Ectopic expression of the p23 silencing suppressor of *Citrus tristeza virus* differentially modifies viral accumulation and tropism in two transgenic woody hosts

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SUMMARY

Citrus tristeza virus (CTV), a phloem-restricted closterovirus infecting citrus, encodes three different silencing suppressors (p25, p20 and p23), one of which (p23) is a pathogenicity determinant that induces aberrations resembling CTV symptoms when expressed ectopically in transgenic citrus hosts. In this article, the effect of p23 ectopic expression on virus infection was examined in sweet orange (SwO), a highly susceptible host, and sour orange (SO), which severely restricts CTV cell-to-cell movement. Transgenic plants of both species ectopically expressing p23, or transformed with an empty vector, were graft inoculated with the mild CTV isolate T385 or with CTV-BC1/GFP, a clonal strain derived from the severe isolate T36 carrying the gene for the green fluorescent protein (GFP). CTV distribution in infected tissues was assessed by direct tissue blot immunoassay and fluorescence emission, and virus accumulation was estimated by quantitative real-time reverse transcriptase-polymerase chain reaction. CTV accumulation in p23-expressing and control SwO plants was similar, whereas the viral load in transgenic SO expressing p23 was 10–10⁵ times higher than in the cognate control plants. Although few infection foci composed of a single cell were observed in the phloem of CTV-infected control SO, the number of foci in p23-expressing plants was higher and usually comprised two to six cells, indicating viral cell-to-cell movement. CTV was detected in mesophyll protoplasts and cells from infected SO and SwO expressing p23, but not in similar protoplasts and cells from infected control plants. Our results show that the ectopic expression of p23 enables CTV to escape from the phloem and, in addition, facilitates systemic infection of the resistant SO host. This is the first report of a viral-encoded protein that enhances virus accumulation and distribution in woody hosts.

INTRODUCTION

RNA silencing is a sequence-specific mechanism that mediates plant defence against invading nucleic acids, including viruses. A manifestation of RNA silencing is the recovery phenomenon associated with some resistant plants which, after showing viral symptoms on inoculated and first emerging leaves, do not express symptoms in uppermost leaves, with the virus titre becoming concomitantly low or undetectable (Ratcliff *et al.*, 1997; Wingard, 1928). As counter-defence, viruses encode proteins able to suppress RNA silencing (Csorba *et al.*, 2009).

In nature, mixed viral infections often result in more severe disease symptoms than those caused by individual viruses alone. These synergistic interactions usually result from the silencing suppressor activity of one of the viruses increasing the accumulation of the other virus involved (Pruss *et al.*, 1997; Vance *et al.*, 1995). Co-infection of the phloem-associated *Abutilon mosaic virus* (AbMV) or *Potato leafroll virus* (PLRV) with *Cucumber mosaic virus* (CMV) or *Pea enation mosaic virus 2*, respectively, alleviates their phloem limitation and increases their titre in infected plants, an effect that has been attributed to the silencing suppression ability of the latter viruses (Ryabov *et al.*, 2001; Wege and Siegmund, 2007). Experiments with viruses with mutations affecting their silencing suppressors have shown that suppression is required for cell-to-cell or long-distance movement of *Potato virus X* (PVX) and *Tobacco etch virus*, respectively (Bayne *et al.*, 2005; Kasschau and Carrington, 2001), and to facilitate systemic infection of *Tomato bushy stunt virus* (TBSV) (Qu and Morris, 2002). However, the effects of silencing suppressors in the movement and distribution of viruses infecting woody hosts, which display significant physiological differences from their herbaceous counterparts, have not been examined.

Citrus tristeza virus (CTV), a member of the genus *Closterovirus*, family *Closteroviridae*, causes economically important diseases of citrus trees worldwide. The virus is restricted to phloem cells of most *Citrus* species and some relatives within the subfamily *Aurantioideae*. The host response to CTV infection is

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variable and sometimes strain dependent. Although sweet orange (*Citrus sinensis* L. Osb.), mandarin (*C. reticulata* Blanco) and Mexican lime [*C. aurantifolia* (Christm.) Swing.] normally show systemic infection and relatively high virus titres, uneven distribution and low virus accumulation are observed in infected sour orange (*C. aurantium* L.) and lemon (*C. limon* L. Burn. f.) (Moreno *et al.*, 2008). As with other CTV-resistant species or hybrids, virus accumulation in inoculated sour orange protoplasts is similar to that of inoculated protoplasts from fully susceptible species such as sweet orange or Mexican lime (Albiach-Martí *et al.*, 2004), suggesting that a defect in virus movement is responsible for the low CTV titre observed in sour orange. Indeed, inoculation of sour orange with a clonal CTV T36 strain containing the green fluorescent protein (GFP) gene has demonstrated that the virus is essentially unable to move from cell to cell in this host (Folimonova *et al.*, 2008).

The CTV genome consists of a plus-sense single-stranded RNA (ssRNA) of approximately 19.3 kb organized in 12 open reading frames (ORFs), potentially encoding at least 17 proteins, and 5' and 3' untranslated terminal regions (UTRs) (Karasev *et al.*, 1995). The two 5' proximal ORFs, which are translated from the genomic RNA, encode components of the replicase complex, including papain-like protease (PRO), methyltransferase (MT), helicase (HEL) and RNA-dependent RNA polymerase (RdRP) domains. The ten 3' proximal ORFs encoding p33, p6, p65, p61, p27, p25, p18, p13, p20 and p23 proteins are expressed via 3' co-terminal subgenomic RNAs (Hilf *et al.*, 1995). The p25 and p27 coat proteins, together with p65 and p61, are required for virus assembly (Satyanarayana *et al.*, 2000). The p23 protein is an RNA-binding protein with a putative zinc-finger domain (López *et al.*, 2000) that is involved in the regulation of the balance of plus and minus strands during RNA replication (Satyanarayana *et al.*, 2002). This protein, together with p20 and p25, is a suppressor of RNA silencing in *Nicotiana tabacum* and *N. benthamiana* plants (Lu *et al.*, 2004). In addition, p23 is a pathogenicity determinant of the virus that induces aberrations resembling CTV symptoms when expressed ectopically in transgenic citrus hosts, including Mexican lime, sweet orange and sour orange (Fagoaga *et al.*, 2005; Ghorbel *et al.*, 2001). Moreover, the seedling yellows syndrome, induced by some CTV strains in sour orange and grapefruit (*C. paradisi* Macf.), has been mapped recently at the p23 3' UTR (Albiach-Martí *et al.*, 2010). Finally, the region coding for p23 is a hotspot for RNA silencing because small RNAs from this region accumulate to high levels in CTV-infected Mexican lime, sweet orange and sour orange (Ruiz-Ruiz *et al.*, 2011).

We have shown previously that the ectopic expression of p23 in transgenic Mexican lime does not increase the viral titre in CTV-inoculated plants in comparison with control plants, and that CTV infection does not alter the CTV-like aberrant phenotype exhibited by noninoculated *p23*-transgenic plants, except

for the leaf cupping associated with viral infection (Fagoaga *et al.*, 2005). In this study, we investigated the effect of the ectopic expression of p23 on virus accumulation and distribution in two hosts with different susceptibility to CTV: sweet and sour orange. We showed that the constitutive expression of p23 in transgenic plants: (i) released restrictions to cell-to-cell and long-distance movement of CTV in the sour orange phloem and increased virus accumulation in this host; and (ii) alleviated phloem limitation of CTV in both sour and sweet orange, and allowed infection of mesophyll cells, suggesting constraints to CTV movement in citrus and, especially, in sour orange. To our knowledge, these effects on virus accumulation and distribution, which may, at least in part, be a result of RNA silencing, have not yet been reported in woody hosts.

RESULTS

Ectopic expression of p23 increases CTV accumulation in transgenic sour orange, but not in transgenic sweet orange

We have shown previously that p23 ectopic expression in sweet and sour orange produces aberrant phenotypes, resulting in the death of most transgenic regenerants, with those that survive progressively showing, after 2 years of growth in the glasshouse, normal phenotypes alternating occasionally with aberrant flushes (Fagoaga *et al.*, 2005). To assess whether CTV infection in hosts with different susceptibility could be affected by the ectopic expression of p23, the *p23*-transgenic sour orange line 19 (SO-19) and sweet orange lines 1 and 12 (SwO-1 and SwO-12), accumulating comparable levels of p23 (Fagoaga *et al.*, 2005), plus the controls transformed with the empty vector (SO-C and SwO-C, respectively), were propagated on Troyer citrange and inoculated with the CTV isolate T385. This isolate causes symptomless infection in most citrus species, including sweet and sour orange, with the latter generally showing low virus titre and uneven distribution during the first 2 years after inoculation (Comellas, 2009; Moreno *et al.*, 1993). Three years after CTV inoculation, *p23*-transgenic propagations were analysed for virus titre by quantitative reverse transcription polymerase chain reaction (RT-PCR). CTV accumulation was one to five orders of magnitude higher in SO-19 than in SO-C plants, whereas accumulation in SwO-1, SwO-12 and SwO-C plants was similar (Fig. 1). Interestingly, the virus titres in sweet orange and in *p23*-transgenic sour orange plants were also similar (Fig. 1). Quantitative RT-PCR analysis was repeated the following year on the same plants with essentially the same results, albeit CTV accumulation in *p23*-transgenic sour orange was only one or two orders of magnitude higher than in SO-C controls (Fig. 1). Therefore, the response of sweet and sour orange to the constitutive expression of the p23 protein in their cells was different with

Fig. 1 Estimation of *Citrus tristeza virus* (CTV) T385 cDNA targets in leaf samples from *p23*-transgenic and empty vector control sweet orange (SwO) and sour orange (SO) plants by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Three to seven plants from *p23*-transgenic sour orange line SO-19, sour orange empty vector control line SO-C, *p23*-transgenic sweet orange lines SwO-12 and SwO-1, and sweet orange empty vector control line SwO-C were propagated onto Carrizo citrange rootstock and graft inoculated with the mild CTV isolate T385. Numbers after the line code identify each propagation. The average numbers of CTV cDNA target copies per nanogram of total RNA $\pm$ standard error (SE) were estimated from two different assays, using three replicates per assay, 3 and 4 years post-inoculation.

regard to CTV accumulation, the CTV titre being strongly increased only in sour orange.

Ectopic expression of *p23* increases virus spread in actively growing tissues of transgenic sour orange

The low CTV titre in sour orange, compared with other citrus species, including sweet orange, is mainly a result of the smaller number of infected phloem-associated cells in the former (Folimonova *et al.*, 2008). To investigate whether the increased virus titre in *p23*-transgenic sour orange could result from enhanced virus spread, young leaf blades and petioles were subjected to analysis by direct tissue blot immunoassay–enzyme-linked immunosorbent assay (DTBIA-ELISA). Between 12% and 17% of the SO-C samples tested CTV positive in the vascular region of imprinted sections 3 years after inoculation,

and between 11% and 32% 1 year later (Table 1), indicating low accumulation and uneven distribution of the virus in young shoots. Moreover, the coloured positive spots were generally very small, only visible under the stereomicroscope at the highest magnification. By contrast, SO-19 transgenic plants showed stronger immunoreaction, with a larger number of positive samples and larger spots (Table 1). Samples from SwO-12 and SwO-C plants analysed 4 years after inoculation showed a ratio of DTBIA-ELISA-positive leaf blades similar to that of SO-19 plants, whereas the ratio of positive petioles was higher (Table 1). Overall, these results support the quantitative RT-PCR data and show that the increased virus titre in *p23*-transgenic sour orange, estimated by RT-PCR, may be caused by improved systemic spread of CTV in the phloem of actively growing tissues, whereas spread in *p23*-transgenic sweet orange is not affected.

Table 1 Virus spread in *Citrus tristeza virus* (CTV)-infected *p23*-transgenic or empty vector-transformed plants of sour orange (SO) or sweet orange (SwO), 3–4 years after inoculation, as estimated by direct tissue blot immunoassay-enzyme-linked immunosorbent assay (DTBIA-ELISA) of leaf blade and petiole sections using the monoclonal antibodies 3DF1 and 3CA5.

	<i>p23</i> -transgenic or empty vector-transformed line*	Ratio of (+) leaf blades by DTBIA-ELISA†	Ratio of (+) leaf petioles by DTBIA-ELISA‡
Third year post-inoculation	SO-19 (3)	29/30 (97)	127/150 (85)
	SO-C (3)	5/30 (17)	18/150 (12)
Fourth year post-inoculation	SO-19 (7)	65/70 (93)	247/350 (71)
	SO-C (7)	22/70 (31)	38/350 (11)
	SwO-12 (5)	49/50 (98)	241/250 (96)
	SwO-C (5)	47/50 (94)	210/250 (84)

*Three to seven plants from *p23*-transgenic sour orange line SO-19, sour orange empty vector-transformed line SO-C, *p23*-transgenic sweet orange line SwO-12, and sweet orange empty vector-transformed line SwO-C were assayed. The number of plants tested per transgenic line is shown in parentheses.

†Number of DTBIA-ELISA-positive leaf blade samples/total number of leaves (%).

‡Number of DTBIA-ELISA-positive leaf petiole samples/total number of leaves (%).

Fig. 2 Virus accumulation in *p23*-transgenic and empty vector-transformed sour orange (SO) lines graft inoculated with the CTV-BC1/GFP strain containing an insertion of the green fluorescent protein (GFP) gene between the *p27* and *p25* genes of the *Citrus tristeza virus* (CTV) T36 genome. The number of viral RNA targets in leaf samples was estimated by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Four plants from *p23*-transgenic sour orange lines SO-9 and SO-19, and sour orange empty vector-transformed line SO-C, were propagated onto Carrizo citrange rootstock and graft inoculated with the clonal strain CTV-BC1/GFP derived from CTV T36. Numbers after the line code identify each propagation. The average numbers of estimated CTV cDNA target copies per nanogram of total RNA $\pm$ standard error (SE) were determined, 2 and 3 years post-inoculation (p.i.).

Distribution of CTV is enhanced in *p23*-transgenic sour orange

To elucidate whether improved virus spread in *p23*-transgenic sour orange was limited to young growing tissues or also affected phloem-associated cells in the mature stem, we used a GFP-tagged T36 strain (CTV-BC1/GFP). GFP allows easy localization of CTV-infected cells under a fluorescence stereomicroscope by the green fluorescence emitted on the cambial side of excised bark pieces (Folimonov *et al.*, 2007; Folimonova *et al.*, 2008). However, before using this tool, we examined whether CTV-BC1/GFP, derived from the severe clonal strain CTV T36, behaved as CTV T385 in transgenic sour orange plants. In addition to SO-19, we included in the new assays the transgenic sour orange line 9 (SO-9), which recovered from *p23*-induced aberrations whilst the previous inoculation experiments were being performed. Quantitative RT-PCR analyses during the second and third years post-

inoculation showed that the virus titres in SO-9 and SO-19 were higher than in SO-C plants (Fig. 2). DTBIA-ELISA tests confirmed enhanced virus spread and accumulation in young growing tissues of SO-9 and SO-19 compared with SO-C plants, but not in SwO-1 and SwO-12 compared with SwO-C plants (Table 2). These results corroborate the effect of the constitutively expressed *p23* on CTV accumulation in sour orange, and indicate that this effect appears to be general, irrespective of the CTV strain used.

Observations in two consecutive years of 60–120 stem bark pieces from each of SO-9 and SO-19, and from SO-C plants infected with CTV-BC1/GFP, consistently revealed that the number of GFP-expressing spots was about 10-fold higher in *p23*-transgenic sour orange than in control plants, indicating that *p23* expression in transgenic cells increased significantly the number of infection foci not only in actively dividing cells, but also in mature tissues. The fluorescence intensity in the infected

Table 2 Virus spread in *p23*-transgenic or empty vector-transformed plants of sour orange (SO) or sweet orange (SwO), 2–3 years after inoculation with *Citrus tristeza virus* (CTV)-BC1/GFP, as estimated by direct tissue blot immunoassay-enzyme-linked immunosorbent assay (DTBIA-ELISA) of leaf blade and petiole sections using the monoclonal antibodies 3DF1 and 3CA5.

	<i>p23</i> -transgenic or empty vector-transformed line*	Ratio of (+) leaf blades by DTBIA-ELISA†	Ratio of (+) leaf petioles by DTBIA-ELISA‡
Second year post-inoculation	SO-19 (2)	17/20 (85)	68/100 (68)
	SO-9 (2)	19/20 (95)	89/100 (89)
	SO-C (2)	6/20 (30)	12/100 (12)
Third year post-inoculation	SO-19 (7)	60/70 (86)	210/350 (60)
	SO-9 (7)	57/68 (84)	202/350 (58)
	SO-C (7)	15/70 (21)	44/350 (13)
	SwO-1 (3)	30/30 (100)	132/150 (88)
	SwO-12 (6)	58/60 (97)	253/300 (84)
	SwO-C (6)	56/59 (95)	260/295 (88)

*Two to seven plants from *p23*-transgenic sour orange lines SO-9 and SO-19, sour orange empty vector-transformed line SO-C, *p23*-transgenic sweet orange lines SwO-1 and SwO-12, and sweet orange empty vector-transformed line SwO-C were assayed. The number of plants tested per transgenic line is shown in parentheses.

†Number of DTBIA-ELISA-positive leaf blade samples/total number of leaves (%).

‡Number of DTBIA-ELISA-positive leaf petiole samples/total number of leaves (%).

foci also appeared to be higher in SO-9 and SO-19 than in SO-C plants, suggesting that the ectopic expression of *p23* also enhanced virus accumulation; however, the number of infected cells in control plants was too low to support a strong claim in this respect (Fig. 3). The distribution and intensity of GFP fluorescence were comparable in SwO-1, SwO-12 and SwO-C samples, indicating that *p23* expression in sweet orange had no obvious effect on CTV accumulation (Fig. 3). The fluorescence distribution and intensity in *p23*-expressing SO and SwO plants were also similar, in agreement with previous data on the accumulation of T385 in these hosts (Fig. 1).

Restriction to CTV intercellular movement in phloem-associated tissues of sour orange is released by the ectopic expression of *p23*

Folimonova *et al.* (2008) have reported that essentially no cell-to-cell spread of CTV occurs in sour orange, with virus movement being restricted to sieve tubes and a small proportion of adjacent phloem-associated cells. However, the fluorescence observed in the bark tissue of plants inoculated with the GFP-tagged CTV strain suggested that infection foci in *p23*-transgenic sour orange were composed of more than one cell, as expected if the virus was able to move from cell to cell (Fig. 3). To further elucidate the effect of the constitutive expression of *p23* on CTV movement in the phloem tissue of transgenic sour orange, we examined longitudinal bark sections by confocal microscopy, which allowed for a higher magnification and a more accurate estimation of the number of cells per infection focus. Although CTV was restricted to single cells in SO-C samples (Fig. 4A), infected foci in SO-9 and SO-19 plants usually comprised two to six cells (Fig. 4C,D and Fig. 4E,F, respectively), thereby showing that the constitutive expression of *p23* also alleviated limitations

in cell-to-cell virus movement in the phloem (Fig. 4). Moreover, this experiment confirmed that the number of infection foci and their fluorescence intensity were lower in control sour orange than in its *p23*-transgenic counterparts, further confirming that the ectopic expression of *p23* enhances long-distance spread and virus accumulation in the phloem tissue of infected sour orange (Fig. 4; results not shown). The number and size of infected foci in bark pieces from SwO-C, SwO-1 and SwO-12 plants were similar and comparable with those observed in *p23*-transgenic sour orange (Fig. 4B; results not shown).

Ectopic expression of *p23* in transgenic sour and sweet orange alleviates phloem restriction of CTV and enables virus spread to mesophyll cells

To assess whether the ectopic expression of *p23* not only enhanced the CTV titre and spread in the phloem of sour orange, but could also affect tissue specificity, mesophyll leaf protoplasts from CTV-BC1/GFP-infected sweet and sour orange plants were isolated and examined for GFP expression under a fluorescence microscope. Although no green fluorescent protoplasts were observed in SO-C and SwO-C control samples, GFP-expressing protoplasts were readily observed in all preparations from *p23*-transgenic sour and sweet orange plants. Of the protoplasts counted in at least 50 microscope fields, those emitting fluorescence represented about 15%–20% (Fig. 5A; Fig. S1, see Supporting Information), with this fraction being more dependent on the developmental and physiological stage of the sampled leaves than on the host species. CTV infection in SO-9 and SO-19, and in SwO-1 and SwO-12, mesophyll cells was confirmed by RT-PCR analysis of the viral gene *p27* in total RNA preparations isolated from mesophyll leaf protoplasts (Fig. 5B). To exclude the possibility that part of the fluorescent protoplasts

Fig. 3 Green fluorescent protein (GFP) fluorescence in phloem-associated cells in the cambial face of bark pieces from noninfected sour and sweet orange stems (SO and SwO), and from empty vector-transformed (SO-C and SwO-C) and *p23*-transgenic (SO-*p23* and SwO-*p23*) plants, 3 years after inoculation with the clonal strain CTV-BC1/GFP, containing an insertion of the GFP gene (*gfp*) between the *p27* and *p25* genes in the *Citrus tristeza virus* (CTV) T36 genome (Folimonov *et al.*, 2007). Green fluorescent spots correspond to infected cells or foci. Scale bar, 200 μ m.

could result from sieve elements, invaded by CTV in both *p23*-transgenic and empty vector control plants, the contribution of these potential contaminants was assessed by the isolation of protoplasts from leaf veins. Attempts to find protoplasts in these preparations were unsuccessful, probably as a result of the loss of cell membrane integrity during enzymatic digestion and filtering, thus showing negligible contamination, if any, of mesophyll protoplasts with those from CTV-infected phloem-associated cells. Indeed, as indicated above, no fluorescent protoplasts were observed in SO-C or SwO-C protoplast preparations. This result confirms that the ectopic expression of *p23* was sufficient to strongly modify viral tropism not only in sour orange, but also in sweet orange.

To further confirm the exit of CTV from the phloem-associated cells in *p23*-transgenic sweet and sour orange plants, we examined transverse leaf and petiole cuts by confocal microscopy. GFP fluorescence was clearly visible in a variable fraction of mesophyll cells in SO-9, SO-19, SwO-1 and SwO-12 samples, but not

in SO-C and SwO-C infected and noninoculated controls, reinforcing the concept that CTV-BC1/GFP invades the mesophyll in plants expressing *p23* ectopically. Background autofluorescence, probably caused by lignin accumulation in the wall of some cell types, was generally observed in all infected samples and in noninoculated SO-C and SwO-C controls; however, in SO-C and SwO-C samples, it was clearly restricted to the xylem, fibres and epidermis, whereas no fluorescence was observed in mesophyll cells (Fig. 6).

DISCUSSION

We have analysed the effect of the ectopic expression of *p23* on CTV infection in two natural hosts, sweet and sour orange, with different susceptibility to the virus. Data from quantitative RT-PCR, DTBIA-ELISA and microscopy showed that the virus titre increased in developing and mature phloem tissues from *p23*-transgenic sour orange in comparison with control plants trans-

Fig. 4 Confocal laser scanning microscope images of infection foci, as shown by green fluorescent protein (GFP) fluorescence, in the cambial face of bark pieces from empty vector-transformed sour orange (A), empty vector-transformed sweet orange (B) and *p23*-transgenic sour orange (C and E) plants infected with *Citrus tristeza virus* (CTV)-BC1/GFP. The marked areas in (C) and (E) are shown at higher magnification in (D) and (F), respectively, to reveal foci composed of several cells in *p23*-transgenic sour orange samples.

formed with the empty vector, mainly as a result of enhanced cell-to-cell and long-distance movement. In contrast, the ectopic expression of *p23* did not affect CTV accumulation in sweet orange phloem-associated cells, indicating that the effect of *p23* was host specific, even though *p23*-transgenic expression induces similar phenotypic aberrations in both species (Fagoaga *et al.*, 2005). We have reported previously that ectopic expression of *p23* does not affect the CTV titre in Mexican lime, a highly susceptible host in which this virus incites severe developmental aberrations (Fagoaga *et al.*, 2005).

CTV is a complex virus that accumulates in phloem-associated cells and induces different syndromes depending on the host species, thus suggesting distinct virus–host interactions. Particularly interesting is the CTV–sour orange interaction for the following reasons: (i) the virus is unable to move from cell to cell and accumulates at a very low level (Folimonova *et al.*, 2008); and (ii) severe virus strains induce the seedling yellows syndrome, characterized by stunting, small yellow leaves and growth cessation, with occasional alternate cycles of normal growth after symptom recovery (Moreno *et al.*, 2008). Limited spread of CTV infection and symptom recovery in sour orange could be a result of efficient viral resistance of this species mediated by RNA silencing. The *p23* protein has been character-

ized as an intracellular silencing suppressor in *N. benthamiana* and *N. tabacum* (Lu *et al.*, 2004), and it is most probably a determinant of the seedling yellows syndrome (Albiach-Martí *et al.*, 2010), thus making it of particular interest to examine the effect of this protein in the CTV infection of sour orange. Our finding that the ectopic expression of *p23* in transgenic sour orange enables cell-to-cell movement in phloem-associated cells and enhances systemic infection of CTV presumably results from the suppression of antiviral RNA silencing in this host. In support of this view, mutants of different viruses with dysfunctional silencing suppressors are usually unable to move and establish infections as efficiently as their wild-type counterparts (Bayne *et al.*, 2005; Deleris *et al.*, 2006; Havelda *et al.*, 2003; Kasschau and Carrington, 2001; Qu and Morris, 2002). Unfortunately, this approach cannot be extended to *p23* because it is essential for CTV replication (Satyanarayana *et al.*, 1999).

Alternatively, using an experimental design similar to that reported here, transgenic expression of silencing suppressors in *N. tabacum* and *N. benthamiana* has been shown to enhance virus accumulation and spread. More specifically, using a GFP-tagged Tobacco mosaic virus (TMV) vector, increased spread and enhanced GFP fluorescence have been observed in transgenic *N. benthamiana* expressing the gene AC2 from *African cassava*

Fig. 5 (A) Mesophyll protoplasts isolated from *p23*-transgenic sweet orange line SwO-1, sour orange line SO-9 and the corresponding SwO-C and SO-C empty vector-transformed controls, infected with CTV-BC1/GFP. Images were captured using ultraviolet (UV), blue (B) and green fluorescence (GFP) excitation and filter blocks UV-2A, B-2A and GFP-BP. (B) Reverse transcription-polymerase chain reaction (RT-PCR) amplification products obtained using primers specific to the *Citrus tristeza virus* (CTV) *p27* gene and total RNA from *p23*-transgenic or empty vector control sweet and sour orange protoplasts. Lanes 2, 3, 5 and 6, CTV-infected *p23*-transgenic SwO-1, SwO-12, SO-9 and SO-19; lanes 1 and 4, empty vector-transformed SwO-C and SO-C protoplasts; lanes 7 and 8, no-reverse transcriptase (RT) and no-template (PCR) negative controls; lane 9, PCR amplification product from pMOG-*p27* plasmid used as positive control; lane 10, DNA molecular weight marker IV (Roche Diagnostics GmbH, Mannheim, Germany). Electrophoretic separation was performed in a 1.5% agarose–ethidium bromide gel.

mosaic virus, or increased infection foci only in transgenic *N. benthamiana* expressing the gene *2b* from CMV or the gene *P1* from different sobemoviruses (Siddiqui *et al.*, 2008b). In this same study, expression of the *p19* transgene from TBSV in *N. benthami-*

ana increased the number of TMV-GFP infection foci (Siddiqui *et al.*, 2008b). Moreover, the expression of two other silencing suppressors, HC-Pro and *p25* from *Potato virus Y* (PVY) and PVX, respectively, enhanced accumulation of *Tobacco ringspot virus* in

Fig. 6 Confocal laser scanning microscope images of transverse petiole hand-cuts (approximately 1 mm width) from: (A) *p23*-transgenic sweet orange line SwO-1 and sour orange line SO-9, infected with *Citrus tristeza virus* (CTV)-BC1/GFP; (B) the corresponding SwO-C and SO-C controls transformed with the empty vector, infected with CTV-BC1/GFP; and (C) noninoculated SwO-C and SO-C controls. Images were captured with white light and green fluorescence channels. Mes, mesophyll; Ph, phloem; Xyl, xylem.

transgenic *N. benthamiana* (Siddiqui *et al.*, 2008a), and the expression of a *P1/Hc-Pro* transgene from *Turnip mosaic virus* increased the titre of this potyvirus, but did not affect the spread of a crinivirus (Wang *et al.*, 2009). Overall, these results support the concept that increased CTV accumulation and spread in sour orange phloem cells may result from silencing suppression mediated by p23. Following the same rationale, the lack of detectable effects of ectopically expressed p23 on CTV accumulation and distribution in sweet orange phloem tissues could be a result of less efficient silencing of CTV in this susceptible host, or the need for a co-ordination of p23 with the other two CTV-encoded silencing suppressors (Lu *et al.*, 2004).

Enhanced cell-to-cell movement observed in *p23*-transgenic sour orange may result from higher virus accumulation in infected phloem-associated cells, which would increase the availability of viral proteins involved in CTV movement, thus promoting virus spread to neighbouring cells; however, this hypothesis cannot explain the increased number of infection foci in *p23*-transgenic compared with control sour orange. It is also possible that strong silencing in sour orange cells could drastically limit CTV movement from both sieve tubes to companion cells and from these to neighbouring cells. On virion entry into a new cell and disassembly, stable expression of p23 in transgenic plants would strengthen CTV RNA survival and replication, thus increasing both the number and size of infectious foci. Consequently, the constitutive expression of p23 in sour orange would improve both cell-to-cell and long-distance CTV movement, even if the suppressor activity of p23 in *N. benthamiana* and *N. tabacum* is cell autonomous (Lu *et al.*, 2004).

Although some closteroviruses can eventually infect mesophyll cells in certain host species (Esau *et al.*, 1967), CTV is strictly confined to phloem cells in naturally infected citrus species. However, we have observed CTV infection in mesophyll cells of *p23*-transgenic sweet and sour orange, suggesting that the ectopic expression of p23 releases this phloem limitation in both hosts. Some phloem-limited viruses, including geminiviruses and poleroviruses, can invade mesophyll cells in mixed infections with other viruses, such as umbraviruses, potyviruses or cucumoviruses, with this change in tissue specificity having been attributed, in some cases, to efficient silencing suppression by proteins encoded by the latter viruses (Barker *et al.*, 2001; Ryabov *et al.*, 2001; Wege and Siegmund, 2007). However, these effects may depend on the presence of other virus-encoded proteins in some experimental contexts. Indeed, when the CMV 2b silencing suppressor protein was ectopically expressed in transgenic *N. tabacum* that was subsequently infected with the phloem-associated geminivirus AbMV, the 2b protein was unable to assist AbMV in exiting the vascular tissues, whereas mixed CMV and AbMV infections enabled AbMV to break phloem limitation (Wege and Siegmund, 2007). Similarly, the accumulation of the Hc-Pro silencing suppressor of *Potato virus A* (PVA) in

transgenic *N. benthamiana* plants enhanced the PLRV titre in vascular tissue, but did not alleviate its phloem limitation, whereas PLRV was found to exit the phloem in *N. benthamiana* plants doubly infected with PVA and PLRV, indicating that the phloem exit of PLRV was not necessarily related to PVA-mediated silencing suppression (Savenkov and Valkonen, 2001).

By contrast, our results showed that p23 alone was sufficient to alleviate phloem limitation and allowed CTV to invade mesophyll cells, further supporting a potential role of p23 as a silencing suppressor in its natural hosts. In addition, the finding that the ectopic expression of p23 induced different responses to CTV infection in sweet and sour orange suggests a complex interplay between p23 and other virus- and host-encoded proteins for crossing distinct cell boundaries. Indeed, we cannot discount the possibility that p23 activity unrelated to silencing suppression in citrus may be responsible for or collaborate in CTV egression from the vascular system in both sweet and sour orange hosts. In this regard, agro-expression of the fusion protein p23-GFP in *N. benthamiana* plants results in fluorescence accumulation in the nucleolus and Cajal bodies, as well as in plasmodesmata, suggesting that p23 could be involved in CTV cell-to-cell and long-distance movement (S. Ruiz-Ruiz *et al.*, unpublished results).

As the antiviral and developmental RNA silencing pathways regulated by small interfering RNAs and microRNAs partially overlap (Dunoyer *et al.*, 2004), and p23 binds RNA nonspecifically (López *et al.*, 2000), the CTV-like symptoms observed in p23-transgenic lime, sweet and sour orange, and *Poncirus trifoliata*, but not in *N. tabacum* or *N. benthamiana* (Fagoaga *et al.*, 2005), could result from the interference of p23 with these pathways specifically in citrus. Recently, it has been shown that CTV infection not only incites the accumulation of virus-derived small RNAs, but also affects the host small RNA profile (Ruiz-Ruiz *et al.*, 2011). In a natural context, the decline of sweet orange and other species propagated on sour orange rootstock, the syndrome known as tristeza, results from an incompatibility reaction produced at the bud union line when CTV accumulating at a high titre in a susceptible scion is downloaded into sour orange that is very sensitive to CTV accumulation (Folimonova *et al.*, 2008; Moreno *et al.*, 2008). As the ectopic expression of p23 allows sour orange to accumulate as much CTV as sweet orange, it will be worth testing whether a p23-transgenic sour orange rootstock shows a decline-tolerant response.

EXPERIMENTAL PROCEDURES

Citrus hosts, virus strains and inoculations

Sour orange transgenic lines 9 and 19 (SO-9 and SO-19) and sweet orange transgenic lines 1 and 12 (SwO-1 and SwO-12), carrying the CTV p23 gene, and the corresponding control plants transformed with an empty vector (SO-C and SwO-C, respec-

tively), have been produced and characterized molecularly and phenotypically previously (Fagoaga *et al.*, 2005). Western blot analysis showed that SO-19 and SwO-12, and SO-9 and SwO-1, expressed similar amounts of p23 (Fagoaga *et al.*, 2005; Fig. S2, see Supporting Information). The p23-transgenic plants showed CTV-like symptoms, including vein clearing, epinasty and stunting, albeit with aberrant and normal flushes alternating during plant growth, thus allowing selected lines to survive. SO-9 was recovered from aberrant flushes during the course of this work, and was therefore used only in some inoculation experiments.

In each experiment, three to seven buds from p23-transgenic and empty vector control lines were propagated onto Troyer citrange [*C. sinensis* (L.) Osb. × *P. trifoliata* (L.) Raf.] rootstock, and the plants were maintained in a contained glasshouse at 24–26/15–16 °C day/night temperature with 60%–80% relative humidity and natural light. When the shoots were approximately 40 cm long, the plants were graft inoculated onto rootstock with either the mild CTV strain T385 (Moreno *et al.*, 1993) or CTV-BC1/GFP (kindly provided by Dr W. O. Dawson, Citrus Research and Education Center, Lake Alfred, FL, USA), a clonal strain carrying the GFP gene between the p27 and p25 genes in the CTV T36 genome (Folimonov *et al.*, 2007). Three months after inoculation, one of the two bark chips used to inoculate each plant was removed and the presence of the virus in this tissue was confirmed by RT-PCR using primers specific for the CTV 3' UTR (Domínguez *et al.*, 2002).

Quantitative real-time RT-PCR analysis

Six young leaves were collected from different parts of each infected plant during the first spring flush of the third and fourth years after inoculation with CTV T385, or the second and third years after inoculation with CTV-BC1/GFP. These long periods allowed detection problems derived from the low titre and uneven distribution of CTV T385 and T36 in sour orange to be overcome (Comellas, 2009). Total nucleic acids were extracted from 50 mg of pooled leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and DNA was removed with RNase-free DNase I digestion (Qiagen). RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (TaqMan® Reverse Transcription Reagents, Applied Biosystems International, Foster City, CA, USA).

Primers and fluorogenic TaqMan probe were designed on the gene p27 sequence of CTV T36 (Satyanarayana *et al.*, 2001; accession number AY170468) using Primer Express™ software Version 1.5. The forward primer TM-p27CTV-F was 5'-CTCCGAA CATGAACGAGGTATCA-3' (nucleotide positions: 15 941–15 963), the reverse primer TM-p27CTV-R was 5'-TAGACGTCAA GGTACGAGGAGG-3' (nucleotide positions: 15 992–16 014) and the TaqMan probe was 6FAM-AGCGTCCGAGTCTATGTTAG (nucleotide positions: 15 971–15 990). These primers and probe

also served for quantitative RT-PCR detection of CTV T385 (accession number Y18420). Taq-Man real-time PCR assays were carried out with the ABI Prism 7000 platform using a MicroAmp Optical 96-well reaction plate, and data were analysed with Sequence Detection System software (Applied Biosystems International). The amplification reaction cocktail contained $1 \times$ TaqMan Universal PCR Master Mix, $10 \times$ working stock of Gene Expression Assay Mix containing primers and probe (Applied Biosystems International) and $5 \mu\text{L}$ of cDNA template plus RNase-free water in a total reaction volume of $25 \mu\text{L}$. Reactions comprised an initial step of 2 min at 50°C , followed by 10 min at 95°C , and then 40 cycles of amplification including 15 s at 95°C and 1 min at 60°C .

Plasmid pMOG 180 containing one complete copy of the gene *p27* (pMOG-*p27*) was used to generate a standard curve for CTV cDNA quantification (Pfaffl and Hageleit, 2001) comprising seven log units of CTV *p27* concentration in the linear range. For each run, all samples (including controls without reverse transcription and template, and all points of the standard curve) were analysed at least in triplicate, and data were expressed as the average number of estimated CTV cDNA target copies per nanogram of total RNA $\pm$ standard error (SE).

DTBIA-ELISA analysis

The presence and accumulation of CTV in leaf tissues was checked by DTBIA-ELISA (Garnsey *et al.*, 1993) using a mixture of 3DF1 and 3CA5 CTV-specific monoclonal antibodies (Vela *et al.*, 1986). Ten young leaves (including petioles) were collected from each CTV-infected or noninoculated *p23*-transgenic and empty vector control plant. The antigen was immobilized by carefully pressing fresh leaf blade or petiole sections on $0.45\text{-}\mu\text{m}$ nitrocellulose membranes (Millipore Corporation, Bedford, MA, USA). Immunodetection was performed on imprinted sections using a commercial kit (Plant Print Diagnostics, Valencia, Spain) according to the manufacturer's instructions. Membranes were washed in water, dried and examined under a binocular microscope at $5\text{--}20\times$ magnification.

Examination of fluorescence in plants infected with GFP-tagged CTV

Samples of bark tissue from CTV-BC1/GFP-infected *p23*-transgenic and control plants were cut from branches at the second and third years post-inoculation. The internal surface of the bark pieces was examined for GFP fluorescence using a stereomicroscope equipped with a Leica Fluorescence Module MZ 16FA with a light source HBO 100-W high-pressure mercury bulb, without blocking the red autofluorescence from chlorophyll with any interference filter. Photographs were taken with a Leica

DC500 digital camera using a 3–4-s exposure and processed with Leica IM software (Image Manager Version 4.0-R117). More detailed observations of the infection foci in longitudinal bark sections and in transverse leaf and petiole hand-cuts (approximately 1 mm width) of infected samples were performed using a Leica TCS SLI confocal scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). At least 20 samples per plant and three plants per *p23*-transgenic or control line were analysed in each experiment.

CTV detection in mesophyll protoplasts by GFP observation and RT-PCR analysis

Mesophyll protoplasts from different propagations of SO-9, SO-19, SwO-1 and SwO-12 transgenic lines, and SO-C and SwO-C controls, were isolated according to Grosser and Gmitter (1990) with some modifications (Pensabene-Bellavia, 2009). Briefly, young leaves selected from glasshouse-grown plants were surface disinfected by immersion in 20% commercial bleach for 10 min and rinsed three times with sterile distilled water. Leaves were cut into thin strips with a sharp scalpel, removing major veins, and green slices were incubated in 60-mm Petri dishes with a mixture of 2.5 mL of BH_3 0.6 M medium (Grosser and Gmitter, 1990) and 1.5 mL of enzyme solution (Pensabene-Bellavia, 2009). In a separate experiment, protoplast preparation from leaf veins dissected previously was also attempted. Protoplast purification was carried out by filtering digested leaf tissue mixtures through a $45\text{-}\mu\text{m}$ stainless steel mesh screen, followed by the separation of protoplasts by centrifugation in a 26% sucrose–13% mannitol cushion. The protoplast yield was evaluated with a Fuchs–Rosenthal haematocytometer chamber and the suspension was adjusted to 4×10^6 protoplasts/mL in a 13% mannitol solution. GFP fluorescence in purified protoplasts was examined in a Nikon Eclipse E800 microscope under ultraviolet (UV), blue (B) and green fluorescence (GFP) excitation, using specific filter sets (UV-2A, B-2A and GFP-BP, respectively). The experiment was repeated five times.

For CTV detection by RT-PCR, protoplast aliquots (4×10^6 protoplasts/mL) were centrifuged for 1 min at $11\,100\text{ g}$ and 4°C , and the pellet was used for RNA extraction with the RNeasy™ Plant Mini Kit (Qiagen) following the manufacturer's recommendations. RT-PCR analyses were performed with primers 5'-ATATATGGCCCAGCCGCCATGGCAGGTTATACAG-3' and 5'-GTGCGGCGTCGACCTATAAGTACTTACCCAAAT-3', corresponding to nucleotide positions 15 336–15 351 and 16 039–16 059, respectively, in the CTV T36 sequence. cDNA was synthesized from DNase-treated total nucleic acid preparations with Superscript II Reverse Transcriptase (Invitrogen, Prat de Llobregat, Barcelona, Spain), and PCR amplification included an initial step of 5 min at 94°C , followed by 35 cycles of 30 s at 95°C , 1 min at 54°C and 2 min at 72°C .

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Detail of mesophyll protoplasts isolated from leaves of *p23*-transgenic (SO-19) and empty vector-transformed (SO-C) sour orange observed under ultraviolet (UV) (A) or blue (B) excitation with two different filter blocks (UV-2A and B-2A). Bar, 10 µm.

Fig. S2 Western blot analysis of p23 in *p23*-transgenic sweet orange 1 (SwO-1) and sour orange 9 (SO-9) citrus lines, and in SwO-C and SO-C empty vector-transformed lines. CTV T36 refers to an empty vector-transformed lime infected with CTV T36.

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