ABSTRACT

Citrus tristeza virus (CTV) is the causal agent of the most devastating citrus disease worldwide. It is a viral species of the Closterovirus genus, having a ~20 kb single-stranded and positive sense genomic RNA (gRNA) organized into 12 open reading frames (ORFs) potentially coding 17 protein products, some of which of unknown function. CTV proteins p25 (CP) and p27 (CPm) belong to a gene block involved in virion assembly. It has been demonstrated that p25, along with p20 and p23, are RNA silencing suppressors in some species of *Nicotiana*, being the latter a pathogenesis determinant. CTV host range is very restricted and in nature viral infections are limited to the phloem cells of some citrus species. CTV molecular and biological complexity makes very difficult the study of pathogenic determinants and/or the underlying mechanisms responsible for the most economically important syndromes. Consequently, working with this virus-host pathosystem requires an appropriate experimental host and an efficient CTV genetic system. In our laboratory we have developed a new CTV genetic system based on the agroinfection of *Nicotiana benthamiana* (*N. benthamiana*) plants, a non-natural host, with infectious clones of the T36 isolate. That infection induces characteristic symptoms, some of which correlate with those caused by the virus in citrus plants. Thus, in the present study we have used this pathosystem to achieve two main objectives: i) a preliminary analysis of the virus-host interactions trying to infer the function of some viral proteins, and ii) an evolutionary and adaptation study of CTV to this non-natural herbaceous host.

CTV-*N. benthamiana* interactions are very variable and genotype-dependent, so only some isolates can replicate in this species and T36 is unique causing systemic infections. The differential response of CTV genotypes in this host would allow the analysis of virus-host interaction factors before its latter approach in citrus species. In this work, we studied the function of CTV p20 and p25 proteins from three different isolates differing in its pathogenic characteristics. Transient expression of p20 and p25 fused to fluorescent proteins and subsequent confocal microscopy analysis, showed an identical subcellular localization for both in *N. benthamiana* and citrus. The p20 protein of T36, T318A and T385 isolates localized in cytoplasm and nucleus forming amorphous aggregates associated with perinuclear regions, together with nuclear punctuate inclusions. Moreover, its co-expression with the nucleolar fibrillarin marker, showed a potential p20-fibrillarin interaction and a re-localization of the latter to membrane-associated regions. This localization would be important for p20 to display its pathogenic and suppressor activities.

On the other hand, transient and *in vivo* expression via a heterologous viral infection, showed a differential p25 subcellular localization dependent on the CTV isolate nature. While p25 of T36 and T385 localized in the nucleus, that of T318A did it in cytoplasm. A detailed analysis of the protein regions involved in this subcellular localization unveiled a leucine-rich nuclear export signal (NES) in the N-terminus (Nt) of the protein, in which aminoacid position 31 (where severe isolates, as T318A, have a leucine and milder ones, as T36 and T385, a valine) is essential for the nuclear export. In fact, an infectious T36 clone containing that mutation in its p25 was unable to infect systemically *N. benthamiana* plants. Additional residues in the proximal Nt region of the protein, together with a group

of hydrophobic aminoacids comprising positions 64-74, may also be involved in the regulation of p25 localization.

The pathogenic ability of CTV p20 and p25 in *N. benthamiana* was tested through a PVX heterologous viral infection, showing that p25 was not a pathogenicity determinant in this species, albeit p20 it was. The expression of the latter increased PVX symptoms and necrosis as p23 does, determining the greater pathogenic ability of p20-T36 regarding that of p20-T318A. On the other hand, we have determined that p25 is a weak long distance silencing suppressor in *N. benthamiana*, with p25 of T36 displaying a better suppressor activity than that of the T318A and T385 isolates.

Besides, the interactome of the p25 proteins from isolates T36 and T318A with proteins of *N. benthamiana* was obtained through its transient expression fused to the Strep-*tag* II, follow by purification of plant protein-protein complexes and proteomic analysis. The p25-T36 interactome was more complex and diverse than that of p25-T318A, involving a greater number of metabolic processes, redox activities, homeostasis and cellular transport, biosynthesis and protein degradation, plastid and nucleic acid protein binding, biotic and oxidative stress, jasmonic-mediated defense, methylation, ROS signalling and HSP proteins. On the other hand, most p25-T318A potential interactors were related to transport/localization and stress response, like apoptosis, pathogenesis-related and HSP proteins, Ca^{+2}-binding proteins and redoxins. Both p25 would share a great number of interactors related to photosystems I and II components, cytoskeleton proteins like actins and tubulins, co-chaperones and translation elongation factors eEF1. The p25-T36 showed potential differential interactors with actin depolymerizing factors, translation elongation factors, ATP synthase subunits, aquaporins, and nuclear transcription and proteasome regulation factors. Most of them have been reported to be required for the infection ability of other plant viruses, and could explain, in part, the differential behaviour-response of CTV T36 and T318A isolates in this species.

Regarding the second objective, the experimental evolution of CTV in *N. bethamiana* was achieved through serial passages by graft inoculation and suppressor pre-treatment of plants. The evolution process showed adaptative traits as the increase of graft survival, infectivity rates and viral titers, and a decreased time for the onset of symptom development in this species. The increase in the biological fitness of these evolved virus populations led to the ability of CTV to infect healthy *N. benthamiana* plants by mechanical inoculation without any pre-treatment. Increase in systemic infectivity rates correlated with the number of passages. The adaptation to *N. benthamiana* was also reflected in: i) the detection of higher levels of siRNAs derived from CTV infection in that plants as long as serial passages increase and ii) an enrichment in smaller siRNAs in the profile, similar to what it is observed in natural infections of CTV in citrus.

Adaptive characteristics of evolved viral populations in *N. bethamiana,* were also correlated to their genetic variability and population structure. Two different evolved lineages showed convergent evolution processes reflected also at the molecular level. Along the serial passaging, several mutations accumulated in a specific region of the polyprotein and in the *p23* gene in the consensus sequences of the viral populations. Most of them appeared in P6 and got fixed in both population lineages in a convergent way, with three of them in the polyprotein and five in the *p23* gene, thus suggesting its adaptive character. Also,

in the most evolved populations, de novo single mutations were found in *RdRp*, *p33*, *p61*, *p18* and *p20* genes, and in CTV's untranslated regions.

Phylogenies of evolved populations in *N. benthamiana* displayed its evolutionary history and convergent events. Viral quasispecies structure of *p23* followed the "quasispecies memory" concept, in which the ancestral T36 sequence was maintained as a minor genome in the viral populations along evolution. Two genetic positions in the polyprotein and three in the *p23* gene were detected to be under negative selection pressure, while the other substitutions would follow a purifying selection.

CTV viruses evolved in *N. benthamiana* were found to be less infectious at initial stages of infection when they were back-inoculated to citrus plants, and showed a decreased viral titer during the first year post-inoculation. Viruses from P11 re-adapted faster to citrus, recovering its infectious ability and viral titer during the following two years. This re-adaptation of *N. benthamiana* viral populations back to citrus was correlated, at a molecular level, with the progressive loss of the mutations appeared during its evolution process in *N. benthamiana*. In fact, three years after citrus back-inoculations, the complete full-length consensus sequences of evolved lineages completely reverted to that of the T36 wild-type, which is the better adapted with highest fitness in the natural host.

Biological traits of *N. benthamiana* evolved viruses were also tested when back-inoculated to citrus, showing that the ability of P5 viruses to infect *C. macrophylla*, Mexican lime and sour orange was worse than the ancestral T36 wild-type virus. Conversely, the most evolved P11 viral populations were less pathogenic in resistant hosts like sour orange and grapefruit, thus inducing milder symptoms of seedlings yellows and stunting. Results presented here indicates that CTV evolution and adaptation to *N. benthamiana* species, would have an adaptative cost in the original citrus host, since a loss of infectious ability and pathogenesis induction has been observed in some citrus species.