2010

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The pathogenicity determinant of *Citrus tristeza virus* causing the seedling yellows syndrome maps at the 3′-terminal region of the viral genome

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SUMMARY

Citrus tristeza virus (CTV) (genus *Closterovirus*, family *Closteroviridae*) causes some of the more important viral diseases of citrus worldwide. The ability to map disease-inducing determinants of CTV is needed to develop better diagnostic and disease control procedures. A distinctive phenotype of some isolates of CTV is the ability to induce seedling yellows (SY) in sour orange, lemon and grapefruit seedlings. In Florida, the decline isolate of CTV, T36, induces SY, whereas a widely distributed mild isolate, T30, does not. To delimit the viral sequences associated with the SY syndrome, we created a number of T36/T30 hybrids by substituting T30 sequences into different regions of the 3′ half of the genome of an infectious cDNA of T36. Eleven T36/T30 hybrids replicated in *Nicotiana benthamiana* protoplasts. Five of these hybrids formed viable virions that were mechanically transmitted to *Citrus macrophylla*, a permissive host for CTV. All induced systemic infections, similar to that of the parental T36 clone. Tissues from these *C. macrophylla* source plants were then used to graft inoculate sour orange and grapefruit seedlings. Inoculation with three of the T30/T36 hybrid constructs induced SY symptoms identical to those of T36; however, two hybrids with T30 substitutions in the p23-3′ nontranslated region (NTR) (nucleotides 18 394–19 296) failed to induce SY. Sour orange seedlings infected with a recombinant non-SY p23-3′ NTR hybrid also remained symptomless when challenged with the parental virus (T36), demonstrating the potential feasibility of using engineered constructs of CTV to mitigate disease.

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INTRODUCTION

The 2000-nm-long filamentous virions of *Citrus tristeza virus* (CTV) (genus *Closterovirus*, family *Closteroviridae*) contain a single-stranded, positive-sense RNA genome of 19.3 kb consisting of 12 open reading frames (ORFs). ORF 1 makes up approximately the 5′ half of the genome (Karasev *et al*., 1995). ORF 1a encodes two papain-like protease domains plus type I RNA methyltransferase-like and RNA helicase-like domains. Translation occasionally continues by a +1 frameshift through the RNA-dependent RNA polymerase-like domain of ORF 1b. The 10 3′ proximal CTV ORFs are translated from 10 overlapping 3′-coterminal subgenomic mRNAs (sgRNAs), which differ by their time course of synthesis during cell infection and their accumulation levels (Hilf *et al*., 1995; Navas-Castillo *et al*., 1997). The characteristic *Closteroviridae* 'quintuple gene module' (Dolja *et al*., 2006) is composed of ORFs p6, HSP70h (homologue of heat shock protein 70), p61 and the minor (CPm) and major (CP) coat proteins (Satyanarayana *et al*., 2000, 2004). The p20 protein is a major component of characteristic viral amorphous inclusion bodies (Gowda *et al*., 2000) and the p23 protein has a zinc finger domain with the capacity to bind RNA (López *et al*., 2000) and regulates the asymmetrical accumulation of positive to negative strands of both genomic and sgRNA during CTV replication (Satyanarayana *et al*., 2002b). The CP, p20 and p23 proteins are suppressors of viral RNA silencing in *Nicotiana benthamiana* plants. Protein p23 inhibits intracellular silencing, CP suppresses intercellular silencing and p20 suppresses both (Lu *et al*., 2004). The p33, p18 and p13 genes are involved in infection and movement in some hosts (Tatineni *et al*., 2008).

Phenotypically, CTV is a complex virus, with a myriad of different symptom combinations amongst a range of different citrus genotypes (Bar-Joseph and Dawson, 2008; Moreno *et al*., 2008). Although different sequence-related groups have been

identified (Hilf *et al.*, 2005; Sambade *et al.*, 2003), which we now recognize as strains, individual isolates within a strain often have the full range of phenotypes. In addition, CTV populations normally have a mixture of different sequence variants, recombinants and defective RNAs (D-RNAs) that modulate symptom expression (Albiach-Martí *et al.*, 2000b; Mawassi *et al.*, 1995; Vives *et al.*, 2005; Weng *et al.*, 2007).

CTV has caused or threatens to cause serious economic damage to all citrus industries. Depending on the virus isolate and the variety/rootstock combination, CTV can cause severe economic losses as a result of 'decline' or 'stem pitting'. Decline results in the death of sweet orange [*Citrus sinensis* (L.) Osb.], mandarin [*C. reticulata* Blanco] or grapefruit [*C. paradisi* Macf.] varieties grafted on sour orange [*C. aurantium* (L.)] rootstocks as a result of a virus-induced bud union incompatibility. During the last century, CTV-induced decline destroyed entire citrus industries, leading to the substitution of the popular sour orange rootstock with other rootstocks that are tolerant to decline, but that are susceptible to other pathogens or show a worse horticultural performance. Stem pitting results from abnormal vascular differentiation and development and, when severe, substantially reduces the vigour and yield of lime, sweet orange and grapefruit trees (Bar-Joseph and Dawson, 2008; Moreno *et al.*, 2008). In contrast with decline, stem pitting diseases caused by CTV affect citrus cultivars regardless of the rootstock used, and often limit the choice of varieties that can be grown commercially. A third syndrome caused by some CTV isolates is 'seedling yellows' (SY). SY is characterized by stunting, leaf chlorosis and, sometimes, complete cessation of growth with small yellow leaves, when sour orange, grapefruit or lemon [*C. limon* (L.) Burn. f.] seedlings become infected (Fraser, 1952; McClean, 1960). The physiological mechanism associated with the induction of SY symptoms is not understood. The SY reaction may sometimes be transient (Wallace and Drake, 1972) and, after a few months, trees may resume normal growth. Although SY is not important economically, it can be assayed in the glasshouse much more easily than can decline and stem pitting. Some CTV strains are symptomless or very mild in almost all varieties, including those propagated on sour orange rootstocks, even though the virus multiplies to high titres. These mild isolates are common in Florida and many other citrus growing areas, although their presence is frequently masked when they are present in mixed infections with more severe isolates (Bar-Joseph and Dawson, 2008; Moreno *et al.*, 2008).

The control of CTV diseases constitutes a continuous challenge. Although a few commercial citrus industries are free of CTV, many contain various levels of infection by indigenous virus isolates that are either relatively mild or so limited in distribution that they do not prevent economic production. However, these areas are continually threatened by the possible introduction of more virulent exotic CTV isolates (Roistacher and Moreno,

1991). In other areas, stem pitting isolates are endemic and chronically limit the profitable growth of different varieties, making cross-protection necessary. Mild strain cross-protection consists of the deliberate inoculation of trees with a mild isolate of CTV that prevents or reduces disease caused by a more virulent isolate (Fraser, 1998). This strategy has been used in South America, South Africa and Australia (Broadbent *et al.*, 1991; Costa and Müller, 1980; Van Vuuren *et al.*, 1993). However, the protection of sweet orange trees grafted onto the sour orange rootstock against decline and death has not been effective and remains an important goal, because of the superior agronomic qualities of the sour orange rootstock (Bar-Joseph *et al.*, 1989). To reduce the risks derived from the introduction and dispersal of virulent isolates, and to properly monitor cross-protection, methods are needed to rapidly discriminate virulent from mild isolates. Many attempts have been made to develop rapid diagnostics for specific CTV syndromes. Although some correlations have been established between various serological and molecular markers (Hilf *et al.*, 2005; Pappu *et al.*, 1993; Sambade *et al.*, 2003), direct linkage of these markers to symptoms has not been established. Thus, there is considerable interest in the mapping of disease determinants of CTV to develop methods which would specifically discriminate between severe, disease-causing isolates and mild or symptomless isolates, and promote the development of molecular-based strategies to control CTV diseases. The development of methods to construct an infectious clone of CTV and to re-establish this in citrus (Satyanarayana *et al.*, 1999, 2001) has created new opportunities for the mapping of disease determinants.

In this article, we report the first mapping of a disease determinant of CTV, the determinant associated with the induction of SY in sour orange and grapefruit, by creating a series of recombinant hybrids of T36, an SY-inducing strain, and T30, which is symptomless. Substitution of the p23 gene and the 3′ nontranslated region (NTR) from T30 into an infectious clone of T36 resulted in a hybrid virus that not only did not induce SY, but whose presence effectively protected citrus seedlings from SY when challenged by the parent T36 virus.

RESULTS

Generation of the T36/T30 hybrid virus constructs

The recombinant virus pCTV9, which was obtained from an infectious cDNA clone of the type strain of T36 (Satyanarayana *et al.*, 1999, 2003), and the original wild-type T36 isolate produce identical SY symptoms in sour orange and grapefruit seedlings (Satyanarayana *et al.*, 2001). T30, the type isolate of the widely distributed mild strain (T30 strain), does not induce SY and consists of one genotype and its quasispecies (Albiach-Martí *et al.*, 2000c). The sequences of T30 and T36 are
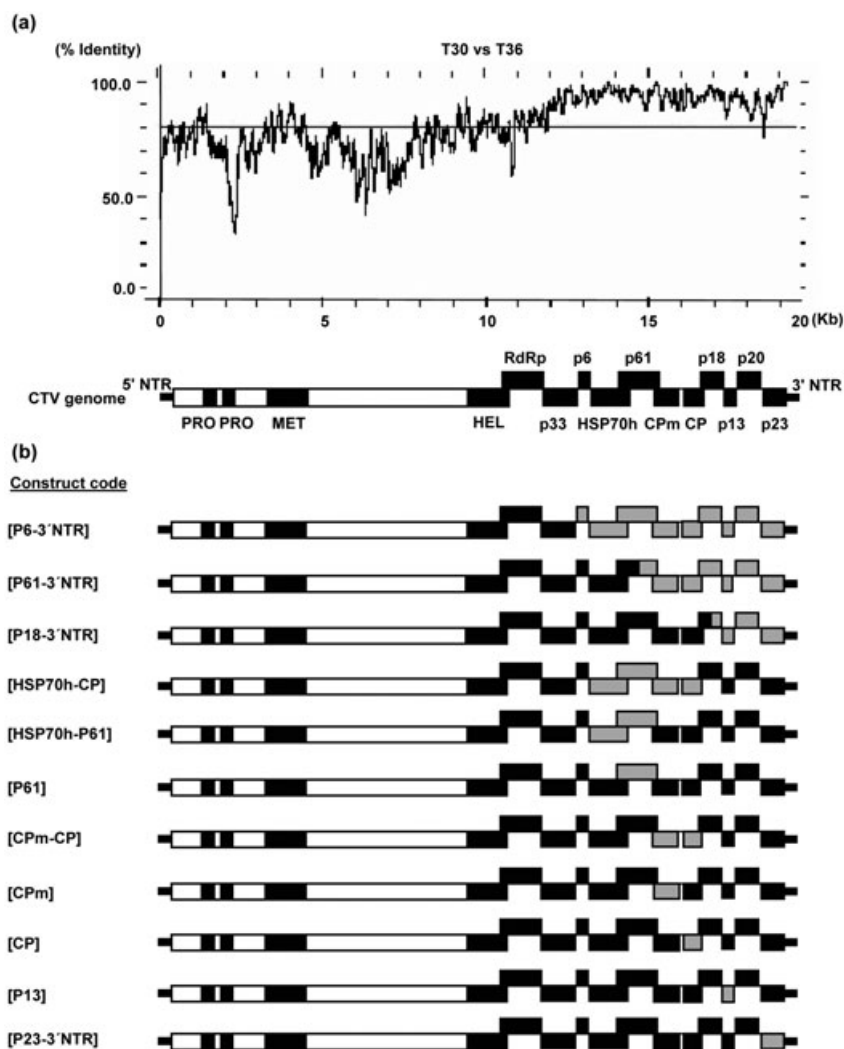
Fig. 1 (a) Graphic of the nucleotide identity between the T36 and T30 genomes and schematic representation of the genome organization of wild-type *Citrus tristeza virus* (CTV) (Karasev *et al*., 1995). PRO, MT, HEL and RdRp indicate protein domains of papain-like protease, methyltransferase, helicase and RNA-dependent RNA polymerase, respectively. HSP70h, CPm and CP indicate open reading frames (ORFs) encoding a homologue of heat shock protein 70 and the minor and major coat proteins, respectively. (b) Schematic representation of the T36/T30 hybrid constructs. Black boxes indicate the T36 sequence and grey boxes indicate the T30 sequences substituted within the T36 genome.

about 90% similar in the 3′ half of their genomes, but this similarity progressively decreases towards the 5′ terminus to as little as 42% within the 5′ NTR (Albiach-Martí *et al*., 2000c; Fig. 1a). Eleven T36/T30 hybrids were generated by substituting T30 sequences for homologous T36 sequences located in the 3′ moiety of pCTV9 (Fig. 1b, Table 1). To preclude any uncertainty about the substitution process as a result of the similarity of the T30 and T36 sequences within the 3′ portion of the genome (Fig. 1a), we first deleted the appropriate region in pCTV9, and then the corresponding T30 DNA sequences were inserted into the deletion areas within pCTV9 (Fig. 2). A T36 self-replicating deletion mutant pΔ6-20 was generated to assemble the full-length hybrids in two steps. This pΔ6-20 construct replicated in *N. benthamiana* protoplasts (Fig. 2b), indicating that it retained functional replicase ORFs. Eight of the 11 T36/T30 hybrids generated chimeric CTV proteins (Fig. 1b, Table 1). All T36/T30 hybrid regions were confirmed by sequencing.

In the absence of the ability to transmit the virus directly to citrus plants using RNA transcripts, the inoculation of *N. benthamiana* protoplasts has been routinely used to analyse the infectivity of T36 CTV constructs (Satyanarayana *et al*., 1999). As not all strains of CTV replicate in these protoplasts, including isolates of the T30 strain (M. R. Albiach-Martí *et al*., unpublished results), the ability of the T30/T36 hybrids to replicate in these conditions was examined. *In vitro* RNA transcripts of each construct were used to inoculate protoplasts, which were then analysed for RNA accumulation by Northern blot hybridization employing positive-stranded, RNA-specific riboprobes homologous to the 3′ end of the T36 genome. As shown in Fig. 3 (lanes T), transcripts of all hybrids were infectious and replicated in the protoplasts.

As several hybrids resulted in chimeric proteins of HSP70h, p61, CPm and CP (Fig. 1b, Table 1), which could prevent virion assembly (Satyanarayana *et al*., 2000, 2004), the ability of each

Table 1 T30 genomic regions exchanged in the T36 infectious clone CTV9 to generate T36/T30 hybrid constructs.

T36/T30 hybrid code	T30 sequences exchanged in T36	Location of the region exchanged†
[P6-3′NTR]	Complete p6, HSP70h, p61, CPm, CP, p18, p13, p20 and p23 ORFs, and the 3′NTR	11872–19296
[P61-3′NTR]	61 nt of p61 ORF and complete CPm, CP, p18, p13, p20 and p23 ORFs, and the 3′NTR	15248–19296
[P18-3′NTR]	88 nt of p18 and complete p13, p20 and p23 ORFs, and the 3′NTR	17094–19296
[HSP70h-CP]	Complete HSP70h, p61, CPm and CP ORFs, and 34 nt of p18 ORF*	12041–16826
[HSP70h-P61]	Complete HSP70h and p61 ORFs, and 26 nt of CPm ORF*	12041–15364
[P61]	Complete p61 ORF and 26 nt of CPm ORF*	13757–15364
[CPm-CP]	Complete CPm and CP ORFs, and 26 nt of p61 ORF and 34 nt of p18 ORF*	15339–16826
[CPm]	Complete CPm ORF and 26 nt of p61 ORF*	15339–16061
[CP]	Complete CP ORF and 34 nt of p18 ORF*	16155–16826
[P13]	Complete p13 ORF	17329–17686
[P23-3′NTR]	Complete p23 ORF and the 3′NTR	18394–19296

CP, major coat protein; CPm, minor coat protein; HSP70h, homologue of heat shock protein 70; nt, nucleotide; NTR, nontranslated region; ORF, open reading frame.
*As a result of overlapping between *Citrus tristeza virus* (CTV) ORFs. Only the overlapped regions with sequence differences between T36 and T30 genomes are indicated.
†Referred to T36 sequence (GENBANK accession no. U16304).

of the hybrids to form viable virions was assayed by inoculating a second set of protoplasts with crude extracts from the transcript-inoculated protoplasts. Incomplete virions do not withstand this procedure (Satyanarayana *et al.*, 2001). Viral RNAs from the second set of protoplasts were analysed at 4 days post-inoculation (dpi) by Northern blot hybridization. Although five hybrids were passaged to the next set of protoplasts, hybrid constructs [P6-3′NTR], [P61-3′NTR], [HSP70h-CP], [CPm-CP], [CPm] and [CP], which carried exchanges of T30 CP and CPm into the T36 genome, failed to passage (Fig. 3, lanes P), possibly because of deficient heteroencapsidation. Because significant amplification of intact virions in protoplasts is necessary to infect citrus plants (Robertson *et al.*, 2005), these hybrids could not be examined further.

Examination of the T36/T30 hybrid viruses in citrus trees

All attempts to directly inoculate citrus plants with cloned CTV have failed, and infection of citrus plants with recombinant CTV has involved the amplification of the virus through successive passages in protoplasts of *N. benthamiana* to amounts needed for mechanical inoculation of citrus plants (Robertson *et al.*, 2005; Satyanarayana *et al.*, 2001). Five of the hybrid T30/T36 constructs were sufficiently amplified to allow successful infection of the highly susceptible host *Citrus macrophylla* by stem-slash or bark-flap inoculation (Folimonova *et al.*, 2008; Robertson *et al.*, 2005; Satyanarayana *et al.*, 2001).

As the hybrid constructs contained heterologous sgRNA controller elements, their capability to generate the characteristic CTV sgRNA pattern (Hilf *et al.*, 1995) was analysed by Northern blot hybridization. Analysis of total RNAs indicated that the T36/T30 hybrids replicated in *C. macrophylla* plants, producing relative amounts of the different sgRNAs similar to those of

isolate T36 (Fig. 4) (Hilf *et al.*, 1995), which demonstrated that these hybrids were able to infect and move systemically in *C. macrophylla* as the parental viruses. Occasionally, D-RNAs are seen, which usually are visible as additional bands (Albiach-Martí *et al.*, 2000b; Mawassi *et al.*, 1995). Isolate T30 usually generates elevated concentrations of several small D-RNAs (Fig. 4) during replication in some species of citrus plants. Some of the T36/T30 hybrids infecting *C. macrophylla* (Fig. 4) also accumulated D-RNAs, which did not appear to affect the T36/T30 hybrid replication in *C. macrophylla* (Fig. 4, arrows and data not shown).

Estimation of the capacity of the T36/T30 hybrids to induce SY in sour orange and grapefruit plants

Tissues from *C. macrophylla* plants infected with the hybrid constructs [P23-3′NTR], [P13], [P61], [P18-3′NTR] and [HSP70h-P61], as well as plants infected with controls T36 and T30, were used to graft inoculate groups of five sour orange and Duncan grapefruit seedlings to test for SY syndrome development (Fig. 5a). SY testing was repeated three times during a 3-year period. Analysis of the SY data indicated no significant differences between these experiments for each of the T36/T30 hybrids and controls. Therefore, SY data were pooled and analysed together. The parental T36 and three of the T36/T30 hybrids induced SY symptoms, whereas hybrid constructs [P23-3′NTR] and [P18-3′NTR] and the wild-type T30 remained symptomless similar to the healthy controls (Fig. 5b,c). All the plants inoculated with hybrid [P13] showed clear SY symptoms, but at a lower intensity than plants inoculated with T36 (Fig. 5c). Similar results were obtained in SY assays in Duncan grapefruit seedlings (data not shown), with the overall conclusion that the SY determinant maps within the p23-3′NTR region.

Analysis of total RNA extracts by Northern blot hybridization showed that all T36/T30 hybrids yielded the typical CTV sgRNA
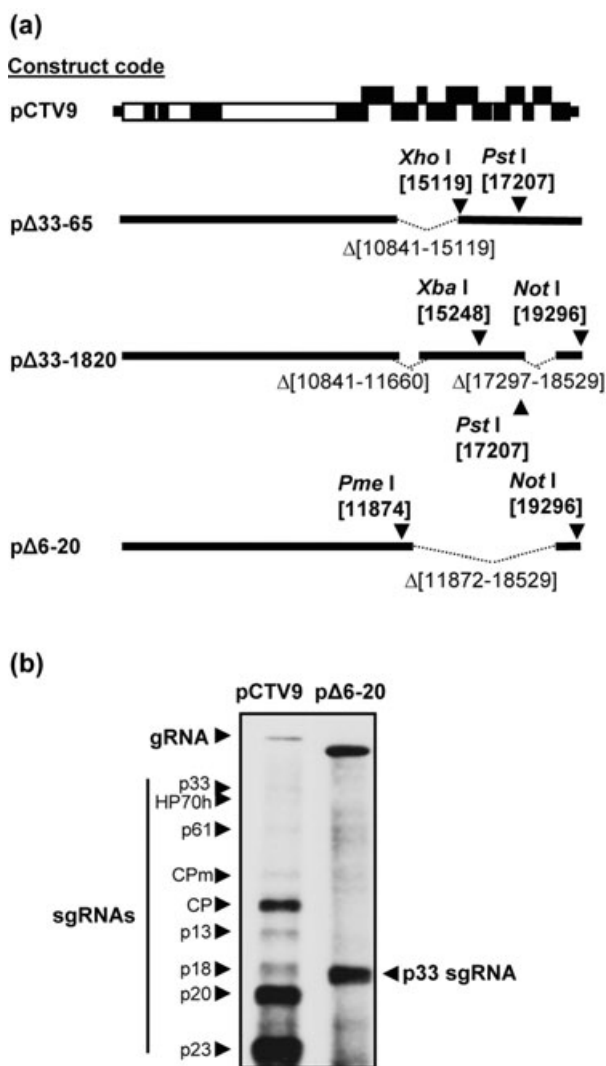
(a)

<u>Construct code</u>

Fig. 2 Deletion mutants of *Citrus tristeza virus* (CTV) infectious clone pCTV9 used for the construction of the T36/T30 hybrids. (a) Schematic representation of the pCTV9 infectious clone and the deletion mutants. Arrows indicate the position of the unique restriction endonuclease sites used for cloning purposes. Nucleotide deletions in the pCTV9 sequence are indicated by dotted lines with the location of the deletions indicated in square brackets. (b) RNA accumulation in *Nicotiana benthamiana* protoplasts infected with *in vitro* RNA transcripts from pCTV9 and the self-replicating mutant pΔ6-20. Northern blots were performed using positive-stranded, RNA-specific riboprobes to the 3′ end of the T36 genome. gRNA and sgRNAs indicate genomic RNA and 3′-coterminal subgenomic RNAs, respectively.

pattern (Hilf *et al.*, 1995) in sour orange (Fig. 6a). In addition, Western blot analysis indicated that there was no correlation between p23 production and the intensity of the symptoms induced by each of the T36/T30 hybrids and the CTV controls (Fig. 6b).

The p23 sequences of genotypes T36 and T30 have 90.9% and 91.4% nucleotide and amino acid identity, respectively (Albiach-Martí *et al.*, 2000c). These amino acid changes are distributed along the p23 protein, including the zinc finger domain (López *et al.*, 2000), which is required to control CTV negative strand accumulation (Satyanarayana *et al.*, 2002b). To examine whether the hybrid [P23-3′NTR] remained stable in citrus, the hybrid region between p20 and the 3′ end was sequenced after six passages in *N. benthamiana* protoplasts, 4 years in *C. macrophylla*, and 1 year in sour orange plants. Comparison with T30 (Albiach-Martí *et al.*, 2000c) and the T36 CTV9 (Satyanarayana *et al.*, 2003) sequences showed that no amino acid change had occurred in p20 or the 3′ NTR region, and only a single mutation was found in p23. This was a change to a similar amino acid affecting residue 166 of p23 (Phe to Leu), thus leaving intact the T30 zinc finger binding domain (amino acids 46–86) (López *et al.*, 2000) in the hybrid [P23-3′NTR]. These results indicate that the T36/T30 hybrid region of the [P23-3′NTR] construct is stable in citrus.

Evaluation of the ability of the T30/T36 hybrid [P23-3′NTR] to cross-protect against SY

The mild isolate T30 does not cross-protect citrus plants against T36, and only isolates within the T36 sequence group (strain) protect against isolate T36 (Folimonova *et al.*, unpublished results). The ability of the T30/T36 hybrid, which did not induce SY, to protect sour orange plants from the SY syndrome induced by the parent isolate T36 was examined. Sour orange seedlings were inoculated with the T36/T30 hybrid [P23-3′NTR] and incubated in the glasshouse for 2 months for systemic infection to occur. These plants were then challenged with T36 CTV9 by graft inoculation. Sour orange seedlings inoculated with T36 or with the hybrid [P23-3′NTR] and mock-inoculated healthy seedlings served as controls. After the grafts had healed, the seedlings were cut back to force new growth to observe symptom development. Figure 7a shows the pronounced stunting induced by T36 in sour orange plants, in comparison with the healthy appearance of plants inoculated with the hybrid [p23-3′NTR] or pre-inoculated with the hybrid [p23-3′NTR] and then challenged with T36. In addition, the seedlings pre-inoculated with hybrid [P23-3′NTR] did not develop other typical SY symptoms (Fig. 5a) when challenged with T36, whereas unprotected control plants developed clear SY (Fig. 7b).

DISCUSSION

In the current study, we have demonstrated that SY is mapped to the region encompassing the p23 gene and the 3′ NTR of the CTV genome by examining hybrid constructs of T36 (SY-positive) and T30 (SY-negative) isolates. We need to further map the
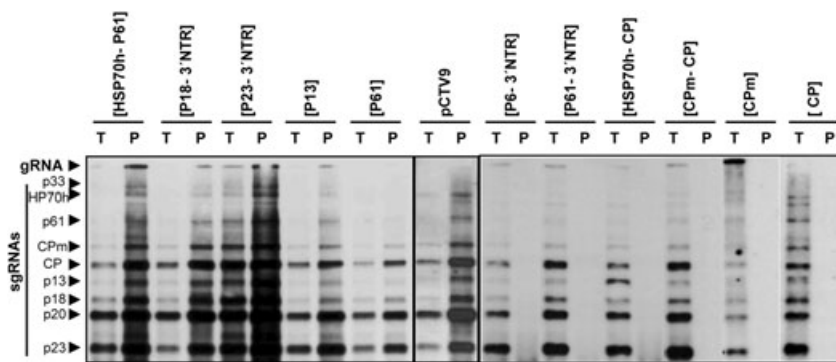
Fig. 3 Accumulation of viral RNA in *Nicotiana benthamiana* protoplasts inoculated with *in vitro* transcripts of *Citrus tristeza virus* (CTV) T36 infectious clone CTV9 and the 11 T36/T30 hybrid constructs (line T), and accumulation of viral RNA in a second set of protoplasts inoculated with the virion extracts from transcript-infected protoplasts of line T (line P). Northern blots were performed using positive-stranded, RNA-specific riboprobes to the 3′ end of the T36 genome. gRNA and sgRNAs indicate genomic RNA and 3′-coterminal subgenomic RNAs, respectively.
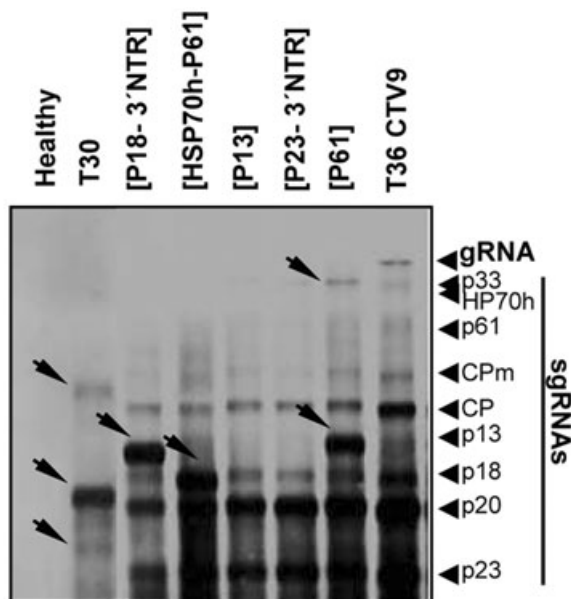

Fig. 4 Accumulation of *Citrus tristeza virus* (CTV) RNA in bark extracts from *Citrus macrophylla* plants infected with isolate T30, the T36 infectious clone CTV9 and five T36/T30 hybrid constructs. Northern blot hybridization was performed using positive-stranded, RNA-specific riboprobes to the 3′ end of the T36 genome. gRNA, sgRNAs and D-RNAs (arrows) indicate genomic RNA, 3′-coterminal subgenomic RNAs and defective RNAs, respectively.

et al., 2002b). p23 specifically inhibits intracellular silencing in *N. benthamiana* plants (Lu *et al.*, 2004), and has been used in attempts to produce transgenic plants searching for resistance to CTV (Batuman *et al.*, 2006; Fagoaga *et al.*, 2006).

When ectopically expressed in transgenic citrus, p23 induces virus-like symptoms that are even more intense than those induced by CTV infection (Fagoaga *et al.*, 2005; Ghorbel *et al.*, 2001). In addition to intense vein clearing in leaves, transformed Mexican lime plants develop chlorotic pinpoints in leaves, stem necrosis and collapse (Ghorbel *et al.*, 2001), which usually are not symptoms associated with CTV infection. Transgenic sour orange plants expressing p23 also develop vein clearing, leaf deformation, defoliation and shoot necrosis (Fagoaga *et al.*, 2005). These transgene-induced symptoms differ substantially from the virus-induced SY symptoms in sour orange. In transgenic limes, symptom severity parallels the accumulation levels of p23, regardless of the source or sequence of the transgene (Fagoaga *et al.*, 2005; Ghorbel *et al.*, 2001), whereas the symptom intensity in CTV-infected limes depends on the pathogenicity characteristics of the virus isolate. This difference in the host response could be related to the fact that, in transgenic plants, p23 is produced constitutively in most cells, whereas, in nature, p23 expression associated with virus infection is limited to phloem tissues.

If the symptoms induced by CTV in sour orange are determined by p23, they should be related to the p23 sequence and not to protein expression levels, as there was no correlation between the amount of p23 and the intensity of the SY symptoms induced by T36 or by the T36/T30 hybrids, which did not induce SY in sour orange plants. As p23 is a suppressor of RNA-mediated gene silencing, it potentially could disrupt micro-RNA metabolism (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004), thus inducing SY syndrome. Several viral suppressors of RNA-mediated gene silencing have been identified as pathogenicity determinants (Qu and Morris, 2005), and p23 could be the logical candidate for the CTV determinant of SY syndrome development in sour orange and Duncan grapefruit seedlings. However, as a viral 3′ NTR has also been related to symptom

determinant of this phenotype to either the p23 gene or the 3′ NTR, and even to specific subdomains if possible. However, the amplification and inoculation of trees with a new series of hybrids and the performance of a series of SY assays will require an additional 2–3 years. The 3′ NTR is highly conserved among all CTV genotypes (López *et al.*, 1998) and contains the recognition signal for the replicase complex (Satyanarayana *et al.*, 2002a). The p23 gene product, based on the relative levels of sgRNAs, is one of the most highly expressed CTV proteins (Navas-Castillo *et al.*, 1997); it is an RNA-binding protein responsible for asymmetrical replication, resulting in an excess of positive genomic and sgRNAs (López *et al.*, 2000; Satyanarayana
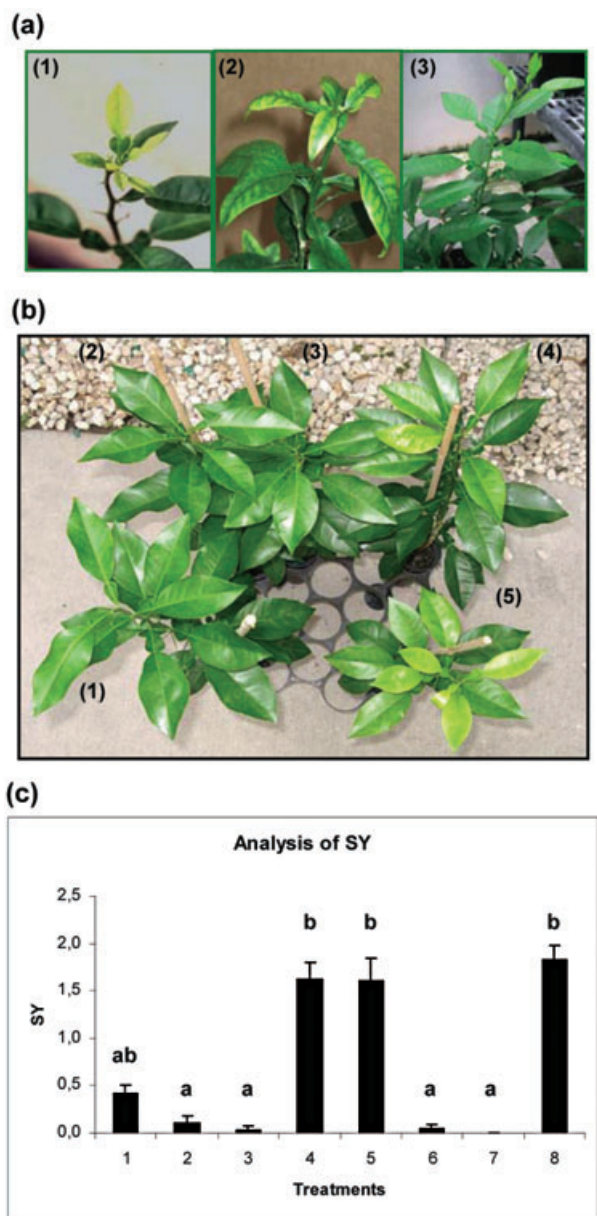
Fig. 5 Development of seedling yellows (SY) syndrome in *Citrus tristeza virus* (CTV)-infected plants. (a) SY symptoms in sour orange (1) and Duncan grapefruit (2) seedlings compared with a healthy sour orange plant (3). (b) SY symptoms in T36/T30 hybrid [P23-3'NTR] (1), isolate T30 (2), healthy (3) and T36 infectious clone CTV9 (4) and T36/T30 hybrid [HSP70h-P61] (5) sour orange seedlings. (c) Average intensity of SY symptoms (plus standard error of the mean) rated on a scale of 0–4 in sour orange seedlings infected with T36/T30 hybrids [P13] (1), [P18-3'NTR] (2), [P23-3'NTR] (3), [P61] (4) and [HSP70h-P61] (5), and controls healthy (6), T30 isolate (7) and T36 CTV9 (8). Bars accompanied by the same letter were not statistically significantly different (Mann–Whitney *U*-tests and sequential Bonferroni correction).
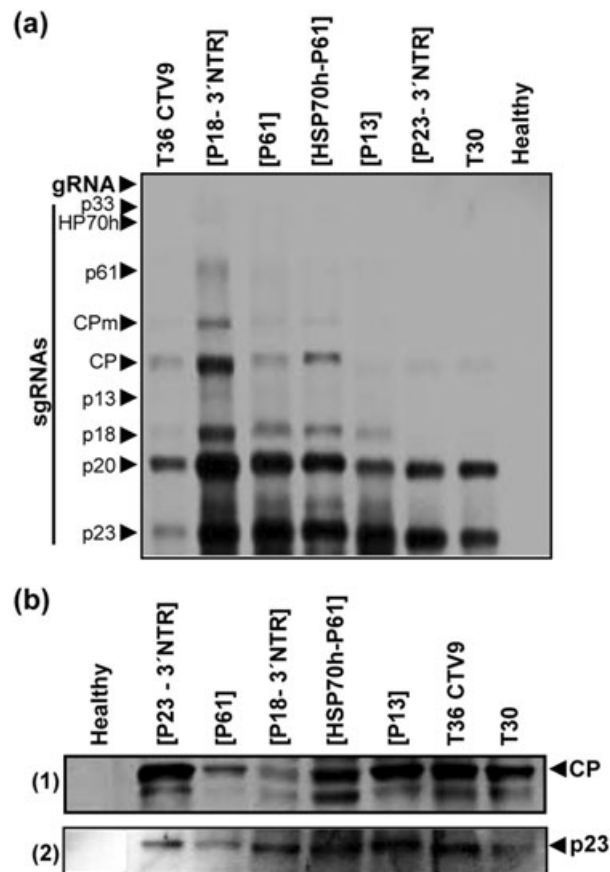

Fig. 6 Viral RNA and protein accumulation in bark extracts from sour orange plants infected with *Citrus tristeza virus* (CTV) isolates T30, T36 CTV9 and five T36/T30 hybrid constructs. (a) Analysis of CTV RNA accumulation using Northern blot hybridization with a positive-stranded, RNA-specific riboprobe of the 3' end of the T36 genome. CTV gRNA and sgRNAs indicate genomic and 3'-coterminal subgenomic RNAs, respectively. (b) Immunoblot analysis of CTV coat protein (CP) (1) and p23 protein (2) obtained from each of the T36/T30 hybrids and controls. The protein band under CP corresponds to a truncated CP, which was generated during the protein extraction process (Albiach-Martí *et al*., 2000a).

development (Rodríguez-Cerezo *et al*., 1991), we cannot yet conclude that the p23 protein directly induces SY.

The SY reaction is specific to only certain citrus hosts of CTV, such as lemons, sour orange and grapefruit, indicating that there are specific host factors involved in its expression in addition to the isolate-specific factors identified here. Although we were able to map a determinant of the SY syndrome in T36, there is a need to determine whether this determinant is common to other CTV genotypes that also induce SY, or if there are other possible SY determinants. A pressing need also remains to map the decline and stem pitting determinants of CTV, which are more important economically. It is possible that determinant(s) for the decline disease map similarly to that of SY. A strong correlation
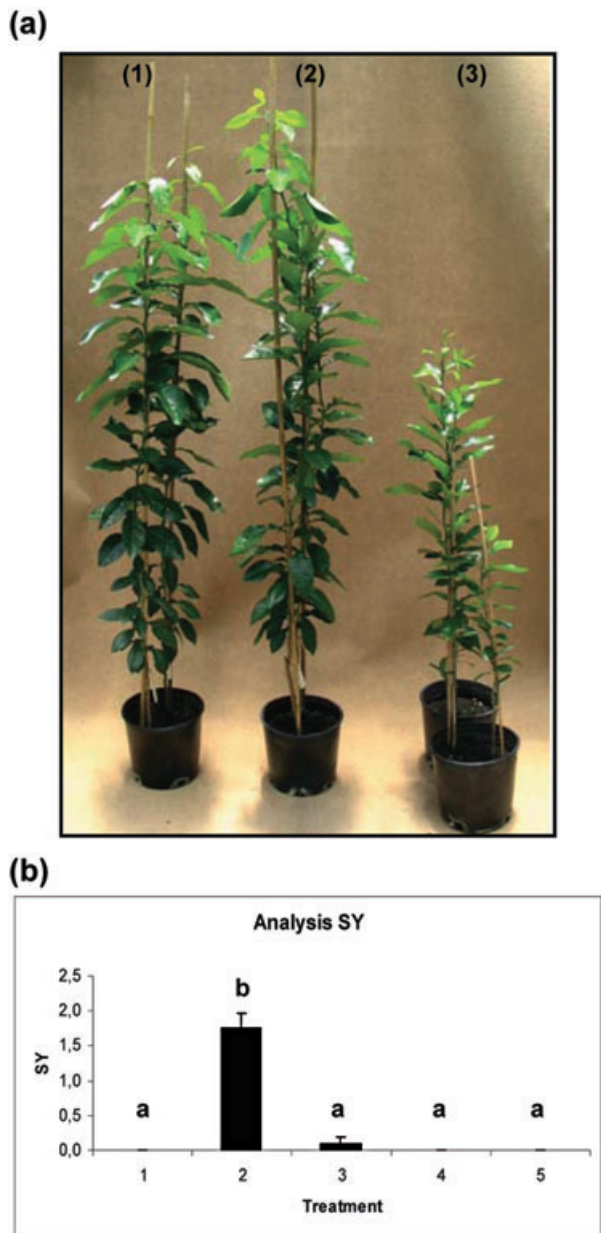
(a)

(b)

Fig. 7 Ability of the T36/T30 hybrid [P23-3'NTR] to protect against the development of seedling yellows (SY) symptoms in sour orange seedlings when challenged with the SY-inducing isolate T36 CTV9. (a) Symptoms in plants inoculated with T36/T30 hybrid [P23-3'UTR] (1), hybrid [P23-3'UTR] and then challenged with T36 CTV9 (2) and T36 CTV9 (3). (b) Average SY symptom intensity (plus standard error of the mean) rated on a scale of 0–4 in sour orange plants, healthy (1) or inoculated with T36 CTV9 (2), isolate T30 (3), hybrid [P23-3'NTR] (4) and hybrid [P23-3'UTR] and then challenged with T36 CTV9 (5). Bars accompanied by the same letter were not statistically significantly different (one-way ANOVA and Bonferroni test).

between SY and decline has been observed in the biological evaluation of a wide range of CTV isolates (Garnsey *et al.*, 2005). However, as some decline-inducing isolates do not produce obvious SY symptoms, we cannot make this conclusion without directly evaluating the T36/T30 hybrids in decline-susceptible grafted combinations of scion and rootstock. Unfortunately, clear decline assays cannot be performed in the glasshouse, but instead need to be conducted for long periods in the field. However, because the hybrids are made by recombinant DNA technologies, these assays require special permits from the plant protection and environmental safety authorities.

Wider application of natural mild strain cross-protection to control CTV severe isolates has been limited by the difficulty in finding mild isolates of CTV that effectively protect against more severe isolates (Bar-Joseph *et al.*, 1989; Roistacher and Dodds, 1993). Another problem is that natural mild CTV isolates may contain minor severe stem pitting variants which, on aphid transmission, could become prevalent (Moreno *et al.*, 1993). Recently, it was found that only isolates within a closely related sequence group (which we refer to as a strain) will cross-protect against each other (S. Y. Folimonova *et al.*, unpublished results). Thus, naturally occurring mild T30 isolates would not protect against disease-inducing isolates from other genotypes. A valuable outcome of the mapping process using genome substitution is that hybrid constructs with a disease determinant removed could potentially be used in mild strain cross-protection strategies for disease control. Our recombinant mild hybrid virus is able to efficiently protect citrus trees from SY caused by the parental virus (T36), and their hybrid genomic sequences are highly stable in citrus plants. Thus, the use of recombinant hybrids, such as the [P23-3'NTR] construct, could offer a mechanism to custom engineer isolates that are both protective and free of disease induction potential. The stability noted in the T30/T36 constructs is also important for application. This means that, if naturally occurring mild strains cannot be found for stem pitting or decline diseases, it would be possible to map the disease determinant, remove it by recombinant DNA technology and use the recombinant mild virus as a protecting strain.

EXPERIMENTAL PROCEDURES

Virus isolates and indicator plants

CTV isolates T30 and T36 were used as viral RNA sources for the generation of the T36/T30 hybrid constructs and as viral controls in the plant experiments. The T30 isolate was obtained originally from a naturally infected sweet orange tree (Albiach-Martí *et al.*, 2000c) and was maintained in Madam Vinous sweet orange and *C. macrophylla* plants. The T36 infectious clone pCTV9 (Satyanarayana *et al.*, 1999, 2001, 2003) and the hybrid T36/T30 constructs were maintained in *C. macrophylla* plants.

Table 2 Primers used for polymerase chain reaction (PCR) amplification.

Primer code	Primer sequence†	Location‡	CTV genotype	CTV region
C274(+)	GCACTCGAGGTTGTTATGGGTACTAACGTTGACG	17170	T36	p18
C279(−)	TATAGCGGCCGCAGGCCTTTGGACCTATGTTGGCCCC	19296, **19259**	T30 = T36	End 3′NTR
C311(−)	gcactcgagaagttgccacgtctctgctc	**15253**	T30	p61
C312(+)	gcaatgcatgtgcaattagctaaaactcacc	**11794**	T30	p33/p6*
C321(+)	gcactcgagttgtttagctgtcacgtcgg	**17094**	T30	p18
C322(+)	ccaccattcaatcgacttag	**15173**	T30	p61
C327(−)	atgacttcgtgtctaagtcgcg	**17247**	T30	p18
C538 (−)	CGTGTCTAAGTCACGCTAAACAAAGTG	17274	T36	p18
C579(+)	GCACTCGAGGTCTCTCCGTATATCG	11674	T36	p33
C871(−)	aacccagaagtac**cat**ACCGCTTAGACAGTC	**12004**/12034	T30/T36	Start HSP70h
C870(+)	GACTGTCTAAGCGGT**atg**gtacttctgggtt	12034/**12004**	T36/T30	Start HSP70h
C873 (−)	TTTCTTTATCATCGGTT**tta**ggaagtgttgtataa	15365/**15302**	T36/T30	End p61
C872(+)	ttatacaacacttcc**taa**AACCGATGATAAAGAAA	**15302**/15365	T30/T36	End p61
C925(−)	tgatgagacga**cat**CGATATCGTTT	**13712**/13746	T30/T36	Start p61
C924(+)	AAACGATATCG**atg**tcgtctcatca	13746/**13712**	T36/T30	Start p61
C867(−)	agtgttgtataacctgc**cat**GATAAGGTAAATTAAACC	**15294**/15321	T30/T36	Start CPm
C866(+)	GGTTTAATTTACCTTATC**atg**gcaggttatacaacact	15321/**15294**	T36/T30	Start CPm
C922(−)	TCCCCAAACCAA**cta**caagtacttaccca	16062/**16000**	T36/T30	End CPm
C923(+)	tgggtaagtacttg**tag**TTGGTTTGGGGA	**16000**/16062	T30/T36	End CPm
C920(−)	ttcgtcgtccatAATTCAAACCTA	**16116**/16142	T30/T36	Start CP
C921(+)	TAGGTTTGAATT**atg**gacgacgaa	16142/**16116**	T36/T30	Start CP
C868(−)	CGGAACGCAACAGA**tca**acgtgtgttaaattt	16824/**16770**	T36/T30	End CP
C869(+)	aaatttaacacacg**tga**TCTGTTGCGTTCCG	**16770**/16824	T30/T36	End CP
C913(−)	acgcgtcgaatact**cat**TGCAGCAATTGAATCC	**17290**/17313	T30/T36	Start p13
C912(+)	GGATTCAATTGCTGCA**atg**agtattcgacgcgt	17313/**17290**	T36/T30	Start p13
C915(−)	TACTACTGAGTAGACT**cta**gttatcgcaaggt	17689/**17634**	T36/T30	End p13
C914(+)	accttgcgataac**tag**AGTCTACTCAGTAGTA	**17634**/17689	T30/T36	End p13
C907(−)	cgctagtataatc**cat**TGTAACTCGCAGACTT	**18356**/18778	T30/T36	Start p23
C906(+)	AAGTCTGCGAGTTACA**atg**gattatactagcg	18778/**18356**	T36/T30	Start p23
C851(+)	ACGTTTAAACATCGATGCTTTGATACGGAAGAATAG	11872/18529	T36	p33-p6*/p23

CP, major coat protein; CPm, minor coat protein; HSP70h, homologue of heat shock protein 70; ORF, open reading frame.

*Intergenic region between p33 and p6 ORFs.

†T36 sequence is written in capitals and T30 sequence in lower case. The start and stop codons of the *Citrus tristeza virus* (CTV) ORFs are indicated in bold.

‡T30 nucleotide numbering (GENBANK accession no. AF260651) is indicated in bold and T36 numbering (GENBANK accession no. U16304) in normal type.

Glasshouse-grown seedlings of sour orange and Duncan grapefruit were used for SY assays. All virus-inoculated citrus plants were kept in an insect-free, temperature-regulated glasshouse at the Citrus Research and Education Center, Lake Alfred, FL, USA.

Generation of the CTV hybrid T36/T30 constructs

The T36 and T30 nucleotide numbering and sequences of the primers used in this study are according to Karasev *et al.* (1995) (GENBANK accession no. U16304) and Albiach-Martí *et al.* (2000c) (GENBANK accession no. AF260651), respectively. Polymerase chain reaction (PCR) to amplify cDNA fragments was performed with a proofreading thermostable polymerase (*Pfu* Turbo™, Stratagene, La Jolla, CA) following the supplier's recommendations. The full-length cDNA clone pCTV9 and deletion mutants pΔ33-1820 and pΔ33-65 have been described previously (Satyanarayana *et al.*, 1999, 2002b) (Fig. 2b). The T36 self-replicating deletion mutant pΔ6-20 (Fig. 2b) was obtained by PCR amplification of a T36 DNA fragment (nucleotides 18 529–19 296) using the primers C851(+) and C279(−) (Table 2), and by

ligating the PCR product into pCTV9 between *Pme*I (T36, nucleotide 11 872) and *Not*I (T36, nucleotide 19 278) restriction endonuclease sites. The CTV T30 cDNA sequences were obtained according to Albiach-Martí *et al.* (2000c) from viral dsRNA (Moreno *et al.*, 1990), except that the reverse primers C327(−) and C279(−) (Table 2) were used.

The T36/T30 hybrids [P6-3′NTR], [P61-3′NTR] and [P18-3′NTR] were generated by exchanging sequences between restriction endonuclease sites common to both T36 and T30. In order to obtain hybrid [P6-3′NTR], plasmid pT30DIK was generated by amplifying three T30 RT-PCR fragments, covering the 3′ terminal 8 kb of the T30 genome, with the primer pairs C312(+)/C311(−), C322(+)/C327(−) and C321(+)/C279(−) (Table 2), which were sequentially assembled into pUC119 between *EcoR*I and *Hind*III restriction endonuclease sites [which were previously blunt-ended with T4 DNA polymerase (New England Biolabs, Beverly, MA)]. Hybrid [P6-3′NTR] was synthesized by digesting plasmid pT30DIK with *Pme*I (T30, nucleotide 11 827) and *Not*I (T30, nucleotide 19 258) restriction endonuclease enzymes and cloning it into pΔ6-20. The [P61-3′NTR] and [P18-3′NTR] con-

structs were generated by PCR amplification of DNA fragments using the pair of primers C322(+)/C279(−) and C321(+)/C279(−), and ligating these into pΔ33-1820 between restriction sites *Xba*I/*Not*I and *Pst*I/*Not*I, respectively (Fig. 2b). Both T36/T30 hybrid regions were *Pme*I and *Not*I excised from pΔ33-1820 and cloned into pΔ6-20 (Fig. 2b) to obtain a full-length hybrid cDNA clone.

The T30 fragments for exact ORF exchanges integrated into the remaining eight T36/T30 hybrid constructs were generated by overlap extension PCR. Synthesis was performed using, as template, CTV PCR fragments that were amplified with the T36/T30 hybrid primers listed in Table 2. Each of the hybrid overlap extension PCR products included in [HSP70h-p61], [HSP70h-CP], [P61], [CPm-CP], [CPm] and [CP] constructs were generated using the primers C579(+) and C538(−), and ligated into pΔ33-65 between *Xho*I and *Pst*I restriction sites (Fig. 2b). The hybrid overlap extension PCRs included in the [P13] and [P23-3′NTR] constructs were amplified with the pair of primers C274(+)/C279(−), and cloned into pΔ33-1820 between *Pst*I and *Not*I restriction sites (Fig. 2b). Finally, each of the T36/T30 hybrid fragments were *Pme*I and *Not*I excised from vectors pΔ33-65 and pΔ33-1820, and cloned into pΔ6-20 between the unique restriction sites *Pme*I and *Not*I (Fig. 2b) to obtain full-length cDNA clones comprising the T30 sequences (Table 1).

Transfection of *N. benthamiana* protoplasts and mechanical inoculation of *C. macrophylla* plants

Protoplasts were isolated and purified from *N. benthamiana* leaves and inoculated with CTV-capped *in vitro* transcripts, as described previously (Navas-Castillo *et al.*, 1997; Satyanarayana *et al.*, 1999) with some modifications. RNA transcripts were synthesized with the *Not*I- or *Stu*I-linearized plasmid cDNAs, and the transfection efficiency was improved by treating the CTV-capped *in vitro* transcripts (30 μL) with 169 μL of 0.6 M mannitol solution [0.6 M mannitol, 6.25 mM 2-(*N*-morpholino) ethanesulphonic acid (MES), pH 5.8] and 1 μL of SuperFect® transfection reagent (Qiagen, Valencia, CA), followed by 10 min incubation on ice, prior to protoplast inoculation.

To test for protoplast infection by the transcripts, RNA was extracted from an aliquot of CTV-inoculated protoplasts at 4 dpi and Northern blots were performed as described by Satyanarayana *et al.* (1999). Membranes were hybridized with positive-stranded, RNA-specific, digoxigenin-labelled riboprobes corresponding to the 3′-terminal 904 nucleotides of the CTV-T36 genomic sequence. The progeny virions from the remaining transcript-inoculated protoplasts were extracted and further amplified by serial passage in *N. benthamiana* protoplasts (Satyanarayana *et al.*, 2001). After the sixth passage, the virions were partially purified and used to mechanically inoculate *C. macrophylla* plants, as described by Robertson *et al.* (2005).

Inoculated plants were tested for infection 4–8 weeks post-inoculation by double antibody sandwich indirect enzyme-linked immunosorbent assay indirect (DASI-ELISA) (Satyanarayana *et al.*, 2001). Plants that tested positive were retained as virus sources.

Indexing for SY syndrome development and cross-protection assays

Groups of five sour orange and five Duncan grapefruit seedlings were graft inoculated with *C. macrophylla* tissue infected by each of the T36/T30 hybrids or with the T30 or T36 CTV9 controls. Additional groups of five plants were left as noninoculated controls. After the grafts had healed, the plants were cut back to force new growth and, approximately 2 months later, this new growth was assessed for SY symptoms. SY development was rated visually on a scale of 0–4. Overall comparisons between treatments were analysed by Kruskall–Wallis analysis of variance (ANOVA) (Siegel and Castellan, 1988), and pairwise *post-hoc* comparisons were performed with the Mann–Whitney *U*-test plus sequential Bonferroni correction.

To test for cross-protection, 10 sour orange seedlings were graft inoculated with tissue from [P23-3′NTR]-infected *C. macrophylla* plants and five additional seedlings were left as healthy controls. Once infection by the candidate protecting isolate was confirmed by DASI-ELISA (approximately 2 months post-inoculation), five of the [P23-3′NTR]-infected plants were challenged by graft inoculation with tissue from the T36 CTV9 source plants. The other five [P23-3′NTR] plants were left uninoculated. Five sour orange seedlings inoculated only with CTV9 were also included as controls. Evaluation of CTV SY syndrome development was as described above. Overall comparisons in the cross-protection experiments were analysed by one-way ANOVA, and *post-hoc* comparisons were performed with the Bonferroni test.

Analysis of viral RNA and protein accumulation in infected tissues

Total RNA was extracted from 1 g of tissue collected from CTV-infected *C. macrophylla* or sour orange plants following the procedure described for protoplast total RNA extraction (Satyanarayana *et al.*, 1999). Gels were loaded with 3 μg of total RNA and Northern blot hybridization was performed as indicated above.

For protein analysis, total proteins were extracted from 1 g of bark tissue by grinding in liquid nitrogen and then extracting in 1 vol of 2 × Laemmli buffer. After CTV protein separation by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA), immunoblot analysis was performed as described by Satyanarayana *et al.* (2002b) using the polyclonal antibodies 908-15 and P23 (López *et al.*,

2000) as the primary antibodies for CP and p23, respectively. The same amount of protein sample was used for both CP and p23 immunoblot analysis.

Sequence analysis of the hybrid regions of the T36/T30 hybrid constructs

For the verification of hybrid sequences prior to the inoculation of protoplasts, the hybrid regions were PCR amplified using *Pfu* Turbo™ DNA polymerase (Stratagene). The primer pairs of C274/C279 or C579/C538 (Table 2) were used to amplify the hybrid regions of all constructs except [P6-3′NTR], which was amplified with the pairs of primers C279/C321, C322/C327 and C579/C311 (Table 2). The PCR fragments were sequenced in both directions by an ABI PRISM DNA Sequencer 377 (PE Biosystems, Foster City, CA). The chromatograms were assembled with the Staden Package to obtain a consensus sequence, which was aligned to T30 (GenBank AF260651) and T36 CTV9 (GenBank AY170468) sequences using BLAST 2 SEQ (Tarusova and Madden, 1999). Multiple sequence alignments were performed using the CLUSTAL W program (Thompson *et al*., 1994).

In order to analyse the stability of the sequence of the hybrid region of [P23-3′NTR] constructs in sour orange plants, dsRNA was extracted from citrus bark tissue and cDNAs from the hybrid region were synthesized by RT-PCR with Superscript™ II Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA), *Pfu* Turbo™ DNA polymerase and the C279(−) and C274(+) pair of primers (Table 2). The hybrid CTV RT-PCR products were sequenced and analysed as indicated above.

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