Post-transcriptional gene silencing of the p23 silencing suppressor of *Citrus tristeza virus* confers resistance to the virus in transgenic Mexican lime

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**Abstract**

Previously, we have shown that most Mexican limes (*Citrus aurantifolia* (Christ.) Swing.) expressing the p23 gene of *Citrus tristeza virus* (CTV) exhibit aberrations resembling viral leaf symptoms. Here we report that five independent transgenic lines having normal phenotype displayed characteristics typical of post-transcriptional gene silencing (PTGS): multiple copies of the transgene, low levels of the corresponding mRNA, methylation of the silenced transgene, and accumulation of p23-specific small interfering RNAs (siRNAs). When graft- or aphid-inoculated with CTV, some propagations of these silenced lines were immune: they neither expressed symptoms nor accumulated virions and viral RNA as estimated by DAS-ELISA and Northern blot hybridization, respectively. Other propagations were moderately resistant because they became infected later and showed attenuated symptoms compared to controls. The susceptible propagations, in addition to symptom expression and elevated virus titer, accumulated p23-specific siRNAs at levels significantly higher than immune or non-inoculated propagations, and showed transgene demethylation. This variable response among clonal transformants indicates that factors other than the genetic background of the transgenic plants play a key role in PTGS-mediated resistance.

**Introduction**

Citrus (namely species and hybrids of genera *Citrus, Fortunella*, and *Poncirus*) are the most important fruit tree crop in the world, with a cultivated surface of more than 7 million Ha distributed in about 100 countries, mainly in tropical and subtropical areas, and a fruit production of more than 100 million tons per year (FAO, 2005). *Citrus tristeza virus* (CTV) causes the most destructive viral disease and is considered a major threat for the citrus industry worldwide (Bar-Joseph et al., 1989). CTV produces decline and death of scion varieties, except lemons (*Citrus limon* (L.) Burm.), grafted on sour orange (*C. aurantium* (L.)). More than 85 millions trees grafted on this rootstock have been destroyed along the last 70 years in Argentina, Brazil, California, Florida, Israel, Venezuela and Spain, and the disease continues to spread into new areas, either by propagation of infected buds or by different aphid species, mainly *Aphis gossypii* and...
Toxoptera citricida. This has led to a progressive substitution of sour orange by CTV-tolerant rootstocks. However, tree performance on these rootstocks is often worse than on sour orange. Additionally, severe CTV strains cause stem pitting, stunting, low yield and poor fruit quality of some varieties of sweet orange (C. sinensis (L.) Osb.), limes (C. aurantifolia (Christm.) Swing.; C. latifolia Tan.) and grapefruits (C. paradisi Macf.), regardless the rootstock used (Roistacher and Moreno, 1991). This is a largely unsolved problem in most citrus areas. In some countries, cross-protection with mild CTV strains is being used to avoid the effects of severe stem pitting strains (Costa and Müller, 1980; Van Vuuren et al., 1993).

CTV has a plus-strand RNA genome of ~20 kb organized in 12 open reading frames (ORFs) and two 5′ and 3′ untranslated regions (UTRs) (Karasev et al., 1995). The 5′ half of the genome encompasses two ORFs encoding proteins associated with viral replication that are expressed from the genomic RNA. The ten 3′ proximal ORFs are expressed via 3′ co-terminal subgenomic RNAs (Hilf et al., 1995; Ayllón et al., 2003) and they encode the minor and major coat proteins (CPs) of 27 and 25 kDa (p27 and p25), respectively, and several other proteins (p33, p6, p65, p61, p18, p13, p20 and p23) (Pappu et al., 1994; Karasev et al., 1995). Both CPs, together with p65 and p61, are involved in virion assembly (Satyanarayana et al., 2000). Additionally, p27 has been shown to initiate encapsidation of the genomic RNA from its 5′ end (Satyanarayana et al., 2004). Protein p20 accumulates in amorphous inclusion bodies of CTV-infected cells (Gowda et al., 2000). Protein p23, which has no homologue in other closteroviruses, binds in vitro to RNA in a non-sequence-specific manner (López et al., 2000), and it is involved in regulating the balance of plus and minus RNA strands during replication (Satyanarayana et al., 2002). Recently, p23, p20 and p25 have been found to act as RNA silencing suppressors in Nicotiana tabacum and N. benthamiana plants (Lu et al., 2004). The small hydrophobic p6 may operate as a membrane anchor (Satyanarayana et al., 2000), with the function of p33, p13 and p18 remaining unknown.

We have previously reported that ectopic expression of the p23 gene from mild or severe CTV strains in Mexican lime (Citrus aurantifolia (Christm.) Swing.) induces aberrations resembling viral leaf symptoms, the intensity of which is independent on the pathogenicity of the CTV strain but correlates with p23 accumulation. Transformation with p23 of other CTV-susceptible and -resistant citrus genotypes (sweet orange, sour orange, and trifoliate orange) also leads to CTV-like symptoms that correlate with the levels of p23 transcripts, although p23 is barely detectable in these hosts. In contrast, transgenic expression of p23 in CTV non-host N. tabacum and N. benthamiana does not induce any phenotypic aberration albeit they consistently accumulate p23. Altogether, these results indicate that p23 is an important CTV pathogenicity determinant that interferes with plant development specifically in citrus species and relatives (Ghorbel et al., 2001; Fagoaga et al., 2005).

In the course of these experiments, 3 out of 60 lines of Mexican lime carrying the p23 transgene of the severe CTV isolate T36, and 2 out of 20 lines carrying the p23 transgene of the mild isolate T317, were visually normal and developed similarly to controls transformed with the empty vector or non-transformed plants. Here we report that the p23 transgene was silenced in these five lines and that bud propagations thereof, challenged with CTV by grafting or aphid feeding, showed no symptoms, undetectable levels of viral RNA and virions, and accumulation of the viral-specific small interfering RNAs (siRNAs) associated with post-transcriptional gene silencing (PTGS).

Materials and methods

Generation of transgenic Mexican lime plants

Transgenic Mexican limes carrying the p23 gene from CTV isolates T36 or T317 were generated as described previously (Ghorbel et al., 2001; Fagoaga et al., 2005). The transgene was cloned in the binary plasmid pBin19-sgfp under the control of a modified Cauliflower mosaic virus (CaMV) 35S promoter containing a duplicated enhancer region and the nopaline synthase terminator (nos-ter). The p23 expression cassette was flanked by the selectable markers neomycin phosphotransferase II gene (nptII), between the nos promoter (nos-pro) and the nos-ter, and synthetic green fluorescent protein gene (sgfp) (Chiu et al., 1996), between the 35S