Preliminary Molecular Characterization of Some *Citrus Tristeza Closterovirus* Isolates Infecting Croatian Citrus

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Abstract

Citrus tristeza Closterovirus (CTV) is widespread in major citrus-growing regions of the world often causing destructive diseases. Citrus samples were taken from orchards in the Croatian coastal region. CTV was detected in two symptomless field trees of Satsuma mandarins and one diseased lemon tree. Double-stranded RNA was isolated from the field trees and the dsRNA patterns were compared in polyacrylamide gels. The same dsRNA extracts were used as templates in RT-PCR experiments amplifying the CTV coat protein sequence. Amplicons were subjected to SSCP and RFLP analyses. The results indicate greater similarity between CTV isolates from Satsuma mandarins than between these two and the lemon isolate.

Keywords: *Citrus tristeza Closterovirus* (CTV); dsRNA; RFLP; RT-PCR; SSCP

INTRODUCTION

Citrus tristeza Closterovirus (CTV) is one of the most important pathogens of citrus. CTV virions are phloem-limited, exous filaments of 2000 × 11 nm in size (BAR-JOSEPH *et al.* 1979). With its monopartite, single-stranded, positive-sense RNA genome of 19.3 kb organized into 12 open reading frames (ORFs), CTV is considered to be the largest single-stranded RNA plant virus characterized so far. Infected plants contain relatively large amounts of double-stranded (ds) replicative form (RF) RNAs corresponding to the genomic and 9–10 subgenomic RNAs (KARASEV *et al.* 1997). Aphids naturally spread the virus but it is also easily transmitted by grafting. Depending on a virus strain, scion cultivar and rootstock, CTV can induce one of the three main syndromes: "quick decline", "stem pitting" and "seedling yellows". Mild CTV strains could even remain unnoticed in the field. Although biological indexing is necessary, using molecular techniques can save time in detection and differentiation of CTV isolates from field trees. DsRNA pattern comparison, reverse transcriptase polymerase chain reaction (RT-PCR), restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) analyses have been performed in this research in attempt to characterize 3 CTV isolates.

MATERIALS AND METHODS

Virus isolates

CTV isolates were obtained from three different orchards in Dalmatia, one of the Croatian coastal regions. The source trees were: two symptomless Satsuma mandarins (*Citrus unshiu* Marc. cv. Zorica Rana and cv. Ichimaru) and one lemon (*C. limon* L., type Lisbon) grafted on *Poncirus trifoliata* (L.) Raf. The lemon tree displayed rootstock bark shelling, severe yield reduction and decline.

dsRNA purification and analysis

Young bark, leaf midrib and petiole tissue from field trees (2 g) were used for dsRNA purification and analysis in 5% polyacrylamide gel (DODDS & JARUPAT 1991). Gels were silver-stained for greater sensitivity of detection.

cDNA synthesis and PCR amplification

Two-step RT-PCR was performed by using primers for the CTV coat protein (CP) gene (GILLINGS *et al.* 1993). The cycle profile was: 1', 94°C; 1', 94°C, 1', 40°C, 1', 72°C, 30 cycles; 5', 72°C. PCR products were analyzed by gel electrophoresis (1.2% agarose) and ethidium bromide staining.

Restriction analysis of the coat protein gene

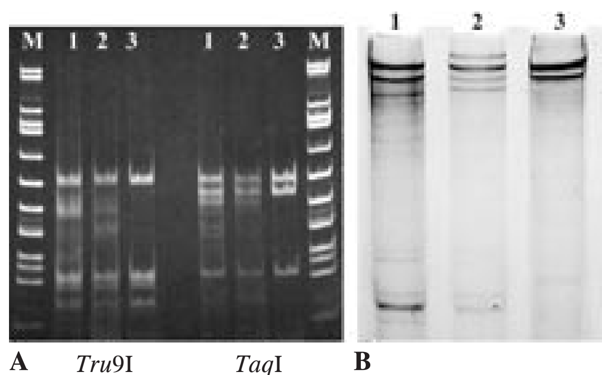
RFLP on the CTV-CP amplicons was performed separately using 4 enzymes: *Hinf*I, *Alu*I, *Tru*9I and *Taq*I. RFLP-patterns were compared in 5% polyacrylamide gel in TBE buffer (const. 10V/cm, 1.5 h).

Single-strand conformation polymorphism analysis

SSCP analysis (14% PAGE, TBE, const. 20 V/cm, 4 h, 4°C, silver staining) was performed on the resulting PCR products, as well (PALACIO & DURAN-VILA 1999).

RESULTS

All 3 extracts obtained directly from the field trees showed the presence of CTV-like dsRNAs. RT-PCR reactions templated by these dsRNAs gave strong amplification signals corresponding to the 670bp-long cDNA of *CTV-CP* gene. When these amplicons were separately digested with restriction enzymes, slightly different RFLP patterns were observed (Figure 1A). SSCP analysis (Figure 1B) also showed differences in the *CP* gene of CTV isolated from 2 different Satsuma mandarins and lemon.



A *Tru*9I *Taq*I B

M – molecular weight marker VIII (Roche); 1 – *Citrus unshiu*, cv. Zorica Rana; 2 – *C. unshiu*, cv. Ichimaru; 3 – *C. limon*, type Lisbon

Figure 1. A – Electrophoretic patterns generated upon digestion of the amplified CTV coat protein (CP) gene with *Tru*9I and *Taq*I restriction enzymes. B – SSCP patterns of *CTV-CP* gene

DISCUSSION

CTV exists as a large number of strains some of which may not express severe symptoms especially if the rootstock is *P. trifoliata*. *Tristeza* has the most devastating effects on sweet orange and mandarine varieties grafted on sour orange. The first report of CTV in ex Yugoslavia (presumably Croatia) was by Italian authors (DAVINO & CATARA 1988) who detected CTV in Satsuma mandarine budsticks. The material had been introduced to Croatian nurseries in 1980's from Japan (ŠARIĆ & DULIĆ 1990). Most of the Croatian Satsuma trees stem from this material, over 90% is grafted on *P. trifoliata* and show no symptoms which is also the case with the two mandarins included in this study. However, dsRNA and amplification of *CTV-CP* gene clearly indicated the presence of CTV in both Satsuma trees. The severely diseased lemon from this study also harbored CTV but the symptoms were more indicative of *Citrus exocortis viroid* presence which, together with several other citrus viroids, had indeed been identified (ŠKORIĆ *et al.* 2001). RFLP patterns of CTV coat protein gene showed differences between mandarins and lemon sample which, in case of *Hinf*I digests, were similar to those of strains T30 and PB14, respectively (GILLINGS *et al.* 1993). SSCP corroborates the existance of different strains in Satsumas and lemon, but also indicates certain differences between the two Satsuma isolates. Further molecular and biological assays should enable classification of these CTV strains.

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