as templates for RT-PCR, mixtures with different ratios (e.g., 0, 1/10, 2/10, …, 1) of dsRNA from two CTV isolates with a readily identifiable SSCP pattern. The dsRNA concentration in both preparations was previously adjusted by comparing serial dilutions in a polyacrylamide gel stained with ethidium bromide. When the RT-PCR products of these mixtures were analyzed by SSCP (Fig. 1), the intensity of the DNA bands characteristic of each isolate was proportional to the concentration of the correspondent dsRNA in the template mixture. It was observed that the presence of 10% of dsRNA from one isolate in the mixture was enough to yield a detectable DNA band in the SSCP pattern.

When T317 and the group of subisolates obtained from it by different host changes (e.g., T317L, T317D, T317M, T318, and T305) were compared by SSCP analysis of the p20 gene, changes in the population of allelic variants of this gene were observed (Fig. 2). For example, T317 and T317M clearly differed by their SSCP pattern, which suggested that transmission from citron to sweet orange had changed the original population of T317. This was supported by the finding that graft transmission of T317M back to citron caused reversion to the SSCP pattern of T317. Analysis of T317D at 6-mo intervals after graft inoculation to sweet orange, showed a progressive evolution from the pattern characteristic of T317 in citron to that previously found in sweet orange. T317L in sweet orange showed a hybrid pattern that contained the bands characteristic of T317 in both citron and sweet orange. Finally, T318 and T305 in sweet orange had similar SSCP patterns, differing from those of the other isolates. The pattern of T318 remained unchanged when this isolate was graft inoculated to citron.

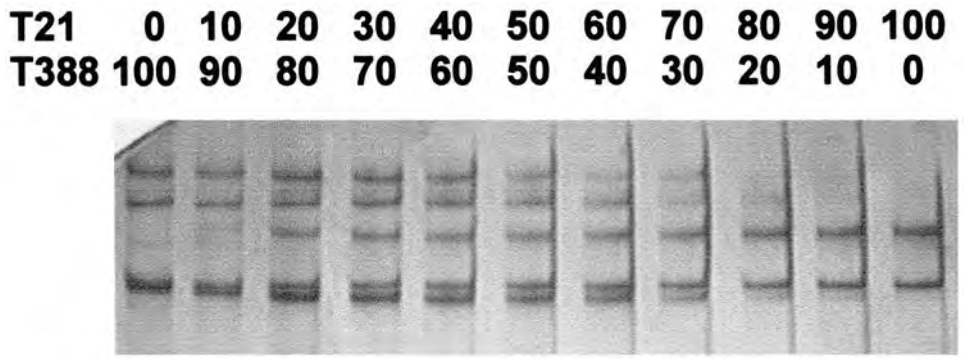

Fig. 1. Single-strand conformation polymorphism profiles of the gene p20 obtained analyzing the reverse transcriptase polymerase chain reaction product synthesized from a composite of dsRNA from two citrus tristeza virus isolates at different ratios.

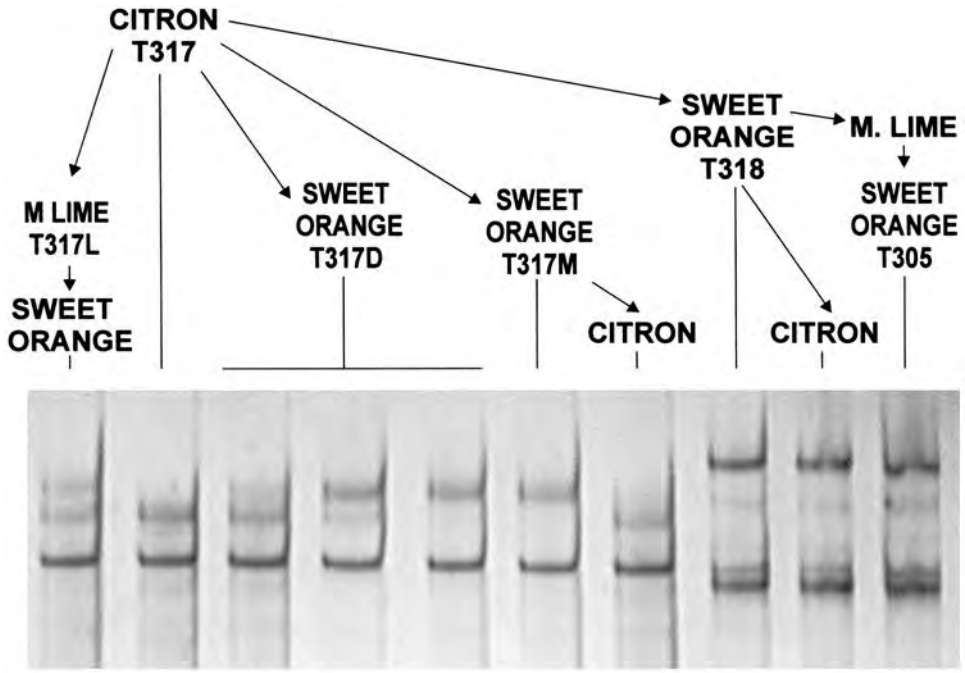

Fig. 2. Effect of host change on the citrus tristeza virus population as detected by single-strand conformation polymorphism analysis of the gene p20.

DISCUSSION

SSCP analysis is a simple and powerful tool to detect even single-nucleotide mutations in cloned DNA fragments, without any need for sequencing (19). Here, we found that this technique allows a quick characterization of the population of genomic variants in CTV isolates by directly analyzing the RT-PCR product of a selected genomic region.

The presence of different RNA variants in the template mixture apparently did not alter the efficiency at which each component was retro-transcribed and amplified. Thus, when different ratios of dsRNA from two CTV isolates were used as template for RT-PCR and the DNA synthesized was analyzed by SSCP, the intensity of the DNA bands characteristic of each RNA population were proportional to the concentration of the corresponding dsRNA in the mixture. This was an indication that the DNA population was a faithful reflection of the RNA population and that RNA variants representing only 10% of the total population could be readily detected in the SSCP pattern. Strong hybridization of each dsRNA extract with a probe prepared with the RT-PCR product or with a clone of the most abundant cDNA variant provided additional evidence that the major sequence variants in the RNA population were proportionately represented in the cDNA population. However, some of the minor RNA variants might have been excluded by primer mismatching during retro-transcription.

This new procedure to characterize CTV genomic RNA populations enabled us to detect variations in these populations induced by host change. Transmission of isolate T317 from citron to sweet orange (isolates T317L, T317D and T317M) caused the virus population to shift to a new predominant allelic variant of gene p20. This change, which seemed to

be gradual, as detected by periodical SSCP analysis of the T317D population, may be due to new constraints acting on the population which likely selected those allelic variants that were more efficient in the new host. The finding that transmission of T317D back to citron apparently reestablished the population of T317 supports this hypothesis. The final populations in T317D and T317M were indistinguishable, suggesting that graft or mechanical inoculation did not affect the allelic variants selected. Contrasting with these isolates, the SSCP pattern of T317L showed the major components detected in both citron and sweet orange. This might result from previous selection of certain allelic variants performed by Mexican lime.

The SSCP pattern of T318 (and that of T305) also contained the bands characteristic of T317 in sweet orange, but its major component was different, although this isolate was also obtained by transmission of T317 to sweet orange. As indicated above, this transmission was performed soon after obtaining T317, whereas T317M, T317D, and T317L were obtained at least four to six yr later. It is conceivable that transmission of T317 to obtain T318 was performed before its population was fully stable, which could give rise to a different virus population. The pathogenic characteristics of T318 (and T305) also differed dramatically from those of T317M, T317D or T317L.

The results presented here indicate that host change often induce changes in the CTV genomic RNA populations. This may explain, in part, the wide biological and molecular variability observed among isolates of this virus. They also show that SSCP analysis of the cDNA populations obtained by RT-PCR is a simple and powerful method to quickly characterize CTV populations and to monitor changes occurred under different circumstances.

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