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Transformation of Mexican lime with an intron-hairpin construct expressing untranslatable versions of the genes coding for the three silencing suppressors of *Citrus tristeza virus* confers complete resistance to the virus

Nuria Soler[1], Montserrat Plomer[1], Carmen Fagoaga[1], Pedro Moreno[1], Luis Navarro[1], Ricardo Flores[2] and Leandro Peña[1,*]

[1]*Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain*
[2]*Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, Valencia, Spain*

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**Correspondence* (Tel +34 963424000; fax +34 963424001; email lpenya@ivia.es)

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Summary

Citrus tristeza virus (CTV), the causal agent of the most devastating viral disease of citrus, has evolved three silencing suppressor proteins acting at intra- (p23 and p20) and/or intercellular level (p20 and p25) to overcome host antiviral defence. Previously, we showed that Mexican lime transformed with an intron-hairpin construct including part of the gene *p23* and the adjacent 3′ untranslated region displays partial resistance to CTV, with a fraction of the propagations from some transgenic lines remaining uninfected. Here, we transformed Mexican lime with an intron-hairpin vector carrying full-length, untranslatable versions of the genes *p25*, *p20* and *p23* from CTV strain T36 to silence the expression of these critical genes in CTV-infected cells. Three transgenic lines presented complete resistance to viral infection, with all their propagations remaining symptomless and virus-free after graft inoculation with CTV-T36, either in the nontransgenic rootstock or in the transgenic scion. Accumulation of transgene-derived siRNAs was necessary but not sufficient for CTV resistance. Inoculation with a divergent CTV strain led to partially breaking the resistance, thus showing the role of sequence identity in the underlying mechanism. Our results are a step forward to developing transgenic resistance to CTV and also show that targeting simultaneously by RNA interference (RNAi) the three viral silencing suppressors appears critical for this purpose, although the involvement of concurrent RNAi mechanisms cannot be excluded.

Introduction

Citrus is the most economically important fruit tree crop worldwide, with more than 7.6 million hectares grown and about 110 million tons of fruit produced in 2009 (FAO 2010, http://www.fao.org/es/esc/common/ecg/243/es/bull2006.pdfs). *Citrus tristeza virus* (CTV), a member of the genus *Closterovirus*, family *Closteroviridae*, is the causal agent of devastating epidemics that have changed the course of the citrus industry (Moreno *et al.*, 2008). CTV only infects phloem-associated tissues of species of the genera *Citrus* and *Fortunella* within the family *Rutaceae* (Bar-Joseph *et al.*, 1989). In plants propagated on sour orange (*Citrus aurantium* L.) rootstock, CTV produces in some cases a bud-union disease known as tristeza, which has caused decline and death of about 100 million citrus trees grown on this rootstock. Some CTV isolates incite the seedling yellows syndrome, consisting of stunting, yellowing and growth cessation of infected sour orange, lemon (*Citrus limon* (L.) Burn. f.) or grapefruit (*Citrus paradisi* Macf.) seedlings. Others may cause stem pitting on sweet orange (*Citrus sinensis* (L.) Osb.), grapefruit and Mexican lime (*Citrus aurantifolia* (Christ.) Swing.) or Tahiti lime (*Citrus latifolia* Tan.) scion varieties regardless of the rootstock, reducing vigour, yield and fruit quality (Moreno *et al.*, 2008).

Citrus tristeza virus virions are filamentous particles of about 2000 × 11 nm in size that are composed of two capsid proteins of 25 and 27 kDa and a single-stranded, plus-sense genomic RNA (gRNA) of approximately 19.3 kb, organized in 12 open reading frames (ORFs) potentially encoding at least 17 protein products, and two 5′ and 3′ untranslated regions (UTRs) (Karasev *et al.*, 1995). The two 5′-proximal ORFs (1a and 1b) encoding replication-related proteins are translated directly from the gRNA, and the ten ORFs located in the 3′ portion of the genome are expressed through a set of 3′ coterminal subgenomic RNAs (Hilf *et al.*, 1995) that encode proteins p33, p6, p65, p61, p27, p25, p18, p13, p20 and p23 (Karasev *et al.*, 1995; Pappu *et al.*, 1994). Proteins p6, p65, p61, p27 and p25 are part of a block conserved in all closteroviruses that is involved in virion assembly and movement (Dolja *et al.*, 2006). The small hydrophobic protein p6 is proposed to act as a transmembrane anchor, and p25 and p27 are the major and minor coat proteins, respectively. While p25 encapsidates about 97% of the gRNA, the 5′-terminal 650 nucleotides are encapsidated by p27 (Febres *et al.*, 1996; Satyanarayana *et al.*, 2004), in cooperation with p65 and p61 (Satyanarayana *et al.*, 2000). The p20 protein accumulates in amorphous inclusion bodies of CTV-infected cells (Gowda *et al.*, 2000). The p23 protein is a

RNA-binding protein with a Zn-finger domain (López *et al.*, 2000) that regulates the balance of plus and minus RNA strands during replication (Satyanarayana *et al.*, 2002). Additionally, p23 is a pathogenicity determinant likely involved in the seedling yellows syndrome (Albiach-Martí *et al.*, 2010) that when ectopically expressed in transgenic citrus plants induces aberrations resembling CTV symptoms in some hosts (Ghorbel *et al.*, 2001; Fagoaga *et al.*, 2005), facilitates CTV escaping from the phloem in sweet and sour orange and increases virus accumulation in the latter host (Fagoaga *et al.*, 2011). Proteins p23, p20 and p25 act as RNA silencing suppressors in *Nicotiana tabacum* and *Nicotiana benthamiana*, with p25 acting intercellularly, p23 intracellularly and p20 at both levels (Lu *et al.*, 2004). Proteins p33, p13 and p18 are required to systemically infect some citrus hosts but not others (Tatineni *et al.*, 2008, 2011).

Breeding for resistance to CTV in scion varieties has been largely ineffective, mainly because of the complex reproductive biology of citrus. The only successful results in this respect are the hybrid rootstocks citranges [sweet orange × *Poncirus trifoliata* (L.) Raf.] and citrumelos (grapefruit × *P. trifoliata*) widely used by the citrus industry owing to their tolerance to CTV-induced decline. Cross-protection with mild CTV strains is the only available possibility to protect susceptible commercial varieties from CTV isolates inducing stem pitting; however, with the exception of Sao Paulo State (Brazil) (Costa and Müller, 1980) and South Africa (van Vuuren *et al.*, 1993), this protection is variable in most other citrus areas and depends on the citrus scion varieties, the predominant CTV strains and the environmental conditions (Cox *et al.*, 1976; da Graça *et al.*, 1984; Ieki and Yamaguchi, 1988). Pathogen-derived resistance could be a better and more predictable strategy to achieve durable resistance to CTV in transgenic citrus. However, ectopic expression in transgenic Mexican lime of genes *p23* or *p25*, untranslatable or truncated versions of the latter and sense or antisense constructs of the 3′-terminal 549 nucleotides of the gRNA (including part of *p23* and the 3′-UTR) only yielded partial resistance, with a fraction of plants propagated from some lines showing immunity, or attenuated or delayed symptom appearance upon graft or aphid inoculation with CTV (Domínguez *et al.*, 2002a,b; Fagoaga *et al.*, 2006; López *et al.*, 2010). Resistance was associated with RNA silencing of the transgene (Fagoaga *et al.*, 2006; López *et al.*, 2010), and Mexican lime was chosen as a citrus model in these studies because its high sensitivity to CTV, with the potential resistance of transgenic plants being easily assessed by symptom observation in the greenhouse within a year after challenging.

It is generally accepted that RNA silencing explains many cases of genetic defence against viral infection and cross-protection between closely related virus strains (Covey *et al.*, 1997; Dougherty *et al.*, 1994; Lindbo and Dougherty, 1992; Ratcliff *et al.*, 1997, 1999). RNA silencing is induced by double-stranded RNA (dsRNA) or highly structured single-stranded RNA (ssRNA) and ultimately leads to a sequence-specific ssRNA degradation through generation of 21- to 25-nt short-interfering RNAs (siRNAs) by RNase III-like enzymes called Dicers (Bernstein *et al.*, 2001). While one siRNA strand is degraded, the other is incorporated into the Argonaute-containing RNA-induced silencing complex and guides it for cleavage or translational arrest of ssRNAs with sequence complementarity (Csorba *et al.*, 2009; Hammond *et al.*, 2000). RNA interference (RNAi), an approach based on using dsRNA to trigger RNA silencing (Fire *et al.*, 1998), has been exploited in plants by genetic transformation with sense and antisense cDNAs derived from the target viral sequence separated by an intron (intron-hairpin constructs) (Smith *et al.*, 2000).

Using this strategy, virus and viroid-resistant transgenic crop plants have been produced (Prins *et al.*, 2008), as illustrated by some representative examples. Expressing an intron-hairpin construct derived from *Potato spindle tuber viroid* (PSTVd) in transgenic tomato results in resistance to PSTVd infection (Schwind *et al.*, 2009), and targeting by RNAi the gene encoding the nonstructural protein Pns12 of *Rice dwarf virus*, a member of the genus *Phytoreovirus*, provides strong resistance to viral infection in transgenic rice (Shimizu *et al.*, 2009). Within the family *Geminiviridae,* transgenic expression in common bean of an intron-hairpin construct against the replication initiation gene *AC1* of *Bean golden mosaic virus*, genus *Begomovirus*, affords high resistance to virus infection under field conditions (Aragão and Faria, 2009). Finally, RNAi targeting the coat protein gene of *Cassava brown streak Uganda virus* (CBSUV), genus *Ipomovirus,* family *Potyviridae*, results in most lines of transgenic cassava displaying full resistance to virus challenge by graft inoculation (Yadav *et al.*, 2011).

Citrus hosts have developed a strong antiviral response to CTV infection through RNA silencing, as inferred from the high accumulation of CTV-specific small RNAs of 21–25 nt in infected tissues (Fagoaga *et al.*, 2006; Ruiz-Ruiz *et al.*, 2011). As a counterdefence, CTV encodes three silencing suppressor proteins (see above), suggesting complex virus–host interactions in the course of infection. Therefore, searching for RNAi-induced resistance against CTV in transgenic citrus plants has been challenging. Transgenic citrus plants expressing different gene segments (Febres *et al.*, 2007, 2008), or a intron-hairpin construct of gene *p23* (Batuman *et al.*, 2006), failed to provide durable resistance to CTV. Furthermore, over the last 15 years, we have produced more than 300 independent lines carrying different CTV-derived sequences, with resistance to CTV challenge being observed only in some propagations of certain lines (Domínguez *et al.*, 2002a,b; Fagoaga *et al.*, 2006; López *et al.*, 2010). The highest protection (9%–56%) was achieved with an intron-hairpin construct of the 3′-terminal 549 nucleotides of the CTV genome comprising part of gene *p23* and the 3′-UTR. (López *et al.*, 2010). Here, we have extended this approach by transforming Mexican lime with a vector carrying full untranslatable versions of genes *p25, p20* and *p23* plus the 3′-UTR in sense and antisense orientation separated by an intron [Sense-Intron-AntiSense (SIAS)]. This strategy provides the best level of resistance against CTV achieved in citrus so far, because all clonal propagations from some transgenic lines resulted immune when challenged by graft inoculation with homologous CTV strains.

Results

The SIAS construct interferes the silencing suppressor activity of CTV proteins *p20* and *p23* in transient expression assays in *Nicotiana benthamiana*

To get a first insight into the potential of the SIAS construct, the SIAS fragment was cloned into the binary plasmid pCAMBIA 2301 under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) (35S-pro) and the nopaline synthase terminator (*nos*-ter). This expression cassette was flanked by the selectable gene marker neomycin phosphotransferase II (*nptII*), between the 35S-pro and the 35S terminator (35S-ter), and by

the reporter gene β-glucuronidase (*uidA*), between the 35S-pro and the *nos*-ter (Figure 1a). The ability of the SIAS construct to interfere with the silencing suppressor activity of the CTV proteins p23 and p20 was tested by transient expression assays in the transgenic *N. benthamiana* line 16c constitutively expressing the gene for the green fluorescent protein (*gfp*) (Ruiz *et al.*, 1998), essentially as described by Lu *et al.* (2004).

More specifically, we examined *N. benthamiana* 16c leaves after infiltration with a culture of *Agrobacterium tumefaciens* transiently expressing GFP (to induce silencing of the transgene *gfp*) or after co-infiltration with a second culture transiently expressing the CTV silencing suppressors p23 (GFP + p23) or p20 (GFP + p20) alone (to counteract silencing of *gfp*), or plus a third culture transiently expressing the SIAS construct (GFP + p23 + SIAS) or (GFP + p20 + SIAS) (to interfere with intracellular suppression mediated by p23 or p20). At 3 days postinfiltration (dpi), expression of the transgene *gfp* was silenced in *N. benthamiana* 16c agroinfiltrated with only GFP,

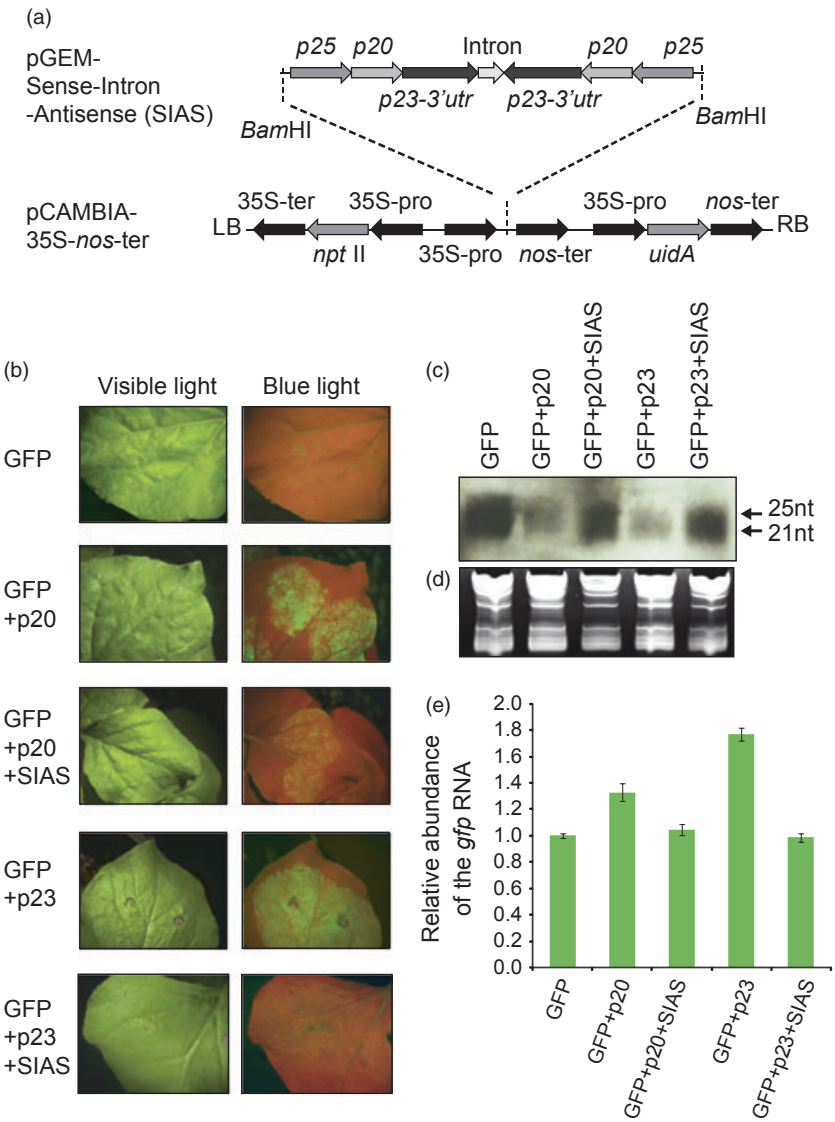

Figure 1 (a) Schematic representation of plasmid pGEM-SIAS carrying the *p25*, *p20* and *p23-3'*UTR fragments of the genomic RNA of CTV-T36 in intron-hairpin configuration, and subcloning of the cassette into the binary vector pCAMBIA-35S-*nos*-ter. The Sense-Intron-Antisense (SIAS) sequence is controlled by the 35S promoter (35S-pro) of CaMV and the *nopaline synthase* terminator (*nos*-ter) and flanked by the gene for *neomycin phosphotransferase II* (*nptII*) between the 35S-pro and 35S terminator (35S-ter) and by the gene for β-glucuronidase (*uidA*) between the 35S-pro and the *nos*-ter. (b) *Agrobacterium tumefaciens* infiltration assay. Leaves of *Nicotiana benthamiana* 16c expressing GFP were infiltrated with equal volumes of *A. tumefaciens* cultures carrying either p35S-GFP + pBin19 (GFP), p35S-GFP + pBin19 + pBin19-p23 (GFP + p23), p35S-GFP + pBin19 + pBin19-p20 (GFP + p20), p35S-GFP + pBin19-p23 + pCAMBIA-SIAS (GFP + p23 + SIAS) or p35S-GFP + pBin19-p20 + pCAMBIA-SIAS (GFP + p20 + SIAS). Images were taken at 3 days postinfiltration (dpi) under visible (left) or blue (right) light. (c) Accumulation of *gfp*-specific siRNAs extracted from the agroinfiltrated leaf areas at 3 dpi, separated by 20% polyacrylamide gel electrophoresis, electroblotted onto a nylon membrane and hybridized with a DIG-labelled riboprobe for detecting the negative strand of gene *gfp*. Arrows indicate the positions of synthetic siRNAs of 21 and 25 nt. (d) Ethidium bromide–stained gel used as control for RNA load. (e) Accumulation of *gfp* RNA in agroinfiltrated leaf areas as determined by qRT-PCR. An expression value of 1 was arbitrarily assigned to leaf areas agroinfiltrated with p35S-GFP, and the other values were referred to it. Data are means ± SD of three experimental replicates. CTV, *Citrus tristeza virus*.

as revealed by the low fluorescence and accumulation of *gfp*-specific siRNAs (Figure 1b,c; GFP). As expected, in leaves co-infiltrated with GFP + p23 or GFP + p20, the fluorescence was intense because of the suppression of *gfp* silencing by p23 or p20 (Figure 1b; GFP + p20 and GFP + p23) (Lu *et al.*, 2004); this enhanced fluorescence was accompanied by reduced accumulation of *gfp*-specific siRNAs (Figure 1c; GFP + p23 and GFP + p20) and increased levels of *gfp* RNA in comparison with leaves agroinfiltrated with only the GFP construct (Figure 1e; GFP + p23 and GFP + p20). When plants were co-infiltrated with *A. tumefaciens* cultures with the SIAS construct and either GFP + p23 or GFP + p20, silencing suppression of the transgene was transiently reversed: at 3 dpi, leaves showed reduced GFP fluorescence (Figure 1b), higher accumulation of *gfp*-derived siRNAs (Figure 1c) and decreased *gfp* RNA levels (Figure 1e), with this reversion of the suppression induced by p23 or p20 becoming almost undetectable at 6 dpi (data not shown). Interestingly, at 3 dpi, *gfp* RNA and siRNA levels and GFP fluorescence in leaves infiltrated with each of the triple mixtures of *A. tumefaciens* cultures were comparable to those observed in leaves infiltrated with the GFP construct alone to trigger GFP silencing. Altogether these results indicated that the SIAS construct efficiently interfered with the intracellular suppression activity of p23 and p20 in *N. benthamiana* and that it might also block these two silencing suppressors in CTV-inoculated transgenic citrus.

Genetic transformation and molecular characterization of transgenic Mexican lime expressing the SIAS construct

Mexican lime internodal stem segments were transformed with *A. tumefaciens* harbouring either pCAMBIA-SIAS or the pCAMBIA 2301 empty vector (EV). Regenerated shoots were selected in a culture medium containing kanamycin and tested for histochemical GUS activity in small tissue fractions, with the explants transformed with the SIAS vector being analysed for transgene integration by PCR with primers P25*mutF/IntronR and IntronF/P25*mutF. Fifteen transgenic plants were recovered for each construct (data not shown), the transgene loci number and integrity of which were evaluated by restriction analysis and Southern blot hybridization with a *p23*-specific riboprobe (Figure S1). DNA restriction with *Eco*RI and *Hin*dIII revealed that ten transgenic lines contained at least one intact copy of the whole expression cassette. Two to six transgene DNA loci integrations were estimated for these lines according to the digestion pattern observed with *Eco*RI and, in at least five of these lines, several copies of the SIAS cassette appeared truncated because bands smaller than 6 kb were detected (Figure S1), a result that was confirmed by digestion with *Eco*RI and *Hin*dIII (data not shown).

The extent of transgene silencing was assessed by Northern blot analysis of siRNAs derived from *p25*, *p20* and *p23*, with most transformants showing high siRNA levels of the three transgene fragments, sometimes close to those shown by EV control limes infected with CTV-T36. The signal intensity generated by siRNAs in the different transgenic lines was similar when hybridized with either of the three probes and also in different propagations of the same transgenic line (data not shown), with the exceptions of line 2, which showed consistently higher *p20*- and *p23*-derived siRNA levels in different propagations and seasons, and lines 39 and 46 that did not accumulate detectable siRNA levels (Figure 2).

The SIAS construct confers immunity against CTV to graft-inoculated transgenic Mexican lime

Transgenic lines SIAS-1, SIAS-2, SIAS-26, SIAS-28 and SIAS-41, accumulating large amounts of transgene-derived siRNAs, and transgenic lines SIAS-39 and SIAS-46, with nondetectable levels of siRNAs (Figure 2), were selected for challenge inoculation with CTV after propagation on Carrizo citrange rootstock. Uniform propagations of the seven transgenic lines and of the corresponding EV transgenic control were graft-inoculated with CTV-T36 on the rootstock. Virus accumulation in young leaves was assessed by indirect DAS-ELISA in three consecutive flushes spanning over a 1-year period, and symptom development in the same flushes was rated in a 0–3 scale (0, no symptoms and 3, very severe symptoms). The 17 EV control propagations inoculated resulted infected and expressed symptoms in the first flush postinoculation, whereas all propagations from lines SIAS-2, SIAS-26 and SIAS-28 (10, 12 and 11, respectively) were resistant, neither accumulating CTV nor developing symptoms. Two of the 11 propagations of line SIAS-1 and one of the ten propagations of line SIAS-41 reacted positively to DAS-ELISA in the first flush postinoculation and their symptoms were comparable to those shown by the EV controls. Additionally, one propagation from each of lines SIAS-1 and SIAS-41 became DAS-ELISA positive and started showing mild symptoms in the third flush, while the remaining propagations of both lines remained unin-

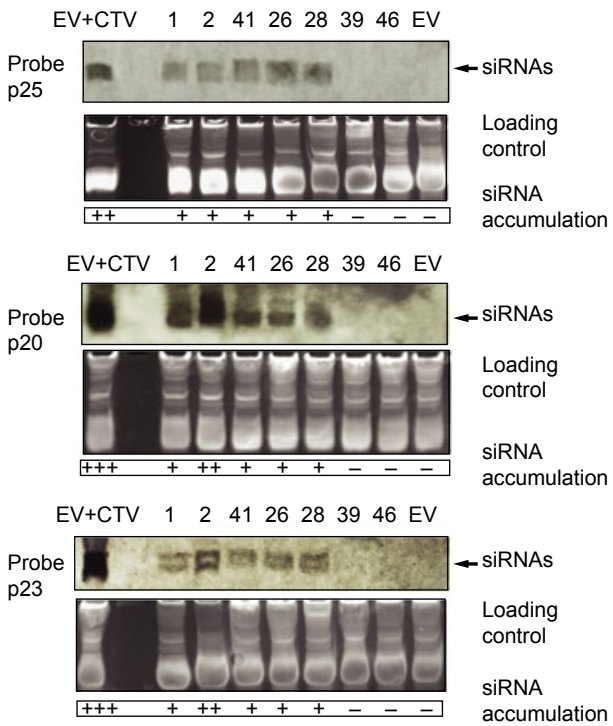

Figure 2 Accumulation of transgene-derived siRNAs in seven Sense-Intron-AntiSense (SIAS) transgenic lines (SIAS-1, SIAS-2, SIAS-41, SIAS-26, SIAS-28, SIAS-39 and SIAS-46). Northern blot analyses were performed with DIG-labelled riboprobes for detecting the positive strand of genes *p23*, *p20* or *p25*. Empty vector (EV) and EV + CTV correspond to noninoculated and CTV-inoculated controls transformed with the EV. siRNA accumulation ranged from undetectable (–) to high (+++). GelRed-stained gels were used as control for RNA loading. CTV, *Citrus tristeza virus*.

fected. The ten propagations from lines SIAS-39 and SIAS-46 showed virus titre and symptoms comparable to those of the EV controls starting from the first flush (Figures 3 and S2). These results indicated that (i) resistance to CTV was associated with the accumulation of transgene-derived siRNAs prior to infection, (ii) the strength of resistance, however, was not directly associated with siRNA levels (Figure 2) and (iii) response to CTV challenge did not depend on either the transgene loci number in the plant genome or the integration of truncated T-DNA copies (Figure S1).

To further characterize the resistance of lines SIAS-2, SIAS-26 and SIAS-28, propagations from these lines and the EV control were graft-inoculated directly in the scion with CTV947R-GFP (kindly provided by Dr W.O. Dawson, University of Florida), a clonal CTV-T36 strain carrying the transgene *gfp* between genes *p27* and *p25* (Ambrós *et al.*, 2011; Folimonov *et al.*, 2007). The biological characteristics of CTV947R-GFP in citrus trees are essentially identical to those of wild-type T36, but it produces GFP fluorescence in infected cells that enables monitoring virus distribution in citrus tissues. GFP foci in the inoculated EV control propagations were detected in the first flush, about 3 weeks after inoculation, and appeared widely distributed in stem bark as well as in young leaves. In contrast, none of the propagations from lines SIAS-2, SIAS-26 and SIAS-28 inoculated with CTV947R-GFP showed GFP foci in stem bark, leaf and petiole tissues (Figure 4). Two months after inoculation, one of the two bark chips used to inoculate each propagation was removed and examined for GFP expression. Fluorescence was intense in all bark chips, thus confirming that a high fraction of phloem-associated cells were virus-infected in the *Citrus macrophylla* donor plants. Moreover, discrete GFP foci were also observed in transgenic tissues in direct contact with the inoculum bark chip, indicating CTV movement to neighbour cells. However, viral infection did not progress further in propagations from these three SIAS lines, as reflected by the lack of GFP fluorescence in more than 50 leaf and stem bark samples per propagation analysed in the second and third flushes after inoculation, contrasting with the wide virus distribution observed in EV control propagations. The absence of GFP fluorescence in all propagations from SIAS-2, SIAS-26 and SIAS-28 transgenic lines strongly supports that they are immune to CTV challenge inoculation.

RNAi-mediated resistance depends on sequence identity between the transgenic construct and the challenging CTV strain

RNAi-mediated resistance to several plant viruses has been shown to rely on nucleotide identity between the challenging virus genome and the transgene-derived RNA (Hamilton and Baulcombe, 1999; Li and Ding, 2006; Lindbo *et al.*, 1993; Prins *et al.*, 2008; Voinnet *et al.*, 1999). The 3′-half of CTV genome, particularly ORFs *p25*, *p20* and *p23*, is relatively conserved, with difference between the most dissimilar strains amounting to 10% (Martín *et al.*, 2009; Mawassi *et al.*, 1996; Pappu *et al.*, 1993). To examine the importance of sequence identity on CTV resistance of the SIAS transformants, the CTV isolate T318A, with nucleotide identities with T36 of 92% (for *p25*) and 91% (for *p20* and *p23*), was used to graft-inoculate propagations of lines SIAS-2 and SIAS-41 showing total or partial protection against CTV-T36, respectively. CTV-T318A is a virulent strain causing very severe symptoms on Mexican lime that include vein corking, stem pitting and pronounced stunting. All propagations of the EV control and SIAS-41 lines resulted infected and showed severe symptoms, indicating that the partial resis-

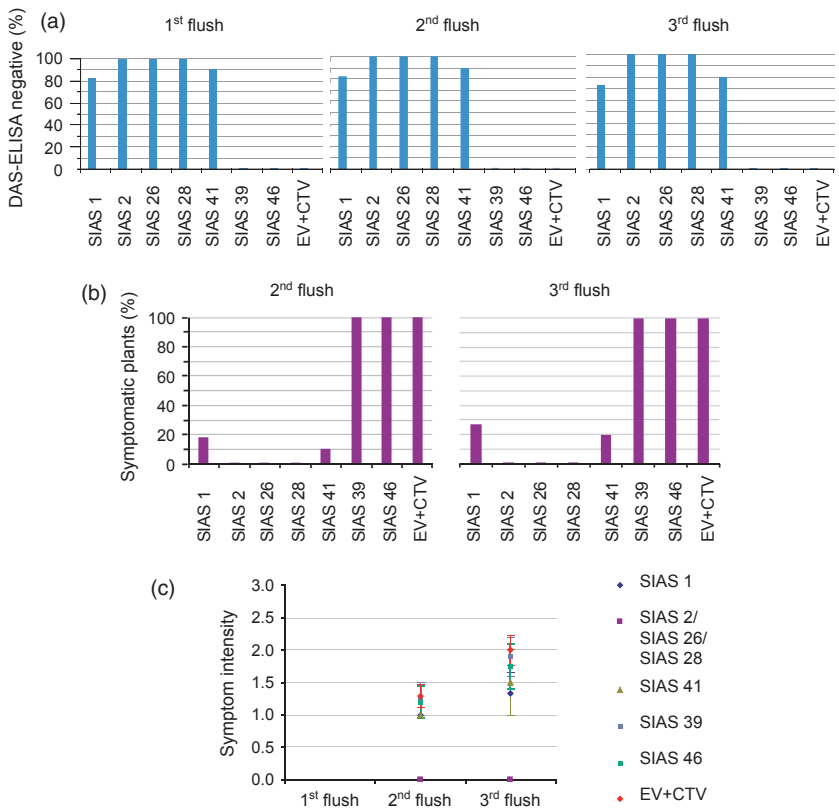

Figure 3 Evaluation of viral resistance in propagations from Sense-Intron-AntiSense (SIAS) transgenic lines 1, 2, 26, 28, 41, 39 and 46, or empty vector (EV) after graft inoculation with the CTV-T36 clonal strain. (a) Fraction (in %) of DAS-ELISA-negative propagations of each transgenic line in three consecutive flushes. (b) Fraction (in %) of symptomatic propagations of each transgenic line. (c) Symptom intensity in CTV-infected plants estimated in a 0–3 scale with 0 indicating the absence of symptoms, 1 mild vein clearing, 2 moderate vein clearing, epinasty of young leaves and leaf cupping of adult leaves and 3 severe symptoms including vein corking and stunting. Vertical bars indicate SE. CTV, *Citrus tristeza virus*.

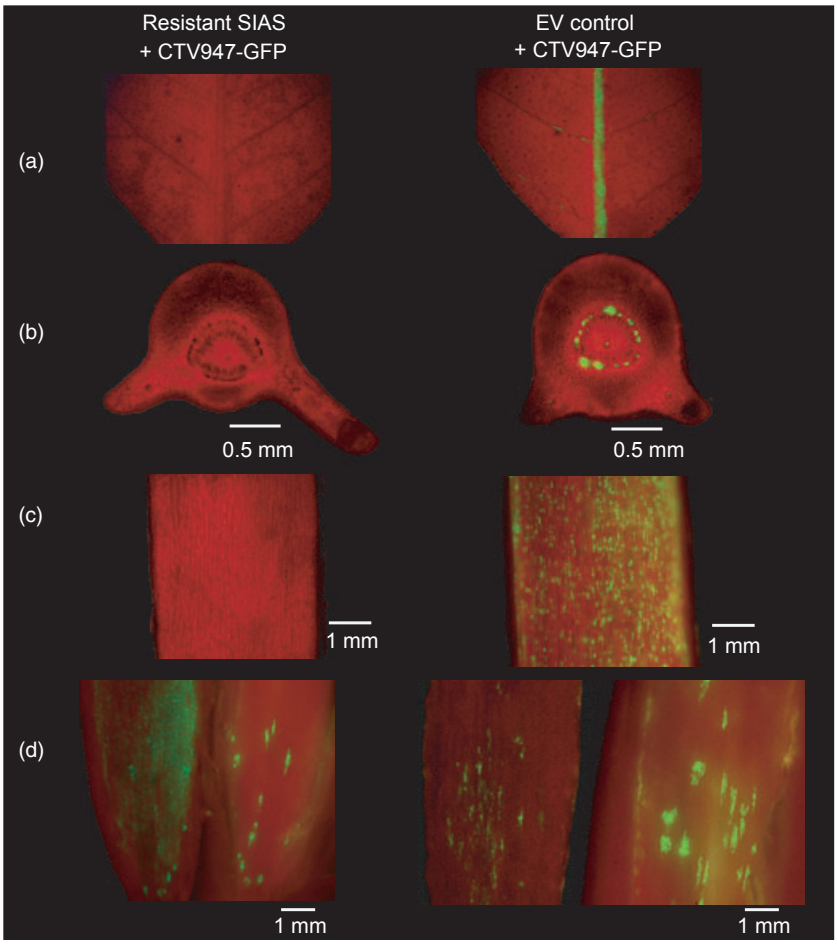

Figure 4 *Citrus tristeza virus* (CTV) distribution as detected by GFP fluorescence in the third flush of propagations of Mexican lime from a resistant Sense-Intron-AntiSense (SIAS) transgenic line (SIAS-2) (left) and from the empty vector (EV) control line (right) after graft inoculation of the scion with the GFP-expressing CTV947R-GFP. Fluorescence is observed in phloem-associated cells from leaves (a), petioles (b) and inner bark surface (c) of the EV control (but not in those of the SIAS line), and on the bark chips used as inoculum source (d, left side). Discrete fluorescence foci are also observed in the inner bark of the SIAS transgenic plant (d, right side) directly in contact with the inoculum bark chip (d, left side).

tance of line SIAS-41 to CTV-T36 was overcome by the divergent T318A isolate. On the other hand, almost half of the propagations of the SIAS-2 line were ELISA-negative and remained asymptomatic in the first flush after inoculation, but part of them became infected later, and 1 year after inoculation, only three of the ten propagations were still fully resistant to CTV-T318A, while the others had become progressively infected (Figure 5a,b). However, none of these symptomatic propagations showed vein corking and stunting, as did all the EV control and SIAS-41 propagations (Figure 5c,d). Therefore, in spite of the partial (91%–92%) sequence identity between the transgenes and their counterparts in CTV-T318A, line SIAS-2 displayed some resistance or tolerance against challenge inoculation with this strain. Collectively, these results indicate that SIAS-induced resistance to CTV in Mexican lime is very much influenced by the sequence identity between the transgene and the challenging CTV strain.

Discussion

RNA silencing has been successfully used to induce resistance to viruses in fruit tree species. The 'SunUp' transgenic papaya resistant to *Papaya ringspot virus* (Gonsalves, 1998) and the 'Honeysweet' transgenic plum resistant to *Plum pox virus* (Marshall, 2010) were generated to express ectopically the CP with the aim of re-encapsidating the cognate virus RNA in the initial stages of infection. However, only transgenic lines with several CP transgene insertions showed strong resistance to the challenging viruses (Gonsalves, 1998; Hily *et al.*, 2004; Ravelonandro *et al.*, 1997; Scorza *et al.*, 1994). Molecular analysis of these lines revealed very low levels of the transgene transcript, undetectable levels of CP and accumulation of transgene-derived siRNAs (Gonsalves, 1998; Hily *et al.*, 2005; Scorza *et al.*, 1994, 2001), indicating that RNA silencing was involved in the resistance achieved.

RNA silencing against CTV has remained an elusive objective in several laboratories (Batuman *et al.*, 2006; Febres *et al.*, 2007, 2008) including ours that has developed more than 300 transgenic lines of Mexican lime expressing ectopically complete and truncated versions of genes *p23* and *p25*, as well as sense, antisense and intron-hairpin constructs of the 3′-terminal 549 nucleotides of the CTV gRNA, including part of *p23* and the adjacent 3′-UTR (Domínguez *et al.*, 2002a,b; Fagoaga *et al.*, 2006; López *et al.*, 2010; our unpublished results). The intron-hairpin construct of the 3′-terminal 549 nucleotides conferred to Mexican lime higher CTV resistance than its sense or antisense counterparts, and this phenotype was associated with the accumulation of transgene-derived siRNAs. However, only 30% of the intron-hairpin transgenic lines showed resistance to the homologous virus, manifested in a fraction of the propagations remaining uninfected and the others being infected and showing symptoms as the EV controls (López *et al.*, 2010). Protection was thus much less efficient than that obtained in other plant-virus systems in which intron-hairpin constructs designed to silence a specific viral region usually confer resistance to 90% or more of the plants inoculated with the homologous virus

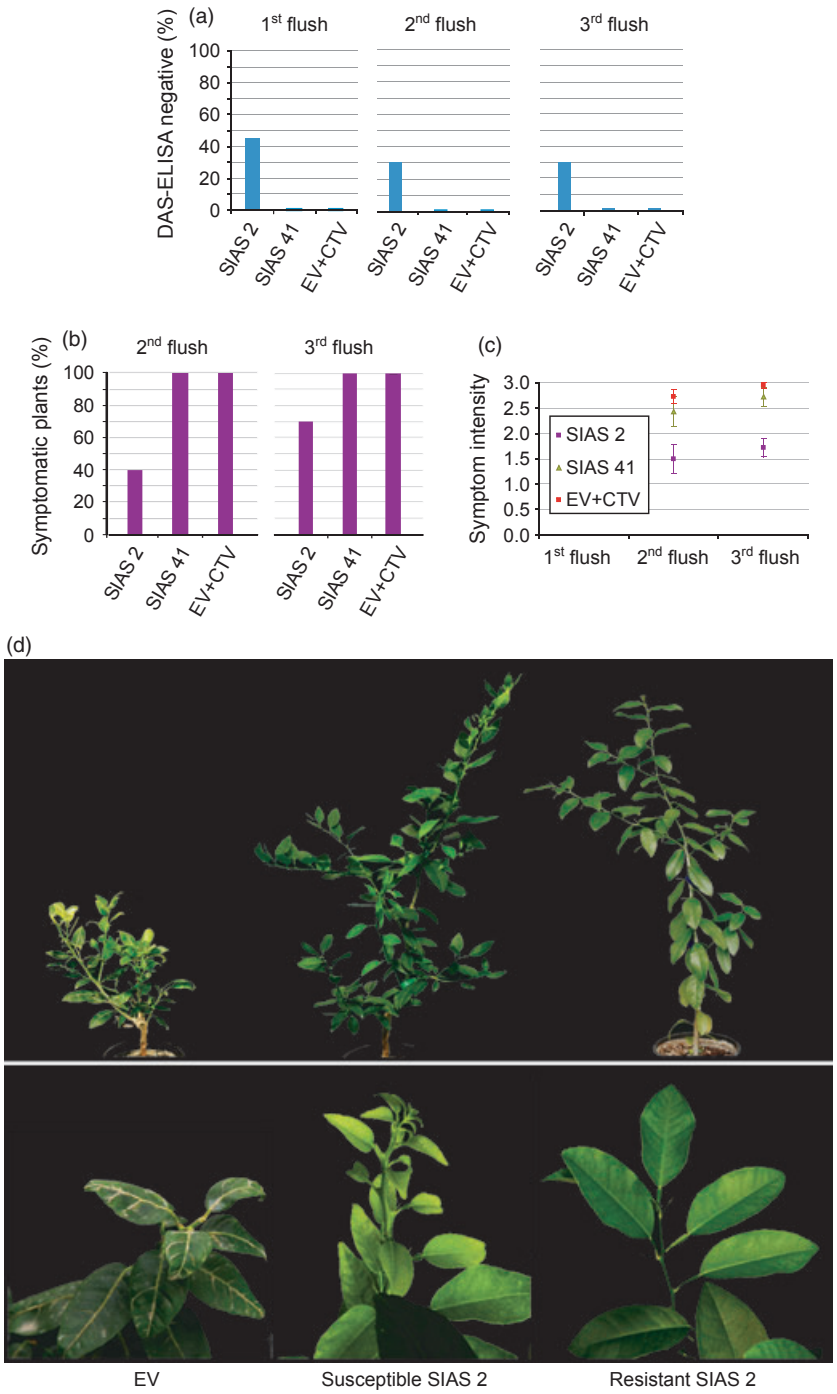

Figure 5 Evaluation of virus resistance in propagations of Mexican lime from the Sense-Intron-AntiSense (SIAS) transgenic lines 2 and 41 and from the empty vector line (EV) graft-inoculated with the heterologous CTV-T318A strain. (a) Fraction (in %) of DAS-ELISA-negative propagations from each transgenic line in three consecutive flushes. (b) Fraction (in %) of symptomatic propagations. (c) Average symptom intensity of infected plants estimated in a 0–3 scale as in Figure 3. Vertical bars indicate SE. (d) Phenotype of SIAS-2 and the EV control lines graft-inoculated with CTV-T318A. SIAS-2 propagations infected with CTV-T318A exhibited either resistance or attenuated symptoms compared with EV control propagations showing stunting (above) and vein corking (below) in the third flush postinoculation (1 year). CTV, *Citrus tristeza virus.*

(Kalantidis *et al.*, 2002; Nomura *et al.*, 2004; Smith *et al.*, 2000; Waterhouse *et al.*, 1998). Challenge by graft inoculation could in part explain the relatively low resistance achieved in Mexican lime transformants, because the constant delivery of high virus doses by graft patches may be sufficient to overcome transgene-derived RNA silencing (Batuman *et al.*, 2006; Domínguez *et al.*, 2002b). Moreover, CTV has unique attributes to counteract antiviral defence because it has evolved to encode three different silencing suppressors acting intra- (p23 and p20) and intercellularly (p20 and p25) (Lu *et al.*, 2004).

Looking for a more efficient strategy, we followed a previous suggestion (Batuman *et al.* (2006) of silencing simultaneously, via an intron-hairpin construct, these three critical genes that are additionally involved in viral encapsidation (*p25*), replication (*p23*) and pathogenicity (*p23* and *p20*) (Satyanarayana *et al.*, 2000, 2002; Ghorbel *et al.*, 2001; Fagoaga *et al.* 2005; Albiach-Martí *et al.*, 2010; our unpublished results). Transient co-expression of *gfp*, the SIAS construct and either CTV *p20* or *p23* by agroinfiltration in *gfp*-transgenic *N. benthamiana* 16c leaves reversed the silencing suppression afforded by either p20 or p23 when they were co-expressed with only GFP, indicating that expression of the SIAS construct in *N. benthamiana* leaf cells counteracted the activity of each of these CTV intracellular suppressors; notably, three of seven independent SIAS transgenic

events in Mexican lime conferred complete resistance to CTV in all propagations, as revealed by the lack of symptoms and negative ELISA reaction in successive flushes. Moreover, CTV947R-GFP, a clonal CTV-T36 strain, was unable to infect scion propagations of these three lines even when graft-inoculated directly, as inferred from the intense fluorescence emitted by the bark inoculum, but not by bark or leaves of the transgenic scion. Although a few discrete fluorescent foci were observed in phloem-associated transgenic cells in direct contact with the inoculum bark chip, the infection had not progressed further 1 year after inoculation, and therefore, the resistance was considered immunity. This result could be epidemiologically relevant, because CTV is dispersed in nature by several aphid species and repeated inoculations are common in long-living citrus trees. Transgenic immunity through RNAi, as shown here, would likely protect against repeated aphid inoculations, at least for CTV genotypes closely related with that serving as source for the transgene.

Other SIAS transformants showed partial protection to CTV because resistance was overcome by the homologous challenging virus in some propagations, while a third group included transgenic lines that, like the EV controls, were fully susceptible to CTV. We have not been able to associate the response to CTV challenge with transgene loci numbers or integration patterns in the Mexican lime transformants, although there is a clear association between resistance to CTV and accumulation of transgene-derived siRNAs, with lines lacking detectable amounts of the latter before CTV challenge being susceptible to infection. The presence of siRNAs is considered a hallmark of RNA silencing (Hamilton and Baulcombe, 1999), and in transgenic plants, it is linked to efficient RNAi-mediated constraint of virus accumulation and resistance (Prins *et al.*, 2008). However, we could not associate the amount of siRNA accumulated in transgenic lines with their level of protection, thus confirming our previous results with Mexican lime transformed with an intron-hairpin construct of the 3′-terminal 549 nucleotides of the CTV genome (López *et al.*, 2010). The mechanism behind the full resistance shown by some SIAS lines, in contrast with the partial protection afforded by previous constructs derived from a single gene, is presently unknown. Although *p23*-derived siRNAs may target sgRNAs of the ten 3′-proximal CTV genes, the larger size of the SIAS construct, or the concurrent presence of siRNAs from *p25*, *p20* and *p23,* may increase silencing efficiency by (i) inactivating more gRNA molecules, (ii) reducing the amount produced of the three silencing suppressor proteins or (iii) affecting the interactions between these proteins and some host factors. Yadav *et al.* (2011) obtained transgenic cassava plants carrying an intron-hairpin construct of the CP gene of CBSUV and found that low siRNA accumulation was sufficient to acquire immunity to graft-inoculated CBSUV in all vegetative propagations of the transgenic lines. While we observed the same situation with some SIAS transgenic lines, we also observed that CTV was able to overcome transgene-mediated RNAi in some propagations from other lines irrespective of their *p25*-, *p20*- and *p23*-derived siRNA accumulation, suggesting that host factors might be important in response to CTV infection.

Challenging immune transformants with a divergent CTV strain resulted in partial breakage of the resistance, thus supporting the notion that efficiency of RNA silencing depends on sequence identity between the RNAi-inducing transgene and the challenging virus genome (Baulcombe, 1996; Mueller *et al.*, 1995; Prins *et al.*, 2008; Waterhouse *et al.*, 1998), with resistance becoming ineffective when this identity differs by 10% or more (Prins *et al.*, 2008). Phylogenetic analyses of the sequences of *p25*, *p20* and *p23* from 18 CTV isolates deposited in GenBank [DQ151548 (T318A); AF001623 (SY568); AB046398 (NUagA); EU937519 (VT); AY170468 (T36); AY340974 (Qaha); DQ272579 (Mexico); EU937520 (T30); Y18420 (T385); JF957196.1 (B301); HQ912022.1 (CTV-D1); HM573451.1 (Kpg3); FJ525435.1 (NZRB-17); FJ525434.1 (NZRB-TH30); GQ454870.1 (HA16-5); GQ454869.1 (HA18-9); HQ912023.1 (CTV-B5); and FJ525436.1 (NZ-B18)] revealed that the most divergent genotypes show nucleotide identities of 90% for *p25*, 88% for *p20* and 87% for *p23*. Our results suggest that it should be possible to control specific CTV strains by transforming plants with intron-hairpin constructs engineered with *p25*, *p20* and *p23* sequences from the corresponding genotypes. A broader resistance, or even general immunity to CTV, might be obtained by fusing in a single chimeric intron-hairpin construct sequences of these three viral genes from divergent strains in order to maximize sequence identity, as it has been carried out to control simultaneously several tospoviruses in transgenic *N. benthamiana* (Bucher *et al.*, 2006; Pang *et al.*, 1997). On the other hand, it will be interesting to test this control strategy in other citrus hosts, like sweet orange, sour orange and grapefruit, in which CTV infects a fraction of phloem-associated cells smaller than in Mexican lime (Fagoaga *et al.*, 2011; Folimonova *et al.*, 2008).

In summary, here we provide the first data showing that it is possible to achieve full resistance to CTV under controlled experimental conditions in a citrus host highly sensitive to the virus by RNAi targeting simultaneously the three viral silencing suppressors. While the complete sequences of the three genes were engineered in the construct to enhance its virus silencing efficiency, their start codons were mutated to make transgenes untranslatable in case of recombination with viral RNA. Although a dissimilar CTV strain partially overcame resistance, it should be possible to use this same strategy with a chimeric intron-hairpin construct showing more than 95% identity with all known CTV genotypes in the three genes. Whether this strategy may provide reliable control of CTV in field-grown commercial citrus varieties remains to be tested.

Experimental procedures

Preparation of intron-hairpin recombinant vectors and citrus transformation

The fragments corresponding to *p25* (nucleotide positions 16152–16823), *p20* (17761–18309) and *p23*-3′UTR (18391–19020) were PCR-amplified from an infectious cDNA clone of the CTV isolate T36 (GenBank accession AY170468) (kindly provided by Dr W.O. Dawson, University of Florida) (Satyanarayana *et al.*, 2001) with AccuPrime *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) and the primer pairs P25*mutF/*Bam*HI (5′-ATAA*GGATCC*ATG-AGACGACGAAACAAAGAA-3′) and P25*mutR/*Xba*I (5′-GCCG-*TCTAGA*TCAACGTGTGTTGAAT-3′); P20*mutF/*Xba*I (5′-ATGA-*TCTAGA*ATGAACGAGCTTACTTTAGTGTTA-3′) and P20*mutR/*Xba*I (5′-ACGA*TCTAGA*CTACACGCAAGATGGAGAG-3′); P23*mutF/*Not*I (5′-ATAC*GCGGCCGC*ATGGATAGGATACTAGCGGACAAA-3′) and P23*mutR/*Not*I (5′-ATTC*GCGGCCGC*TGGACCTATGT-TGG-3′), containing appropriate restriction sites (indicated in

italics). The forward primers p25*mutF, p20*mutF and p23*mutF incorporated nucleotide insertions (underlined) in their respective ORFs, causing frameshift mutations downstream the first AUG resulting in untranslatable RNAs. The three amplification products were separated by electrophoresis in 1% agarose gels, excised and digested with the corresponding restriction enzymes, and then ligated stepwise into plasmid pBluescript II KS+ (Stratagene, La Jolla, CA) to generate the intermediate plasmid pBS p25-p20-p23. On the other hand, the piv2 intron of gene *st-ls1* from *Solanum tuberosum* was PCR-amplified from plasmid p35SGusintron (Vancanneyt *et al.*, 1990) with *Taq* DNA polymerase (Roche Diagnostics Corporation, Indianapolis, IN) using IntronF (5′-TACGTAAGTTTCTGCTTC-3′) and IntronR (5′-TACCTGCACATCAACAA-3′) as forward and reverse primers, respectively, followed by cloning into plasmid pGEM-T according to the manufacturer's instructions (Promega Corporation, Madison, WI). Then, the piv2 intron fragment was obtained by *Sac*II/*Not*I digestion and agarose gel electrophoresis and subcloned into pBS-p25-p20-p23 digested accordingly to generate plasmid pBS-p25-p20-p23-intron with fragment p25-p20-p23 in antisense orientation.

To prepare the intron-hairpin construct, fragments p25-p20-p23-sense and p25-p20-p23-antisense-intron were digested from the corresponding intermediate plasmids with *Sac*II/*Apa*I and *Sac*II/*Pst*I, respectively, electrophoresed and excised from the gel and ligated stepwise into plasmid pGEM-T digested accordingly to produce the intermediate plasmid pGEM-SIAS. In parallel, plasmid pMOG180 was digested with *Hin*dIII/*Eco*RI and the cassette containing the CaMV 35S promoter and the *nos*-ter sequences was inserted into the binary vector pCAMBIA 2301 (GenBank accession AF234316.1), opened between the 35S-pro/*npt*II/35S-ter and the 35S-pro/*uid*A/*nos*-ter cassettes, to generate the intermediate plasmid pCAMBIA-35S-*nos*-ter.

Finally, plasmid pGEM-SIAS was digested with *Bam*HI, and the SIAS fragment was separated by agarose gel electrophoresis and then excised and ligated into the *Bam*HI-digested pCAMBIA-35S-*nos*-ter, generating the final binary vector pCAMBIA-SIAS with the SIAS sequence cloned between the CaMV 35S promoter and the *nos*-ter (Figure 1a). Correct cloning and insert orientation were confirmed by sequencing. Plasmids pCAMBIA-SIAS and the corresponding pCAMBIA 2301 control (EV) were electroporated into *A. tumefaciens* EHA105 and used to transform Mexican lime (Ghorbel *et al.*, 2001).

Southern blot hybridization and siRNA analysis

DNA (15 μg per sample) extracted from leaves (Dellaporta *et al.*, 1983) was digested with *Eco*RI and *Hin*dIII for excising the SIAS expression cassette, or with *Eco*RI that cuts once in the T-DNA (Figure S1). After agarose gel electrophoresis, the DNA was blotted onto a positively charged nylon membrane, fixed by UV irradiation, probed with a digoxigenin (DIG)-labelled fragment of the region coding for *p23* prepared by PCR according to manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany) and detected using the chemiluminescent CSPD substrate (Roche).

For siRNA extraction, 500 mg of transgenic Mexican lime leaves or agroinfiltrated leaf areas of *N. benthamiana* was ground in 4 mL of TRI reagent (Sigma-Aldrich, St Louis, MO) and, after adding 800 μL of chloroform, the mixtures were vigorously shaken and centrifuged at 15 000 ***g***. Total RNA in the supernatant was recovered by isopropanol precipitation, resuspended in 200 μL of sterile distilled water and adjusted to defined concentrations with a NanoDrop®ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE). To detect *gfp* or transgene siRNAs, total RNA (5 μg from *N. benthamiana* or 30 μg from transgenic Mexican lime) was loaded on 20% polyacrylamide gels prepared in 0.25× TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and 8 M urea, separated by electrophoresis, transferred to positively charged nylon membranes (Roche) and fixed by UV irradiation. Hybridization was performed at 35 °C for 14–16 h, using DIG-labelled riboprobes (specific for the negative strand) obtained by the transcription of plasmids pGEM-GFP, pT7-p23, pBS-p20 and pBS-p25. pGEM-GFP was obtained by PCR amplification of gene *gfp* from the binary plasmid 35S-GFP (Ruiz *et al.*, 1998) and cloning into pGEM-T, and CTV genes *p23*, *p20* and *p25* were PCR-amplified from a cDNA clone of CTV-T36 and subsequently cloned into plasmids pT7 (*p23*) or pBluescript II KS+ (*p20* and *p25*). The membranes were washed twice with 2× SSC plus 0.1% SDS for 10 min at room temperature and then with 0.1× SSC plus 0.1% SDS for 15 min at 35 °C. Chemiluminescent detection was performed with the CSPD substrate (Roche).

Agrobacterium infiltration assays

Nicotiana benthamiana plants of the transgenic line 16c, constitutively expressing the gene *gfp* (Ruiz *et al.*, 1998), were used for infiltration assays with *A. tumefaciens* as described previously (Kapila *et al.*, 1997). The CTV genes *p23* and *p20* were PCR-amplified (see above) and then cloned into the binary plasmid pBin19 (GenBank accession U09365) between the 35S promoter and the *nos*-ter (Frisch *et al.*, 1995) to generate pBin19-p23 and pBin19-p20, respectively. These binary plasmids and p35S-GFP (Ruiz *et al.*, 1998), pBin19 and pCAMBIA-SIAS were each transformed into *A. tumefaciens* strain EHA105 by electroporation and used for leaf infiltration. For the co-infiltration experiments, equal volumes of bacterial cultures carrying either p35S-GFP + pBin19 (GFP), p35S-GFP + pBin19-p23 (GFP + p23), p35S-GFP + pBin19-p20 (GFP + p20), p35S-GFP +pBin19-p23 + pCAMBIA-SIAS (GFP + p23 + SIAS) or p35S-GFP + pBin19-p20 + pCAMBIA-SIAS (GFP + p20 + SIAS) were mixed, so that the final concentration for each culture was 0.4 OD_{600}. GFP fluorescence in plant leaves was examined using a Leica MZ16 FA stereomicroscope equipped with a 480/40-nm (460–500-nm) exciter filter, a 510 LP barrier filter and a 100-W high-intensity mercury burner lamp, and photographed with a Leica DC500 digital camera (Leica Microsystems, Wetzlar, Germany). An adjacent Leica L5 FL cold-light fluorescence lamp was also used to intensify the fluorescent images.

qRT-PCR analysis

Total RNA preparations were treated with recombinant DNase I (Roche), and the RNA was precipitated with isopropanol, resuspended in sterile distilled water and accurately quantified in a NanoDrop®ND-1000 spectrophotometer in triplicate. Quantitative real-time reverse-transcription PCR (qRT-PCR) was performed with a LightCycler®480 Instrument (Roche), and fluorescence was analysed using the LightCycler®480 Software. One-step qRT-PCR was carried out on 400 ng of total RNA adding 5 U of SuperScript™ II Reverse Transcriptase (Invitrogen), 8 U of Protector RNase Inhibitor (Roche), 10 μL of Power SYBR® Green PCR Master Mix (2×) (Applied Biosystems) and 750 nM of gene-specific primers in a total volume of 20 μL. Primer pairs GFP4.RT-F: 5′-TAATGGGCACAAATTTTCT-3′(forward) and GFP4RT.R:

5′-TATGATCTGGGTATCTTGA-3′ (reverse) were designed based on the coding sequence of gene *gfp* (GenBank accession U87973) with the Oligo primer analysis software 6.65 and used to amplify a 167-nt fragment. The qRT-PCR cycling conditions included two steps at 48 °C for 30 min and 95 °C for 10 min, respectively, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 60 s. Fluorescence intensity data were acquired during the 72 °C extension step, and the specificity of the reactions was verified by melting curve analysis. To transform fluorescence intensity measurements into relative *gfp* RNA levels, a twofold dilution series of a total RNA preparation from *N. benthamiana* 16c was used as a standard curve, with each point being the mean value of at least three independent analyses. An expression value of 1 was arbitrarily assigned in each experiment to the GFP-infiltrated sample and the rest of the values were referred to it.

Virus resistance analyses

Buds from SIAS or EV transgenic lines were propagated by grafting onto Carrizo citrange seedlings and kept in a greenhouse at 24–26 °C/16–18 °C (day/night), 60%–80% relative humidity and natural light. When new shoots were 30–40 cm long, homogeneous propagations from each transgenic line were graft-inoculated with two bark chips of 0.75–1 cm^2 in size from either a Mexican lime infected with a clonal CTV-T36 strain (Satyanarayana *et al.*, 2001), a Pineapple sweet orange infected with the CTV isolate T318A (Ruiz-Ruiz *et al.*, 2006) or a *C. macrophylla* plant infected with CTV947R-GFP, a clonal strain carrying the *gfp* gene between the genes *p27* and *p25* in the CTV-T36 genome (Ambrós *et al.*, 2011; Folimonov *et al.*, 2007). Bark chips from the CTV-T36- and T318A-infected sources were grafted onto the citrange rootstock of each transgenic plant 1–2 cm below the bud union and, in the first case, graft inoculation was repeated twice at monthly intervals to ensure 100% infection in control plants. Three months after the last inoculation, one inoculum bark chip per challenged plant was removed and the presence of the virus was confirmed by RT-PCR with specific primers (Domínguez *et al.*, 2002b). Bark chips from the CTV947R-GFP-infected source were directly grafted onto the transgenic scion, 1–2 cm above the bud union, and virus infection in the inoculum bark chip was confirmed by the observation of GFP fluorescence in the inner bark side. Virus accumulation in leaves was assessed by DAS-ELISA with the monoclonal antibodies 3DF1 + 3CA5 (Cambra *et al.*, 1990). A plant was considered CTV-infected when the absorbance at 405 nm was at least twofold that of noninoculated controls. CTV symptoms were monitored in at least three consecutive flushes spanning over a 1-year period. Symptom intensity was rated on a 0–3 scale in which 0 indicated a complete absence of symptoms, 1 mild vein clearing, 2 moderate vein clearing with young leaf epinasty and adult leaf cupping and 3 severe symptoms including vein corking and stunting. Young leaves and bark from branches of CTV947R-GFP-infected transgenic plants were examined using a Leica MZ 16 stereomicroscope equipped with a GFP-Plus Fluorescence module and photographed with a Leica DFC490 digital camera.

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Supporting information

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

Figure S1 (a) Diagram of the T-DNA from the binary vector pCambia-SIAS with the SIAS cassette controlled by the CaMV 35S-pro and the *nos*-ter and flanked by the cassettes 35S-pro/*npt*II/35S-ter and 35S-pro/*uid*A/*nos*-ter. Transcription orientation for each cassette is indicated by arrows, and restriction sites *Hin*dIII and *Eco*RI by vertical broken lines. (b,c) Southern blot hybridization of nucleic acid preparations from Mexican lime transformed with the SIAS construct (lines 46, 22, 41, 1, 2, 27, 26, 28, 43 and 39) and with the empty vector (EV). DNA was digested with *Eco*RI (b), which cuts once the T-DNA, or with *Eco*RI and *Hin*dIII (c), which excise the SIAS expression cassette. Size of DNA markers is indicated at the right. Hybridization was with a DIG-labelled DNA probe for detecting the coding region of *p23*. The higher intensity of the two bands in line SIAS-26 suggests multiple integrations as concatamers at the two loci.

Figure S2 Symptoms of Mexican lime propagations from a susceptible (SIAS-39) (a) and a resistant (SIAS-2) (b) SIAS transgenic plants expressed in the third flush after graft inoculating the CTV-T36 clonal strain in the Carrizo citrange rootstock in comparison with a noninoculated EV control (c). Susceptible transgenic SIAS scions showed vein clearing (upper), young leaf epinasty (middle) and adult leaf cupping (lower).

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