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Transgenic resistance against *Citrus tristeza virus* (CTV) and analysis of the viral p23 protein as pathogenicity determinant in citrus

TESIS DOCTORAL

by

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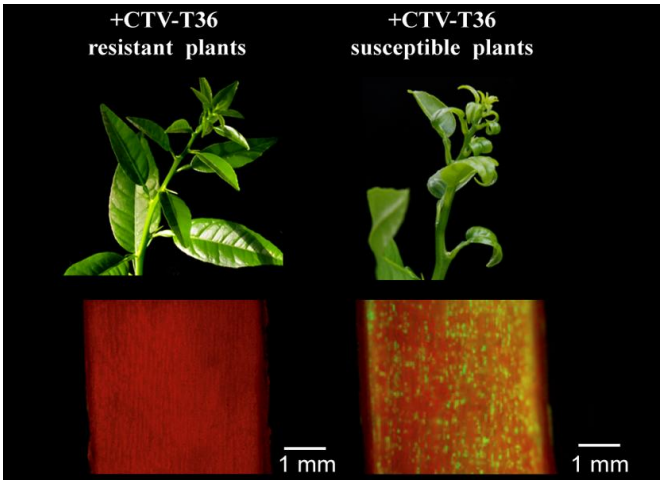

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El Dr. Leandro Peña García, Investigador científico del Instituto Valenciano de Investigaciones Agrarias y la Dra. Carmen Fagoaga, postdoctorado del Instituto Valenciano de Investigaciones agrarias,

CERTIFICAN:

Que Dª **Nuria Soler Calvo**, Licenciado en Biología por la Universidad de Málaga, ha realizado bajo nuestra supervisión la tesis doctoral titulada "**Transgenic resistance against *Citrus tristeza virus* (CTV) and analysis of the viral p23 protein as pathogenicity determinant in citrus"** y constituye su Memoria de Tesis para optar al grado de Doctor en Biotecnología.

Y para que así conste, firmamos la presente

en Valencia, a de 2013

Dr. Leandro Peña Dra. Carmen Fagoaga

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ABSTRACT

Citrus tristeza virus (CTV) is the causal agent of one of the most devastating viral diseases of citrus trees in the world. CTV is phloem-restricted in natural citrus hosts, and has evolved three silencing suppressor proteins acting at intra- (p23 and p20) and inter-cellular level (p20 and p25) to overcome strong host antiviral defense in citrus. RNA interference (RNAi), an approach based on using dsRNA to trigger RNA silencing, has been widely used for generating transgenic plants resistant against viruses. Considering the important role of p23, p20 and p25 in CTV pathogenesis, we have transformed Mexican lime plants with an intron-hairpin vector carrying full untranslatable versions of genes *p25*, *p20*, *p23* and the 3'-UTR from the CTV strain T36, to attempt silencing their expression in CTV-infected cells. Complete resistance to viral infection was observed in three transgenic lines, with all their propagations remaining symptomless and virus-free after graft-inoculation with CTV-T36, either in the non-transgenic rootstock or directly in the transgenic scion. Accumulation of transgene-derived siRNAs was necessary but not sufficient for CTV resistance. Challenging immune transformants with a divergent CTV strain resulted in partial breakage of the resistance, stressing the importance of sequence identity in the underlying RNAi mechanism. This is the first evidence that it is possible to achieve full resistance to CTV in a highly sensitive citrus host by targeting simultaneously its three viral silencing suppressors through RNAi. The p23 protein encoded by the virus is additionally an important pathogenicity factor. Ectopic expression of p23 in

transgenic citrus plants induces developmental aberrations resembling CTV symptoms. To explore in more detail the role of p23 in CTV pathogenesis, the *p23* gene from CTV T36 and three truncated versions thereof under the control of the *Cauliflower mosaic virus* 35S promoter were used to transform Mexican lime. Only the truncated version expressing amino acids 1 to 157 (p23Δ158-209) elicited CTV-like symptoms, similar to, albeit milder than, those incited by expressing the whole p23 protein (209 amino acids), thus delimiting the region responsible for p23 pathogenesis in citrus to a 157 amino acid fragment including the Zn finger and flanking basic motifs of the protein. RNA silencing suppressor activity of p23 in *N. benthamiana* was abolished by all mutants tested, indicating that silencing suppression involves most p23 regions. To better define the role of p23 in CTV pathogenesis, we next restricted the expression of *p23*-derived transgenes to phloem-associated cells in Mexican lime plants by means of using the phloem-specific promoter from *Commelina yellow mottle virus* (CoYMV). Constructions carrying the complete gene *p23* from either the severe T36 or the mild T317 CTV strains, or a fragment comprising the zinc-finger and flanking basic motifs from the former, either under the control of the CoYMV promoter or the constitutive 35S promoter were used for genetic transformation of Mexican lime. Expression of these constructs in the phloem incited aberrations resembling CTV-specific symptoms, but not the unspecific symptoms observed when p23 was constitutively expressed. Moreover, appearance and intensity of the most notorious CTV-like phenotypic aberrations induced by the phloem-specific expression of

the *p23* gene were positively related with the aggressiveness of the source CTV strain used. Additionally, expression in phloem-tissues of the p23 fragment comprising the zinc-finger domain and flanking basic motifs was sufficient to induce CTV-like symptoms, corroborating that the N-terminal region (delimited by amino acids 1 and 157) determines, at least in part, CTV pathogenesis in Mexican lime.

RESUMEN

El virus de la tristeza de los cítricos (*Citrus tristeza virus*; CTV) es el agente causal de unas de las enfermedades virales de los árboles cítricos más devastadoras en el mundo. CTV está restringido al floema en su huésped cítrico natural, y ha desarrollado tres proteínas supresoras de silenciamiento que actúan a nivel intra-(p23 y p20) e intercelular (p20 y p25) para superar la fuerte defensa antiviral del huésped. La interferencia de RNA, una aproximación basada en el uso de dsRNA para desencadenar el silenciamiento de RNA, ha sido utilizada ampliamente para generar plantas transgénicas resistentes a virus. Considerando el importante papel de p23, p20 y p25 en la patogénesis de CTV, hemos transformado plantas de lima Mexicana con un vector intrón-horquilla que porta la secuencia completa en versión no traducible de los genes *p25*, *p20*, *p23* y el extremo 3'-UTR de la cepa T36 de CTV, para intentar silenciar su expresión en células infectadas.

Se ha observado resistencia completa a la infección viral en tres líneas transgénicas, manteniéndose todas sus propagaciones asintomáticas y libres de virus tras ser inoculadas mediante injerto con CTV-T36, tanto en el portainjertos no transgénico como directamente sobre la variedad transgénica. La acumulación de siRNA derivados del transgén fue necesaria pero no suficiente para lograr resistencia frente a CTV en las plantas. Al inocular propagaciones de las líneas transgénicas inmunes con una cepa de CTV divergente, la resistencia fue parcialmente superada, destacando la importancia de la identidad de secuencia en el mecanismo subyacente a la interferencia de RNA.

Este trabajo es el primero en que se consigue resistencia completa a CTV en un huésped cítrico muy sensible, actuando simultáneamente sobre los tres supresores virales de silenciamiento mediante interferencia de RNA. La proteína p23 codificada por el virus es además un importante factor de patogenicidad. La expresión ectópica de p23 en plantas de cítricos induce aberraciones fenotípicas semejantes a síntomas de CTV. Para estudiar en más detalle el papel de p23 en la patogénesis de CTV, se ha sobre-expresado en lima Mexicana el gen *p23* de CTV T36 y tres versiones truncadas del mismo bajo el control del promotor 35S del virus del mosaico de la coliflor (*Cauliflower mosaic virus*). Solo la versión truncada, que expresa los aminoácidos del 1 al 157 (p23Δ158-209) indujo síntomas similares a los producidos por CTV, aunque más suaves que los inducidos por la expresión de la proteína p23 entera (209 aminoácidos), permitiendo delimitar la región responsable de la patogénesis de p23 en cítricos a un fragmento de 157 aminoácidos que incluye el dedo de zinc y los motivos básicos flanqueantes de la proteína. La actividad de p23 como supresor de silenciamiento de RNA en *N. benthamiana* se perdía en todos los mutantes de p23 probados, lo cual indica que la supresión de silenciamiento implica a la mayoría de las regiones de la proteína. Para profundizar más en el papel de p23 en la patogénesis, en un siguiente paso hemos restringido la expresión de transgenes derivados de p23 a células asociadas al floema de lima Mexicana mediante el uso del promotor especifico de floema del virus del moteado amarillo de la comelina (*Commelina yellow mottle virus*, CoYMV). Se transformó lima Mexicana con

construcciones que portaban el gen *p23* completo, ya sea de la cepa agresiva de CTV T36 o de la suave T317, o con un fragmento que comprende el dedo de zinc y los motivos básicos flanqueantes de la primera, todas ellas bajo el control bien del promotor de CoYMV o bien del promotor constitutivo 35S. La expresión de estas construcciones en el floema dio lugar a aberraciones semejantes a los síntomas específicos de CTV, pero no a los síntomas inespecíficos observados cuando se expresaba p23 de forma constitutiva. Por otra parte, la apariencia e intensidad de las aberraciones fenotípicas más notorias similares a síntomas inducidos por CTV generadas por la expresión específica en floema del gen *p23* se relacionó positivamente con la agresividad de la cepa origen utilizada. Además, la expresión en tejidos floemáticos del fragmento de p23 que comprende el dominio de dedo de zinc y los motivos básicos flanqueantes fue suficiente para inducir síntomas semejantes a los producidos por la infección con CTV, confirmando así que la región N-terminal delimitada por los aminoácidos 1 y 157 podría determinar, al menos en parte, la patogénesis de CTV en lima Mexicana.

RESUM

El virus de la tristesa dels cítrics (*Citrus tristeza virus*, CTV) és l'agent causal d'una de les malalties virals dels arbres cítrics més devastadores en el món. CTV està restringit al floema en el seu hoste cítric natural, i ha desenvolupat tres proteïnes supressores del silenciament que actuen a nivell intra (p23 i p20) i intercel·lular (p20 i p25) per a superar la forta defensa antiviral de l'hoste cítric. La interferència de RNA, una aproximació basada en l'ús de dsRNA per desencadenar el silenciament de RNA, ha sigut utilitzada àmpliament per generar plantes transgèniques resistents a virus. Atès l'important paper de p23, p20 i p25 en la patogènesi de CTV, hem transformat plantes de llima Mexicana amb un vector intró-forquilla que porta la seqüència completa en versió no traduïble dels gens *p25, p20, p23* i el 3'- UTR del cep T36 de CTV, per intentar silenciar la seua expressió en cèl·lules de CTV infectades.

S'ha observat resistència completa a la infecció viral en tres línies transgèniques, mantenint totes les seues propagacions asimptomàtiques i lliures de virus després d'inocular mitjançant empelt amb CTV-T36, tant en el portaempelt no transgènic com directament sobre la varietat transgènica. L'acumulació de siRNA derivats del transgèn va ser necessària però no suficient per aconseguir resistència a CTV. En propagacions de les línies transgèniques immunes inoculades amb un cep de CTV divergent, la resistència va ser parcialment superada, destacant la importància de la identitat de seqüència en el mecanisme subjacent al RNAi. Aquesta és la primera

evidència que és possible aconseguir resistència completa a CTV a un hoste cítric molt sensible, actuant simultàniament sobre els tres supressors virals de silenciament mitjançant RNAi. La proteïna p23 codificada pel virus és, a més a més, un important factor de patogenicitat. L'expressió ectòpica de p23 en plantes de cítrics transgènics indueix aberracions fenotípiques semblants a símptomes de CTV. Per estudiar amb més detall el paper de p23 en la patogènesi de CTV, es va utilitzar per transformar llima Mexicana, el gen *p23* de CTV T36 i tres versions truncades de la mateixa sota el control del promotor *Cauliflower mosaic virus* 35S. Només la versió truncada que expressa els aminoàcids de l'1 al 157 (p23Δ158-209) va induir símptomes similars als produïts per CTV, encara que més suaus que els induïts per l'expressió de la proteïna p23 sencera (209 aminoàcids), permetent delimitar la regió responsable de la patogènesi de p23 en cítrics a un fragment de 157 aminoàcids que inclou el dit de zinc i els motius bàsics flanquejants de la proteïna. L'activitat de p23 com supressor de silenciament de RNA en *N. benthamiana* es perd en tots els mutants de p23 provats, indicant que la supressió de silenciament implica la majoria de les regions de la proteïna. Per aprofundir més en el paper de p23 en la patogènesi, en un següent pas hem restringit l'expressió de transgens derivats de p23 a cèl·lules associades al floema de llima Mexicana mitjançant l'ús del promotor específic de floema de *Commelina yellow mottle virus* (CoYMV). Es va transformar llima Mexicana amb construccions que portaven el gen p23 complet, ja siga del cep agressiu de CTV T36 o T317, o un fragment que comprèn el dit de zinc i els motius bàsics flanquejants de

la primera, ja siga sota el control del promotor de CoYMV o del promotor constitutiu 35S. L'expressió d'aquestes construccions en el floema va donar lloc a aberracions semblants als símptomes específics de CTV, però no als símptomes inespecífics observats quan s'expressava p23 de manera constitutiva. D'altra banda, l'aparença i intensitat de les aberracions fenotípiques més notòries similars a símptomes induïts per CTV, generades per l'expressió específica en floema del gen p23 es relacionava positivament amb l'agressivitat del cep d'origen utilitzat. A més, l'expressió en teixits floemàtics del fragment de p23 que comprèn el domini de dit de zinc i els motius bàsics flanquejants va ser suficient per induir símptomes semblants als produïts per la infecció amb CTV, confirmant així que la regió N-terminal delimitada pels aminoàcids 1 i 157 podria determinar, almenys en part, la patogènesi de CTV a llima Mexicana.

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1. INTRODUCTION

RNAi-Mediated Protection Against *Citrus Tristeza Virus* in Transgenic *Citrus* Plants.

In: *Non Coding RNAs in Plants.* RNA TECHNOLOGIES (Erdmann, V.A. and Barciszewski, J., eds), Springer Berlin Heidelberg (2011); pp:447-460.

Nuria Soler, Carmen Fagoaga, Sinda Chiibi, Carmelo López, Pedro Moreno, Luis Navarro, Ricardo Flores, and Leandro Peña.

Abstract

RNA silencing is a sequence-specific mechanism of inhibition of gene expression evolutionarily conserved in most eukaryotes. RNA interference (RNAi), a technology based on the use of doble-stranded RNA (dsRNA) to trigger RNA silencing, can be achieved in plants by genetic transformation with sense and antisense cDNAs derived from target viral sequences separated by an intron (intron-hairpin constructs). Upon transcription, the resulting hairpin RNA transcript usually acts as a strong inducer of RNA silencing. This strategy has been widely used to produce virus-resistant transgenic plants. *Citrus tristeza virus* (CTV) (genus *Closterovirus*, family *Closteroviridae*) is the causal agent of the most devastating viral diseases of citrus trees in the world. It only infects phloem-associated tissues of *Citrus* species and relatives within the family *Rutaceae*. CTV is one of the largest and most complex plant RNA viruses, with a single-stranded, plus-sense RNA genome of 19.3 kb, organized in 12 open reading frames (ORFs), potentially coding for at least 17 polypeptides, and two 5' and 3' unstranslated regions (UTRs). Replication and expression of the genomic RNA results in more than 30 different plus and minus RNA species as well as their corresponding dsRNA forms. Concomitantly, citrus hosts have developed a strong antiviral response through RNA silencing, as inferred from the high level of virus-derived siRNAs observed in infected tissues. As a counterdefense, CTV encodes at least three silencing suppressor proteins acting and/or intercellularly to overcome antiviral defense. Under these circumstances, searching for

RNAi-induced resistance against CTV in transgenic citrus plants becomes a real challenge. We have used intron-hairpin constructs targeting several viral regions, with our present interest focusing on one or the three CTV genes encoding silencing suppressors, or on conserved domains important for viral replication and encapsidation.

1.1. Citrus and Tristeza

Citrus is the most economically important fruit tree crop worldwide, with more than 110 million tons in 2009 produced in more than 7.6 million Ha (FAO 2010). Besides the genus *Citrus* that belongs to the family *Rutaceae,* subfamily *Aurantoideae,* citrus crops include two other genera of economic importance: *Poncirus* and *Fortunella*. There are only three true *Citrus* species: citron (*C. medica* L.), mandarin (*C. reticulata* Blanco), and pummelo (*C. grandis* (L.) Osb.). Since the three ancestral species reproduce only sexually and are original from the same geographical area, Southeast Asia, several hybridizations among these species followed by frequent somatic mutations generated the major citrus types of economic importance, including sweet oranges (*C. sinensis* (L.) Osb.), mandarins (*C. deliciosa* Ten., *C. tangerina* Hort. Ex Tan., *C. clementina* Hort. Ex Tan., *C. nobilis* André non Lour., *C. unshiu* (Mak.) Marc., etc.), lemons (*C. limon* (L.) Burm. f.) and limes (*C. aurantifolia* (Christm.) Swing.; *C. latifolia* Tan.; *C. limonia* (L.) Osb.). Grapefruit is a much more recent type, first described in Barbados in 1750, and originated from a natural hybridization between pummelo and sweet orange

probably followed by introgression with pummelo (Nicolosi et al. 2000).

Another important citrus genotype is sour orange (*C. aurantium* L.). Its use marked the origin of modern citriculture around mid-nineteenth century when bud grafting scion varieties onto sour orange rootstock became a universal practice, mainly due to its resistance to *Phytophthora spp*., but also because of its excellent agronomic attributes, particularly its capacity to induce high fruit yield and quality, and its adaptability to all soils. However, the massive use of sour orange was the basis of the dramatic outcome of several tristeza epidemics that in the last 80 years caused the death of more than 100 million sweet orange, mandarin and grapefruit scion varieties propagated on this rootstock in Argentina, Brazil, California, Florida, Israel, Venezuela and Spain. Moreover, the disease keeps spreading into new areas, either by propagation of infected buds or by different aphid species, mainly *Toxoptera citricida* (Kildaky) and *Aphis gossypii* (Glover). This situation forced the progressive replacement of sour orange by tristeza-resistant or tolerant rootstocks which do not perform as well as sour orange.

Tristeza is a bud union disease and refers to the decline of most scion types propagated on sour orange or lemon rootstocks. Its causal agent is *Citrus tristeza virus* (CTV), a member of the genus *Closterovirus*, family *Closteroviridae*. In nature CTV infects only citrus species and relatives within the family *Rutaceae*, subfamily *Aurantoideae*, and within these hosts it invades only phloem-

associated tissues. Indeed, tristeza decline results from virus-induced necrosis of the rootstock phloem just below the bud union.
Additionally, virulent CTV isolates cause stem pitting on some sweet orange, grapefruit and lime scion varieties regardless the rootstock, resulting in reduced vigor, yield and fruit quality. A third syndrome observed by biological indexing but rarely in the field, is characterized by stunting, small pale or yellow leaves, reduced root system and sometimes complete growth cessation of sour orange, grapefruit or lemon seedlings (Moreno et al. 2008).

1.2. CTV genome

CTV virions are long flexuous particles (2,000 nm X 11 nm) formed by two coat proteins that encapsidate a plus-sense ssRNA of approximately 19.3 kb organized in 12 open reading frames (ORFs) potentially encoding at least 17 protein products and two 5' and 3' unstranslated regions (UTRs) of 107 and 273 nt, respectively (Karasev et al. 1995) . The 5'-proximal ORF 1a encodes a polyprotein containing two papain-like protease domains, plus methyltransferase-like and helicase-like domains. ORF 1b encodes a putative RNA-dependent RNA polymerase that it is thought to be expressed by a +1 frameshift mechanism (Karasev et al. 1995). The 10 ORFs located in the 3' moiety of the genome are expressed through a set of 3'-co-terminal subgenomic (sg) mRNAs (Hilf et al. 1995), and they encode the coat proteins (CPs) of 25 and 27 kDa (p25 and p27), that encapsidate about 97% and 3% of the genome, respectively, and

proteins p33, p6, p65, p61, p18, p13, p20 and p23 (Karasev et al. 1995; Pappu et al. 1994). Both CPs, together with p65 and p61, are involved in virion assembly (Satyanarayana et al. 2000). Additionally, p27 has been shown to initiate encapsidation of the genomic RNA from its 5' end (Satyanarayana et al. 2004). The p20 protein accumulates in amorphous inclusion bodies of CTV-infected cells (Gowda et al. 2000). The small hydrophobic p6 may operate as a membrane anchor (Satyanarayana et al. 2000) and its homologue in the *Beet yellows virus* (BYV), also of the genus *Closterovirus,* is a movement protein (Peremyslov et al. 2004). The protein p23, a RNA binding protein with a Zn finger domain (López et al. 2000), that regulates the asymmetrical accumulation of the plus and minus strands during RNA replication (Satyanarayana et al. 2002), has no homologue counterpart in other closteroviruses, and likely it is the determinant of the seedling yellows syndrome (Albiach-Martí et al. 2010). When ectopically expressed in transgenic citrus plants, p23 induces aberrations resembling CTV symptoms (Ghorbel et al. 2001). Moreover, p23, p20 and p25 act as RNA silencing suppressors in *Nicotiana tabacum* and *N. benthamiana* plants, being p25 intercellular, p23 intracellular and p20 both inter- and intracellular silencing suppressors (Lu et al. 2004). The function of p33, p13 and p18 remains unknown.

1.3. Resistance to CTV

Breeding for resistance to CTV in scion varieties has been largely unsuccessful, mainly due to the complex reproductive biology of citrus. Most genotypes are facultative apomictic, which means that adventitious embryos are generated directly from maternal nucellar cells, precluding the development of the less vigorous zygotic embryos. Although this is the basis for propagation of citrus rootstocks, apomixis seriously limits the recovery of sexual progeny populations in breeding programs. Some important genotypes have total or partial pollen and/or ovule sterility and cannot be used as parents in breeding programs, and there are many cases of cross- and self-incompatibility. Additionally, citrus have a long juvenile period and most species need at least 5 years to start flowering in subtropical areas, and usually several years more to achieve fully mature characteristics. Citrus types have high heterozigosity, and there is a lack of basic knowledge about how the most important horticultural traits are inherited some of which, as those related to fruit quality and maturity time, show quantitative inheritance. All these features together with their large plant size have greatly impeded genetic improvement of citrus through conventional breeding.

The only successful results from breeding for CTV resistance come from the first recorded artificial hybridization, carried out by Swingle and Webber in Florida in 1893 in relation to disease problems. Since a severe freeze destroyed most of the seedlings, they decided to use the cold-hardy relative *Poncirus trifoliata* as a parent in

crosses aimed at incorporating higher cold tolerance to *Citrus* scions. None of the progeny trees combined cold hardiness with good fruit quality. However, the Carrizo and Troyer citranges (sweet orange X *Poncirus trifoliata*) and the Swingle citrumelo (grapefruit X *Poncirus trifoliata*) hybrid rootstocks resulting from these crosses are widely used by the most important citrus industries due to their tolerance to CTV-induced decline.

Resistance to CTV in *P. trifoliata* has been attributed to a single dominant locus (*Ctv*), which has been thoroughly characterized and mapped (Yoshida, 1985, 1993; Gmitter et al. 1996; Mestre et al. 1997; Fang et al. 1998). Because of the complex genetics of citrus, it is extremely difficult to introgress this resistance locus into citrus varieties by conventional breeding. However, its cloning has been attempted in several laboratories (Yang et al. 2003; Deng et al. 2001). A BAC library developed from "Pomeroy" *P. trifoliata*, homozygous for *Ctv*, was used for a 1.2 Mb genome walk spanning the region between *Ctv*-flanking markers. Sequencing of a set of four overlapping BAC clones in this region, using shotgun sequencing and resolution of their ends by sequencing of additional BAC clones, further localized *Ctv* to a 282-kb region comprising 22 predicted genes (Yang et al. 2003). Sequence analysis of the *Ctv* locus in this region identified 61 simple sequence repeats (SSRs) that were used to further narrow down the locus in the *Poncirus* genome to 121 kb, comprising ten genes. Each of these genes has been individually cloned in *Agrobacterium*-based binary vectors and used to transform susceptible grapefruit varieties in Erik Mirkov's laboratory (Rai, 2006). The

transgenic lines expressing any of the ten candidate genes were susceptible to CTV infection, suggesting that more than one gene in the locus is involved in resistance to CTV or that the role of other genomic loci has been overlooked.

In general, citrus genotypes are hosts for CTV, but there is a wide diversity in their response against viral infection, which is strain-dependent. While Mexican lime is a symptomatic host of most CTV strains, which show systemic infection and reach relatively high virus titers, only the aggressive strains induce symptoms in sweet orange and grapefruit systemic hosts. On the contrary, most mandarins are nonsymptomatic but systemic hosts, and sour oranges and lemons hardly tolerate virus spread and show very low CTV titer. Five quantitative trait loci (QTLs) have been associated with the partial resistance of sour orange to certain CTV strains (Asins et al. 2004). There are also species, as pummelo, in which the resistant/susceptible response depends on specific cultivar/strain combinations (Garnsey et al. 1987), with a single dominant gene for resistance to CTV called *Ctv2* having been mapped in "Chandler" pummelo (Fang and Roose 1999).

Nowadays, the only possibility to protect susceptible commercial varieties from severe CTV isolates is classical cross protection with mild CTV strains. This approach has prevented the low yield and small-sized fruits of "Pera" sweet orange in Sao Paulo, Brazil (Costa and Müller 1980) and "Marsh" grapefruit in South Africa (van Vuuren et al. 1993). In both cases protection was based on the search for field CTV isolates causing asymptomatic infection in

the citrus cultivar of interest, and the use of budwood from those trees to propagate pre-inoculated plants for new plantings. The mild strain would then protect the new plants against infections with upcoming severe CTV strains. Considering that "Pera" sweet orange is the main variety of the citrus industry in Sao Paulo, one of the largest in the world, and that all new nursery "Pera" plants are infected with a mild isolate as part of the commercial production system, it is clear that in the last 40 years cross protection has been a tool of paramount importance for the success of the Brazilian citriculture. However, attempts to apply the same strategy in other citrus areas, as Australia, Japan or Florida, have failed because protection was highly dependent on the citrus scion variety, the prevailing CTV strains and the environmental conditions, with the afforded protection being only temporary.

1.4. CTV and RNA silencing

In recent times, RNA silencing has arisen as a mechanism that explains many cases of genetic plant defence against viral infections and cross protection between closely related virus strains (Covey et al. 1997; Ratcliff et al. 1997, 1999). RNA silencing is induced by double-stranded RNA (dsRNA) or highly-structured single-stranded RNA (ssRNA) and results in sequence-specific ssRNA degradation through generation of 21-25 nt short interfering RNAs (siRNAs) by RNaseIII-like enzymes called Dicer (Bernstein et al. 2001). The siRNAs are loaded into an RNA-induced silencing complex (RISC) and one strand

of the siRNA is degraded while the other primes the Argonaute (AGO)-containing RISC active complex that then targets for cleavage of ssRNA sharing sequence similarity with the inducing dsRNA (Hammond et al. 2000). Accumulation of high levels of dsRNA derived from subgenomic RNAs, a characteristic feature of CTV replication (Hilf et al. 1995), could trigger RNA silencing. Moreover, Fagoaga et al. (2006) and Ruiz-Ruiz et al. (2011) have observed high accumulation of viral-specific siRNAs in CTV-infected Mexican lime plants, indicating a strong natural RNA silencing-mediated antiviral response. It is then tempting to speculate that in cross protection the siRNAs generated by the mild CTV isolate could prevent subsequent infections by severe strains through targeting and degradation of their highly homologous genomic and subgenomic RNAs. Even within highly divergent CTV variants, it is easy to find large portions along the CTV genome with more than 24-nt identical.

In principle, pathogen-derived resistance (PDR) would be based on the same mechanism as cross protection, but it could represent a better and more predictable strategy to achieve durable resistance to CTV in citrus. In PDR, introduction and expression in plants of pathogen genes in a dysfunctional form, in excess, or at the wrong developmental stage, could interfere with the pathogen life cycle having minimal effects on the host, and providing resistance to infection (Sanford and Johnston 1985). Since the first demonstration of virus-derived resistance in transgenic plants by using the CP gene of *Tobacco mosaic virus* (TMV) (Abel et al. 1986), this strategy has been proved to be widely applicable. Two of the most successful

examples of CP-mediated protection against viruses in plants refer to fruit tree species, namely the "SunUp" transgenic papaya resistant to *Papaya ringspot virus* (PRSV), which is commercialized in USA since 1999 (Gonsalves 1998; Ming et al. 2008), and the "Honeysweet" transgenic plum, resistant to *Plum pox virus* (PPV), which has been approved for commercial release in USA in 2009 (Marshall 2010). In both cases, transgenic plants were generated with the aim of over-expressing the CP transgene, thus getting an ectopic over-accumulation of the corresponding protein that would reencapsidate the challenging virus soon after initiating infection. However, only unique transgenic events with several CP transgene insertions showed strong resistance to the challenging viruses (Scorza et al. 1994; Ravelonandro et al. 1997), particularly in field trial assays (Hily et al. 2004). Molecular analysis of these events revealed very low transgene mRNA levels and undetectable CP accumulation (Scorza et al. 1994). More detailed analyses showed constitutive transgene methylation and transgene-derived siRNA accumulation (Scorza et al. 2001; Hily et al. 2005). Since all these features are characteristic of RNA silencing, it is clear today that random integration of several foreign DNA copies during genetic transformation was responsible for the resistance in those transgenic lines that never showed transgene CP accumulation but expressed strong RNA silencing.

To generate CP-mediated resistance to CTV in transgenic citrus, we incorporated into Mexican lime a transgene derived from the p25 CP gene from severe and mild CTV strains, with more than 40 independent transgenic lines being produced. Mexican lime was

chosen as a citrus model because it is very sensitive to CTV and the potential resistance could be easily tested by evaluating leaf cupping, vein clearing and stem pitting symptoms in the greenhouse within a few months after challenging. When eight to ten propagations of each transgenic line were graft- and aphid-inoculated with CTV, two types of response to viral challenge were observed: most lines developed CTV symptoms similar to those of the non-transgenic controls, but six of the 40 lines exhibited resistance against the virus. Resistance consisted of a fraction of the propagations, ranging from 10 to 33%, that were immune to CTV, with the rest showing a significant delay in virus accumulation and symptom onset in at least three consecutive flushes (about one year) after inoculation (Domínguez et al. 2002a). These results were reproduced with four of the six transgenic lines in an additional challenge experiment in which propagations were again graft-inoculated with CTV (Figure 1). Since several transgenic lines showed complex T-DNA insertions and undetectable p25 accumulation, but consistent partial resistance, an RNA silencing mechanism was proposed to explain the protection against CTV. In a new set of experiments, Mexican lime plants were transformed with untranslatable versions of the p25 gene, but, in general, the protection achieved was rapidly overcome by the challenging virus (Domínguez et al. 2002b).

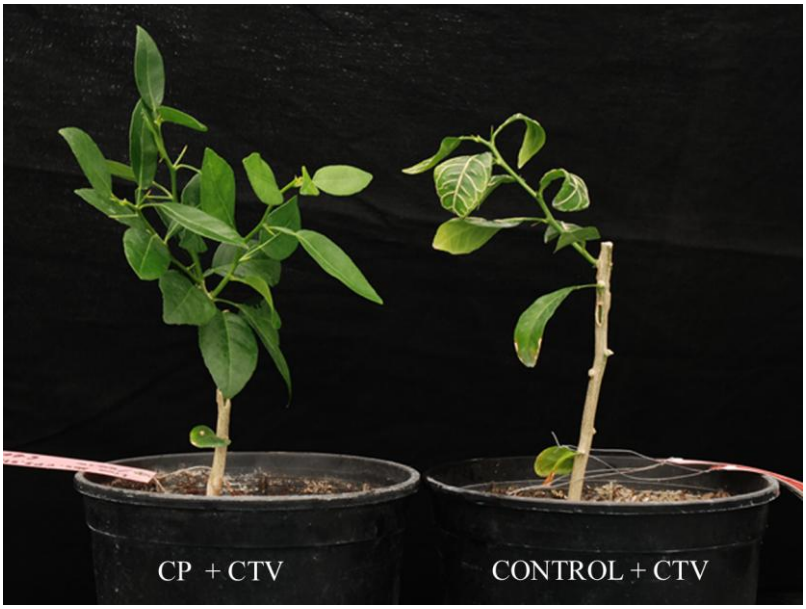

Figure 1 Response to graft inoculation with a severe CTV isolate exhibited by representative Mexican lime plants transformed with the *p25* CP transgene (*left*) and with an empty vector construct (*right*). One year after challenging, the *p25* -transgenic plant shows mild vein clearing symptoms, while the control plant is affected by intense leaf distortion and vein corking.

The 3'-terminal gene of CTV codes for p23, which is an RNA-binding protein (López et al. 2000) involved in regulating the balance of plus and minus RNA strands during replication (Satyanarayana et al. 2002). Considering its regulatory role, we decided to explore whether over-expression of this protein in transgenic citrus could interfere with CTV replication and provide resistance. More than 50 transgenic Mexican lime plants were generated carrying the *p23* gene or a truncated version thereof. Unexpectedly, constitutive expression of p23 induced phenotypic aberrations resembling symptoms incited by CTV in non-transgenic Mexican lime, whereas transgenic plants expressing the p23-truncated version were normal. The onset of CTV-

like symptoms in p23-transgenic plants was associated with the accumulation of p23, and its level paralleled symptom intensity (Ghorbel et al. 2001). Over-expression of p23 in other CTV-susceptible citrus genotypes, including sweet and sour orange, and the CTV-resistant *Poncirus trifoliata*, also led to CTV-like symptoms that were not visible when these plants were transformed with a truncated p23 version (Fagoaga et al. 2005). Altogether, these results indicate that p23 is an important CTV pathogenicity determinant that interferes with plant development in *Citrus* species and relatives. In the course of the experiments to incorporate *p23* into Mexican lime, three out of 60 lines carrying the *p23* gene of the severe strain CTV T36, and two out of 20 lines carrying *p23* from the mild strain CTV T317, were visually normal and developed as controls transformed with the empty vector or non-transformed. These five lines displayed characteristics typical of RNA silencing: multiple copies and methylation of the silenced transgene, low levels of the corresponding mRNA, and accumulation of *p23*-specific siRNAs. When propagations of these silenced lines were graft- or aphid-inoculated with CTV, some were immune since they neither expressed symptoms nor accumulated virions or viral RNA. Other propagations were moderately resistant because they showed delayed expression of leaf symptom and attenuated stem pitting compared to the controls. The susceptible propagations showed normal symptom expression and elevated virus titer, as the empty-vector controls (Fagoaga et al. 2006).

A characteristic of the *p25* and *p23* transgene-mediated RNA silencing is that vegetative propagations from the same transgenic line

showed different responses against CTV, with some propagations being immune and others susceptible to viral challenge. This variable response among clonal transformants carrying viral-derived transgenes indicates that factors other than the genetic background of the transgenic plant, such as environmental conditions or the developmental stage, play a key role in RNA-mediated resistance.

1.5. RNAi against CTV

To further enhance RNA silencing against CTV, a new set of constructs was designed from a highly conserved region (>90% homology) comprising part of the *p23* gene and the 3'-UTR, which is critical for recognition by the replicase complex. Mexican lime plants were transformed with the 3'-terminal 549 nucleotides of the CTV genome in sense, antisense and intron-hairpin formats. Intron-hairpin constructs are strong inducers of RNA interference against plant viruses (Smith et al. 2000) because, upon transcription, they generate a dsRNA molecule that acts as a highly efficient trigger for RNA silencing leading to cleavage and degradation of target complementary viral and transgene-derived RNAs (Fire et al. 1998).

After challenge by graft-inoculation, propagations from all sense, antisense and empty-vector transgenic lines were susceptible to CTV, except for a single sense line with a complex transgene integration pattern that showed transgene-derived siRNAs in association with low levels of the transgene-derived transcript. By contrast, nine of the 30 intron-hairpin lines showed CTV resistance,

with 9%–56% of the propagations (depending on the line) remaining uninfected after graft-inoculation and the others being susceptible. As indicated above, factors other than the genetic background of the transgenic plant, including differences in the physiological and ontological stage of individual propagations, may be critical for the efficiency of RNA silencing-mediated resistance in clonal plants. Resistance was always associated with the presence of transgene-derived siRNAs, but their level in different sense and intron-hairpin transformants was variable irrespective of the response to CTV infection. Empty-vector infected controls also accumulated high levels of siRNAs from the viral 3'-UTR, most likely derived from genomic and subgenomic dsRNAs. Indeed, CTV-infected plants accumulated 1 to 2 orders of magnitude more siRNAs than noninfected intron-haipin transformants, indicating that the virus has evolved very efficient counterdefense strategies based on expression of three different silencing suppressor proteins (Lu et al. 2004), probably targeting different components of the RNA silencing pathway.

In intron-hairpin lines with single transgene integration, CTV resistance was correlated with low accumulation of the transgene-derived transcript rather than with high accumulation of transgene-derived siRNAs (López et al. 2010). As resistance could not be predicted by high transgene-derived siRNA levels, our results suggest that only a fraction of the transgene-derived siRNAs, perhaps those resulting from HEN1-mediated methylation (Yang et al. 2006; Yu et al. 2005) and/or those programming RISC (Omarov et al. 2007; Pantaleo et al. 2007), are competent for RNA silencing, with the other

fraction being quickly degraded, as proposed to occur with most virus-derived siRNAs in infected plants (Qu and Morris 2005). Besides, rather than blocking the biogenesis of siRNAs, CTV silencing suppressors would prevent their loading into the AGO-containing RISC complex or proper functioning of another downstream step in the RNA silencing pathway. The p23 intracellular suppressor has characteristics in common with protein 2b of cucumoviruses and protein P0 of poleroviruses. All the three are pathogenicity factors that induce developmental aberrations when over-expressed in transgenic plants, which are reminiscent of the phenotypes of plants affected in the miRNA pathway (Fagoaga et al. 2005; Lewsey et al. 2007; Bortolamiol et al. 2007). As 2b and P0 target members of the Argonaute family, 2b by binding AGO1 directly to prevent the RISC complex from cleaving its target RNA (Zhang et al. 2006), and P0 by targeting members of the Argonaute family for degradation (Bortolamiol et al. 2007; Baumberger et al. 2007), CTV p23 might act at the same level. Recent results show that miRNA168, which targets AGO1, is upregulated by CTV infection in Mexican lime and other citrus genotypes (Ruiz-Ruiz et al. 2011). It will be worth testing whether p23 is actually targeting AGO1.

The success of RNAi against CTV would depend on whether transgene-induced RNA silencing can substantially attenuate or block virus gene expression and, more specifically, the accumulation of three silencing suppressor proteins (López et al. 2010). To achieve this aim we have designed a transformation vector carrying a cassette comprising the complete untranslatable versions of genes *p25, p20*

and *p23* plus the 3'-UTR in sense and antisense configurations, and separated by the piv2 intron of the potato (*Solanum tuberosum*) gene *st-ls1* under the control of CaMV 35S promoter and the *nopaline synthase* terminator. This construct has been used to transform Mexican lime and the resulting transgenic lines have been propagated and challenged by graft-inoculation with severe CTV strains under greenhouse conditions. The results indicate that this strategy may provide the best level of resistance against CTV achieved so far in this host (Soler et al. 2012).

Another RNAi construct has been designed to target sequences proximal to the 5'-UTR, because the subgenomic dsRNAs derived from this region are much less abundant than those from the 3'-half of the genome (Moreno et al. 2008), and because the siRNAs derived from the 5'-UTR accumulate at much lower levels than those from the 3'-UTR (Ruiz-Ruiz et al. 2011). However, CTV strains show similarities as low as 44% in the 5'-UTR (Gowda et al. 2003), with the corresponding sequences having been classified into three types (I, II and III) based on intragroup sequence identity higher than 88% (López et al. 1998). Consequently, for a construct potentially silencing all known CTV strains, one fragment of at least 50 nt and 100% intragroup sequence identity was chosen for each group. The first fragment (nucleotides 25 to 85 from group I) comprises two stem-loop structures within the 5'-UTR that are required for virus replication (Gowda et al. 2003). The second fragment (nucleotides 105 to 522 from group II) covers the ORF 1a translation initiation and the first part of the papain-like protease PRO I domain. The third fragment

(nucleotides 1531 to 1604 from group III) comprises part of the PRO I and PRO II domains of the polyprotein 1a. The three regions were RT-PCR amplified and cloned as a fragment of 588 nt that was then subcloned in a transformation plasmid in sense and antisense configurations, separated by the intron of the *pyruvate orthophosphate dikinase* gene from *Flaveria trinervia*, under the control of the 35S CaMV promoter and the *octopine synthase* terminator. This construct has been used to transform Mexican lime plants, and several transgenic lines are currently being propagated to be challenged by graft-inoculation with severe CTV strains under greenhouse conditions (Chiibi et al. unpublished results).

In summary, these studies show that RNAi can be extended to CTV in its natural hosts. Whether transgenic citrus plants expressing CTV-derived sequences could be an efficient alternative to cross protection for controlling in the field CTV strains inducing stem pitting remains to be tested. So far, only partial protection to CTV has been achieved in greenhouse experiments with transgenic Mexican lime, but it should be mentioned that this experimental host allows CTV to reach very high titers compared with other citrus species, particularly sour orange, in which CTV is essentially unable to move cell-to-cell (Folimonova et al. 2008). With the aim of developing sour orange rootstocks resistant to the tristeza syndrome, we have transferred to this genotype those constructs providing some level of protection to CTV in Mexican lime. Transgenic sour orange lines carrying *p25*, *p23* and several intron-hairpin constructs are currently being tested for resistance to decline in field trials performed in a

cooperative project (with Catalina Anderson at the Estación experimental INTA-Concordia (Argentina), in an area where the brown aphid (*Toxoptera citricida*) vector and severe CTV strains are prevalent.

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2. OBJECTIVES

The objectives of this work have been:

1. Transformation of Mexican lime (*Citrus aurantifolia* (Christm.) Swing.) with an intron-hairpin construct expressing whole untranslatable versions of the genes coding for the three silencing suppressors of *Citrus tristeza virus* (CTV) to generate transgenic resistance against the virus in a model citrus type highly sensitive to the virus.

2. Investigating which region or regions of the p23 protein from CTV are implicated in the viral pathogenesis through transgenic expression of the whole p23 or truncated versions comprising or not the zinc-finger and flanking basic motifs of the protein under the control of the *Cauliflower mosaic virus* 35S promoter in Mexican lime, and deciphering whether the same region/s are also involved in suppression of RNA silencing in *Nicotiana benthamiana*.

3. Get more insight on the role of the p23 protein from CTV in pathogenesis through transgenic expression in Mexican lime of the complete gene *p23* from either the aggressive T36 or the mild T317 CTV strains, or a fragment comprising the zinc-finger and flanking basic motifs from the former, either under the control of the phloem-specific CoYMV promoter or the constitutive 35S promoter.

3.1. CHAPTER 1

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.

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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.

3.1.1. 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). *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 latofolia* Tan.) scion varieties regardless of the rootstock, reducing vigour, yield and fruit quality (Moreno et al., 2008).

Citrus tristeza virus virions are filamentous particles about 2000x11 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' unstranslated 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' co-terminal 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 X *Poncirus trifoliata* (L.) Raf.] and citrumelos (grapefruit X *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 sensitive 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 21-25 nt short interfering RNAs (siRNAs) by RNase III-like enzymes called Dicers (Bernstein et al., 2001). While one siRNAs is degraded, the other is incorporated into the Argonaute-containing RNA-induced silencing complex and guides it for cleavage or translational arrest of ssRNA 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 an 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.

3.1.2. 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 (*npt*II), between the 35S-pro and the 35S terminator (35S-ter), and by the reporter gene *β-glucuronidase* (*uid*A), 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, as revealed by the low fluorescence and accumulation of *gfp*-specific siRNAs (Figure 1b,c; GFP). As expected, in leaves coinfiltrated 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.

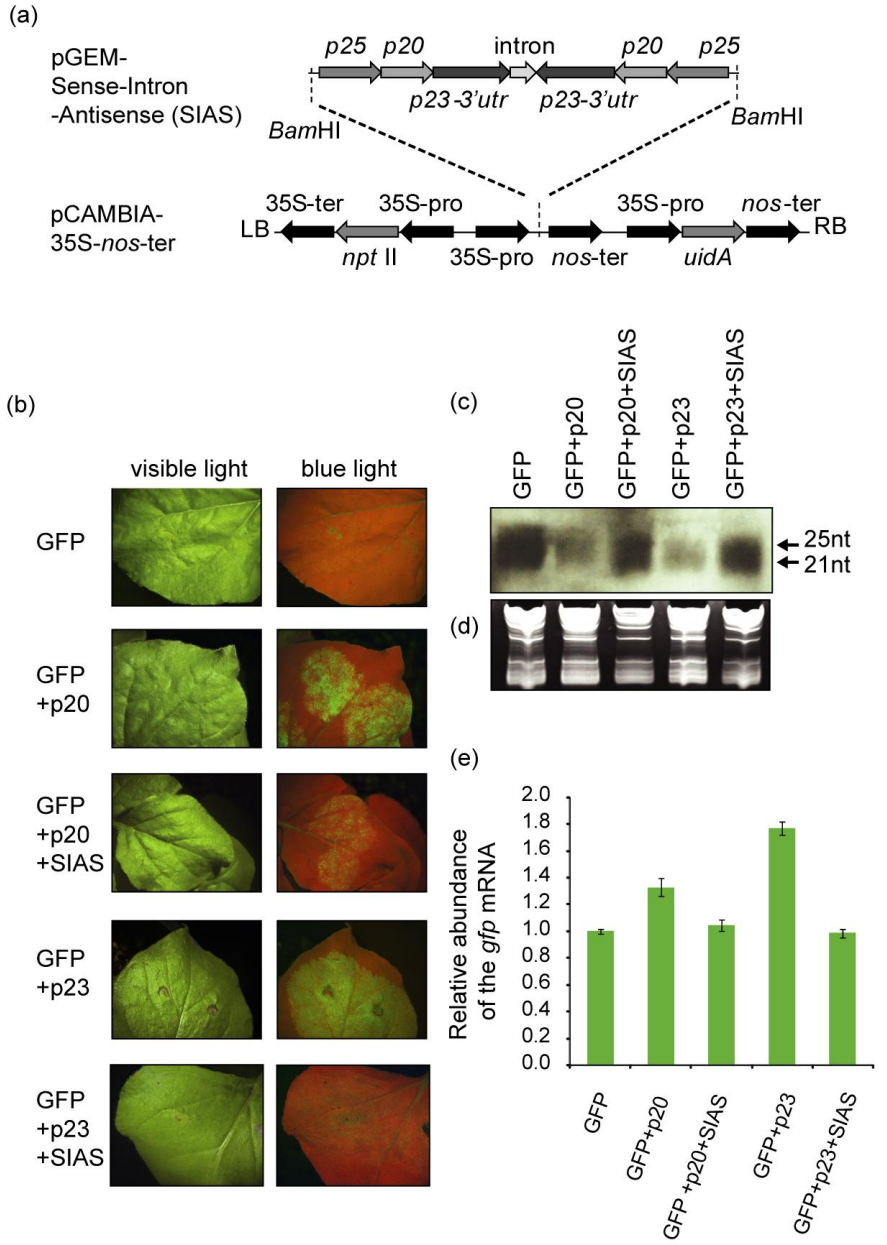

Figure 1 (a) Schematic representation of plasmid pGEM-SIAS carrying the *p25*, *p20* and *p23*-3'UTR fragments of the genomic RNA of CTV-T36 in intron-hairpin configuration, and subcloning of the cassette into the binary vector pCAMBIA-35S-*nos*-ter. The Sense-Intron-Antisense (SIAS) sequence is controlled by the 35S promoter (35S-pro) of CaMV and the *nopaline synthase terminator* (*nos*-ter) and flanked by the gene for *neomycin phosphotransferase* II (*npt*II) between the 35S-pro and 35S terminator (35S-ter) and by the gene for *β-glucuronidase* (*uid*A) between the 35S-pro and the *nos*-ter. (continued)

Figure 1 (continued) (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.*

Fifteen transgenic plants were recovered for each construct (results 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 *Hind*III 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 *Hind*III (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.

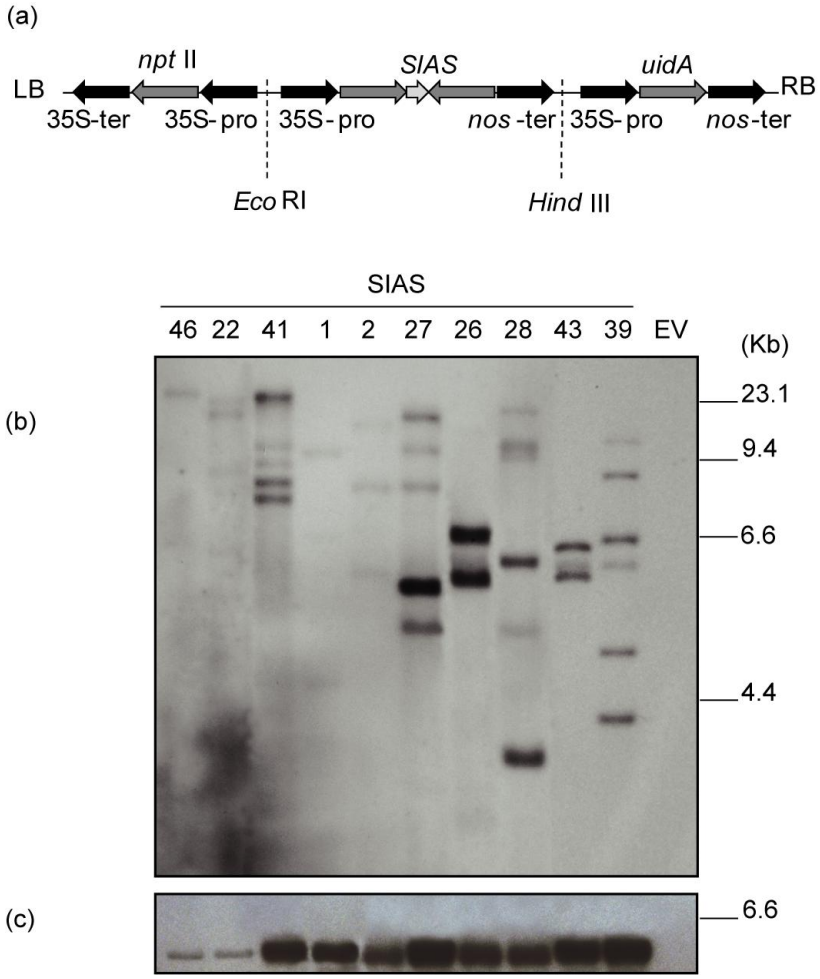

Figure S1 (a) Diagram of the T-DNA from the binary vector pCambia-SIAS with the SIAS cassette controlled by the CaMV 35S-pro and the *nos*-ter and flanked by the cassettes 35S-pro/*npt*II/35S-ter and 35S-pro/*uid*A/*nos*-ter. Transcription orientation for each cassette is indicated by arrows, and restriction sites *Hind*III 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 *Hind*III (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.

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).

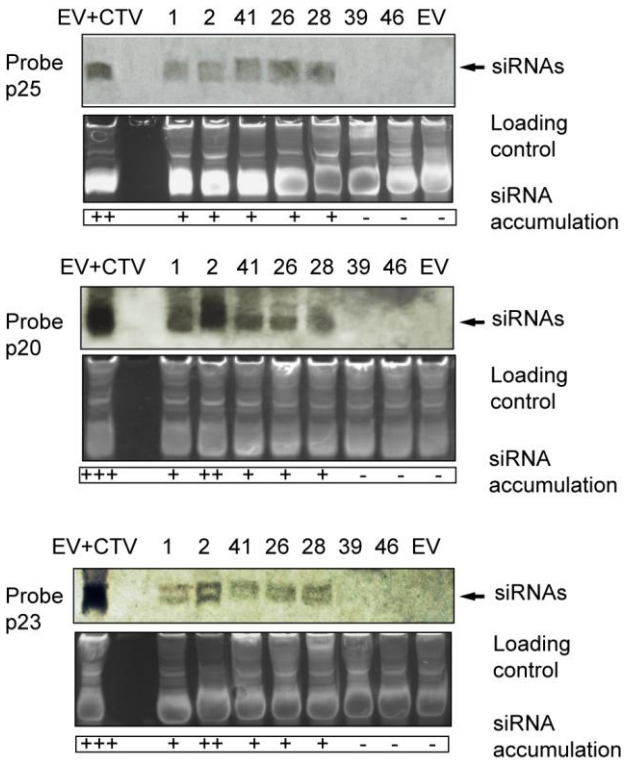

Figure 2 Accumulation of transgene-derived siRNAs in seven Sense-Intron-AntiSense (SIAS) transgenic lines (SIAS-1, SIAS-2, SIAS-41, SIAS-26, SIAS-28, SIAS-39 and SIAS-46). Northern blot analyses were performed with DIG-labelled riboprobes for detecting the positive strand of genes *p23*, *p20* or *p25*. 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*.

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 control. 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 uninfected.

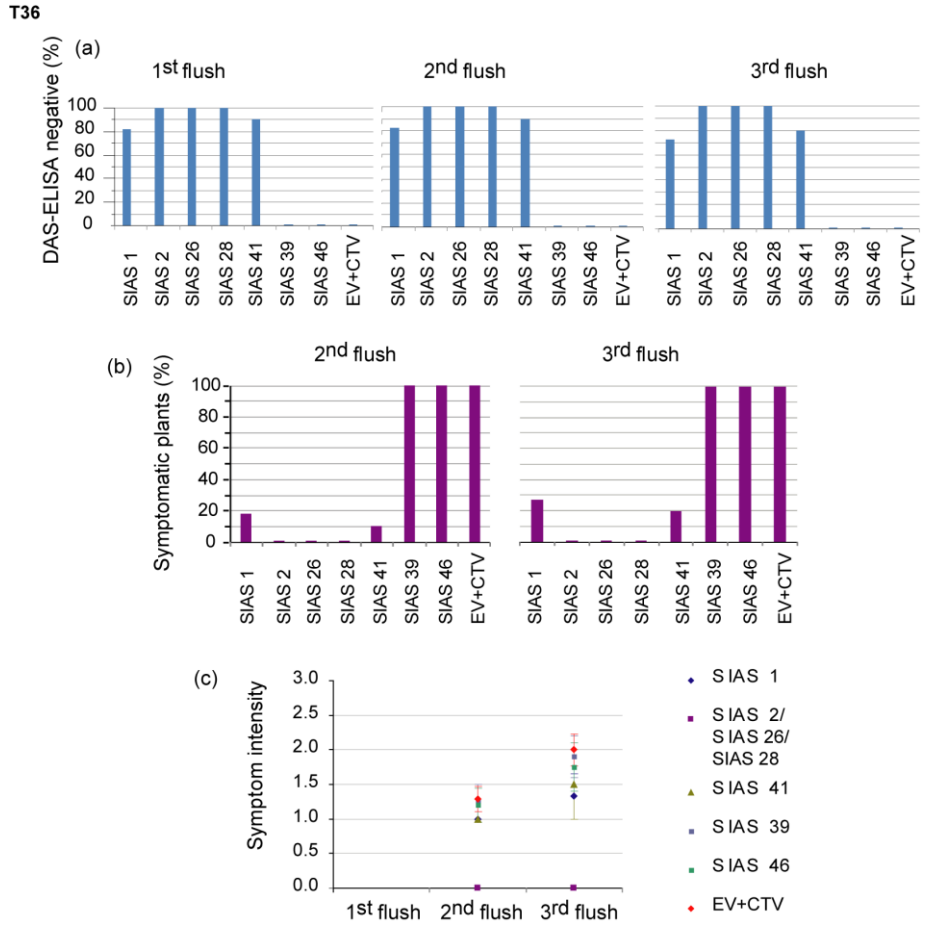

Figure 3 Evaluation of viral resistance in propagations from Sense-Intron-AntiSense (SIAS) transgenic lines 1, 2, 26, 28, 41, 39 and 46, or empty vector (EV) after graft inoculation with the CTV-T36 clonal strain. (a) Fraction (in %) of DAS-ELISA-negative propagations of each transgenic line in three 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*.

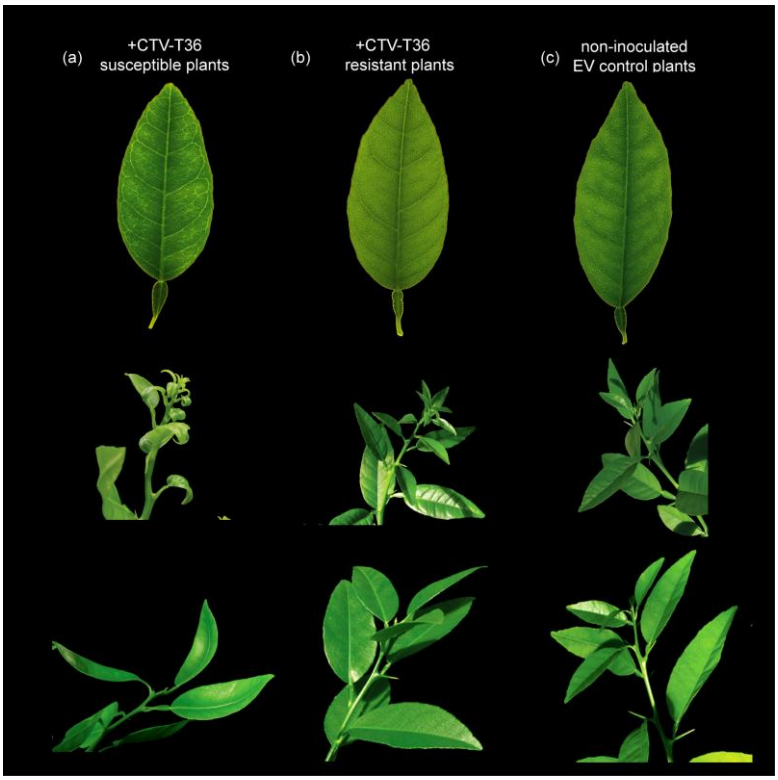

Figure S2 Symptoms of Mexican lime propagations from a susceptible (SIAS-39) (a) and a resistant (SIAS-2) (b) SIAS transgenic plants expressed in the third flush after graft inoculating the CTV-T36 clonal strain in the Carrizo citrange rootstock in comparison with a noninoculated EV control (c). Susceptible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

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 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 of these lines and of 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 the stem bark as well as in the 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 the 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 *C. 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.

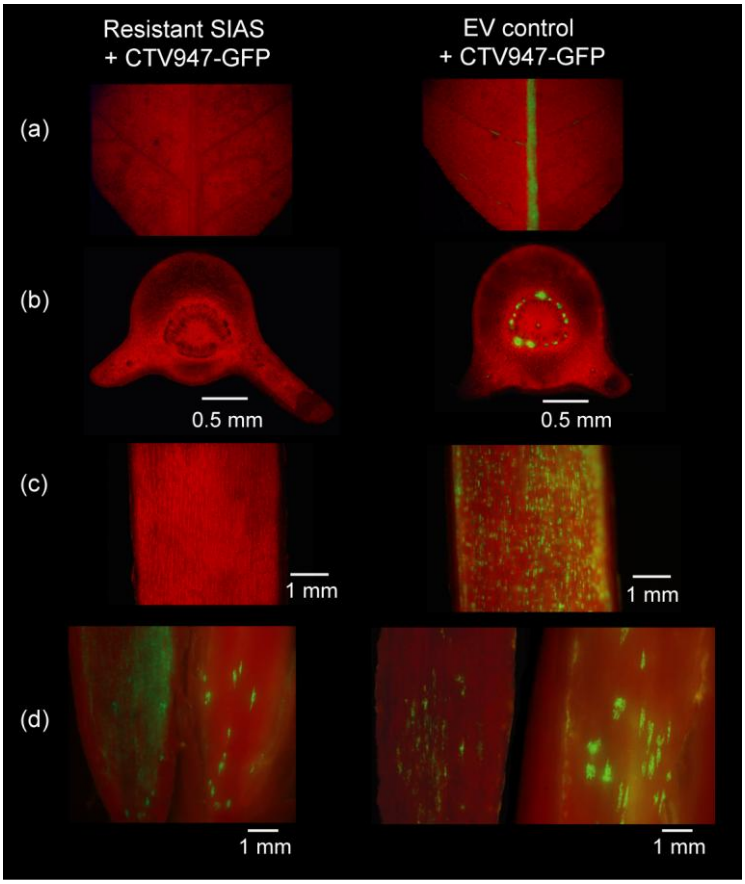

Figure 4 *Citrus tristeza virus* (CTV) distribution as detected by GFP fluorescence in the third flush of propagations of Mexican lime from a resistant Sense-Intron-AntiSense (SIAS) transgenic line (SIAS-2) (left) and from the empty vector (EV) control line (right) after graft inoculation of the scion 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).

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 of 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 resistance 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 CTVT318A, 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.

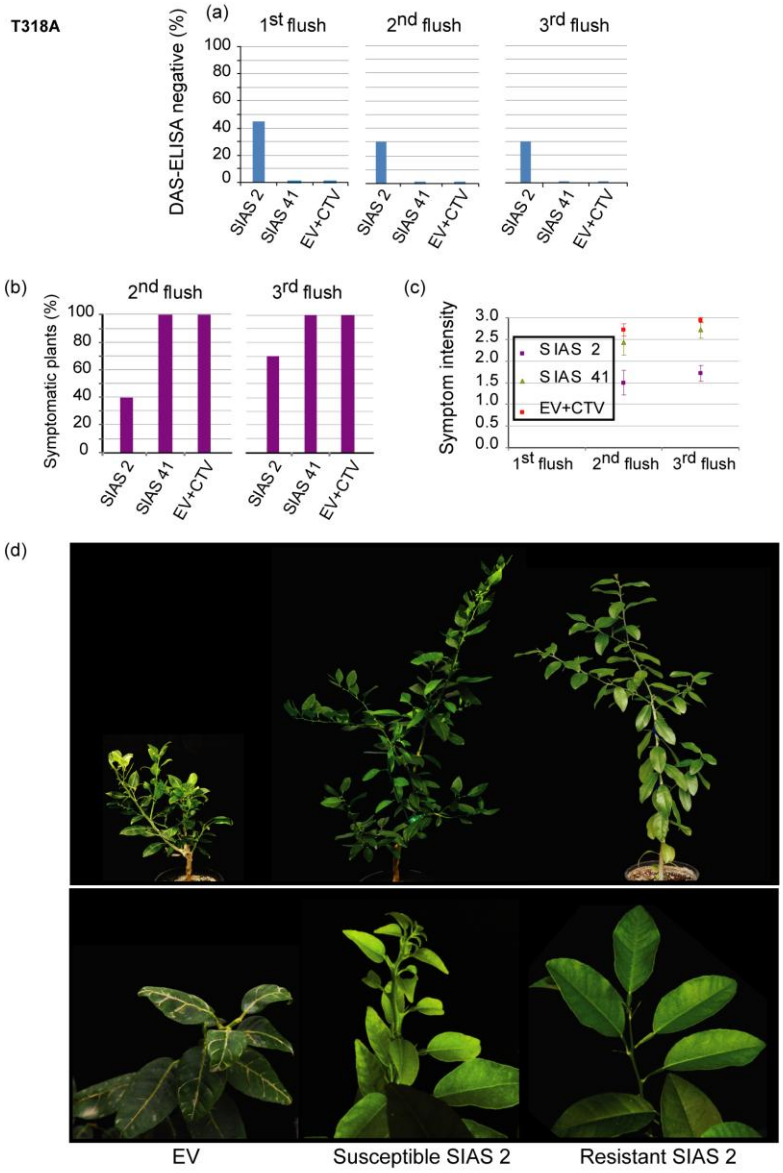

Figure 5 Evaluation of virus resistance in propagations of Mexican lime from the Sense-Intron- AntiSense (SIAS) transgenic lines 2 and 41 and from the empty vector line (EV) graft-inoculated with the heterologous CTV-T318A strain. (a) Fraction (in %) of DAS-ELISA-negative propagations from each transgenic 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*.

3.1.3. 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 (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, CTV947RGFP, 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.

3.1.4. 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) and the primer pairs P25*mutF/*Bam*HI (5'-ATAA*GGATCC*ATGAGACGACGAAACAAAGAA-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*ATGGATAGGATACTAGCGGACA-AA-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-ls*1 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 *Hind*III/*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 *Hind*III 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% polyacrylamidegels prepared in 0.25x 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 ºC 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 2x SSC plus 0.1% SDS for 10 min at room temperature and then with 0.1x SSC plus 0.1% SDS for 15 min at 35 ºC. 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 OD600. 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 lL of Power SYBR® Green PCR Master Mix (2x) (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 ºC for 30 min and 95 ºC for 10 min, respectively, followed by 35 cycles of 95 ºC for 30 s, 53 ºC for 30 s and 72 ºC for 60 s. Fluorescence intensity data were acquired during the 72 ºC 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 two-fold 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 ºC/16–18 ºC (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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3.2. CHAPTER 2

Citrus tristeza virus p23: Determinants for nucleolar localization and their influence on suppression of RNA silencing and pathogenesis.

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Nota: En este trabajo, Nuria Soler ha realizado todos los experimentos de Lima Mexicana además de los experimentos de actividad supresora de p23 sobre *N. benthamiana*, los cuales se realizaron por duplicado en los dos laboratorios.

Abstract

Citrus tristeza virus (CTV) encodes a singular protein (p23, 209 amino acids) with multiple functions, including RNA silencing suppression (RSS). Confocal laser-scanning microscopy of green fluorescent protein (GFP)-p23 agroexpressed in *Nicotiana benthamiana* revealed its accumulation in the nucleolus, Cajal bodies, and plasmodesmata. To dissect the nucleolar localization signal (NoLS) typically associated with basic motifs, seven truncated and 10 point-mutated versions of p23 were assayed. Deletion mutants showed that regions 50 to 86 and 100 to 157 (excluding fragment 106 to 114), both with basic motifs and the first with a zinc-finger, contain the (bipartite) NoLS. Alanine substitutions delimited this signal to three cysteines of the Zn-finger and some basic amino acids. RSS activity of p23 in *N. benthamiana* was abolished by essentially all mutants, indicating that it involves most p23 regions. The necrotic-inducing ability of p23 when launched in *N. benthamiana* from *Potato virus X* was only retained by deletion mutant 158-209 and one substitution mutant, showing that the Zn-finger and flanking basic motifs form part of the pathogenic determinant. Ectopic expression of p23 and some deletion mutants in transgenic Mexican lime demarcated a similar determinant, suggesting that p23 affects related pathways in citrus and *N. benthamiana.* Both RSS activity and pathogenicity of p23 appear related to its nucleolar localization.

3.2.1. Introducction

Citrus tristeza virus (CTV) (family *Closteoviridae*, genus *Closterovirus)* is restricted in natural infections to the phloem of some species of two genera of the family *Rutaceae* (Bar- Joseph et al. 1989; Moreno et al. 2008), with the virus accumulating at significantly higher titers in Mexican lime (*Citrus aurantifolia* (Christm.) Swing.) and sweet orange (*C. sinensis* L. Osb.) than in sour orange (*C. aurantium* L.) (Folimonova et al. 2008; Ruiz-Ruiz et al. 2011). However, when agroinoculated, CTV may also cause systemic infection and symptoms in the presumed non-host *Nicotiana benthamiana* (Ambrós et al. 2011). CTV has the largest (19.3 kb) genome reported for a plant monopartite single-stranded (ss)RNA (+) virus, organized in 12 open reading frames (ORF) potentially coding for at least 17 protein products flanked by 5′ and 3′ untranslated regions (UTR) (Karasev et al. 1995; Mawassi et al. 1996; Vives et al. 1999; Yang et al. 1999). Whereas the genomic (g)RNA directs translation of the two 5′-proximal ORF encoding components of the replicase complex, the 3′-proximal ORF encoding 10 proteins are expressed from 3′-coterminal subgenomic (sg)RNAs (Hilf et al. 1995). Three of these proteins are RNA silencing suppressors (RSS) when expressed in *Nicotiana* spp.: p25 and p23, with an inter- and intracellular mode of action, respectively, and p20 that operates both ways (Lu et al. 2004).

Viruses encode in their genomes one or more RSS to counteract the RNA-based antiviral response of their hosts mediated

by the virus-derived small (vs)RNAs (Csorba et al. 2009; Ding 2010). In plants infected by ssRNA viruses, these vsRNAs result from processing by DICER-like (DCL) enzymes (Jacobson et al. 1999; Qi et al. 2005) of the snap-folded viral ssRNA itself (Molnar et al. 2005) and, particularly, of the double-stranded (ds)RNA generated by the viral RNA replicase or by host RNA-dependent RNA polymerases (RDR) (Dalmay et al. 2000; Voinnet 2008). The vsRNAs then prime and lead an RNase H-like Argonaute protein (AGO) at the core of the RNA-induced silencing complex (RISC) (Hamilton and Baulcombe 1999; Vaucheret 2008) for inactivating their cognate viral ssRNAs (Omarov et al. 2007; Pantaleo et al. 2007).

Like other viral RSS, CTV-p23 (209 amino acids) is a multifunctional protein because, in addition to suppressing RNA silencing, it has been involved in regulating the asymmetrical accumulation of CTV RNA strands (Satyanarayana et al. 2002) and in eliciting CTV-like symptoms when expressed ectopically as a transgene in several *Citrus* spp. (but not in *Nicotiana* spp.) (Fagoaga et al. 2005; Ghorbel et al. 2001), with recent data supporting the idea that, most likely, it is also the CTV determinant of the seedling yellows syndrome in sour orange and grapefruit (*C. paradisi* Macf.) (Albiach-Martí et al. 2010). Moreover, the ectopic expression of p23 enhances systemic infection (and virus accumulation) in sour orange and enables CTV to escape from the phloem of sweet and sour orange, suggesting that constraints to CTV movement in citrus, particularly in sour orange, may result, at least in part, from RNA silencing or from p23 involvement in virus movement (Fagoaga et al. 2011). From a

biochemical viewpoint, p23 is an RNA-binding protein with a putative zinc-finger domain and some basic motifs (López et al. 2000); and, from an evolutionary perspective, p23 is unique to CTV, with no homologues found in other closteroviruses, including the type species of the genus *Beet yellows virus* (BYV), despite CTV and BYV sharing multiple homologous genes (Dolja et al. 2006). Therefore, p23 might have evolved for specific interactions of CTV with its citrus hosts.

Here, we report that p23 accumulates preferentially in the nucleolus, being the first closterovirus protein with such a subcellular localization, as well as in plasmodesmata. By deleting and substituting specific regions and amino acids of p23 we have i) mapped its bipartite nucleolar localization signal (NoLS) and ii) determined the effects of these deletions and substitutions in suppressing RNA silencing in *N. benthamiana* and in eliciting a pathogenic reaction, when expressed as a sRNA of *Potato virus X* (PVX) in this species and as a transgene in a sensitive citrus host.

3.2.2. Results

CTV-p23 accumulates preferentially in the nucleolus and Cajal bodies and in plasmodesmata

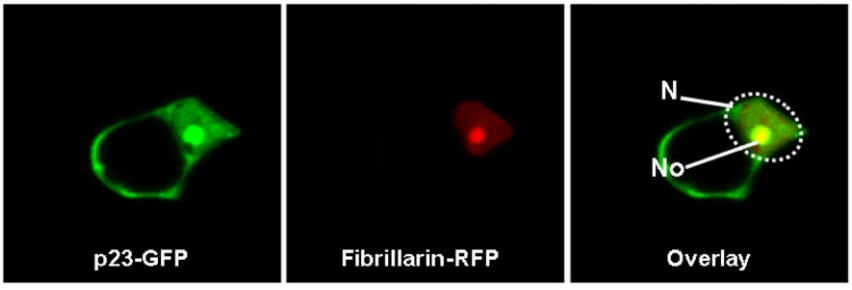
The presence of p23 in CTV, with no homologue in other closteroviruses, made it particularly interesting to determine its major subcellular accumulation sites. For this purpose, the 3′ terminus of gene *p23* from CTV isolate T36 was fused to the 5′ terminus of the green fluorescent protein (GFP) gene and this construct, under the

control of the 35S promoter from *Cauliflower mosaic virus* (CaMV) and the *nopaline synthase* terminator (NOS-t), was agroinfiltrated in leaves of *N. benthamiana*. Leaf examination by confocal laser-scanning microscopy at 2 days post agroinfiltration (dpai) revealed the preferential accumulation of the p23-GFP fusion protein in the nucleus and, more specifically, in the nucleolus and nucleolar bodies resembling Cajal bodies, while free GFP was found in the nucleus and cytoplasm (but not in the nucleolus) in the agroinfiltrated control (Figure 1). To further confirm these observations, the construct expressing the fusion protein p23-GFP was co-agroinfiltrated with another construct in the same vector expressing fibrillarin fused to the red fluorescent protein (RFP), which specifically marks the nucleolus and Cajal bodies (Barneche et al. 2000; Kim et al. 2007). Overlay of both images revealed a perfect match (Figure 2), thus substantiating the accumulation of p23 in these subnuclear compartments. This perfect match and the lack of any apparent cellular aberration made unlikely an artefactual localization for p23-GFP, a point further confirmed by mutational analyses and by the use of an alternative expression vector (discussed below). Moreover, co-agroinfiltration of p23-GFP and fibrillarin-RFP in young leaves of *C. macrophylla* also showed the preferential accumulation of p23 in the nucleolus of this sensitive natural host of CTV (Supplementary Figure S1).

On the other hand, p23 was also detected in agroinfiltrated leaves in punctuated structures at the cell wall that could represent plasmodesmata (Figure 1). To corroborate this localization, the

construct expressing p23-GFP was co-agroinfiltrated with a second construct in the same vector expressing the movement protein of *Tobacco mosaic virus* (TMV-MP) fused to the RFP, which should mark plasmodesmata specifically (Tomenius et al. 1987). However, overlay of both images showed p23-GFP in the nucleolus as well as in peripheral nuclear aggregates of both fusion proteins, suggesting an interaction between them, with TMV-MP-RFP being dragged by p23-GFP to its predominant subcellular localization site (data not shown). To circumvent this problem, we co-agroinfiltrated two constructs expressing p23-RFP and the movement protein of *Prunus necrotic ringspot virus* (PNRSV-MP) fused to the yellow fluorescent protein (YFP), a marker of plasmodesmata (Herranz et al. 2005). Overlay of the resulting images supports localization of p23 also in plasmodesmata (Figure 2).

Altogether, these results strongly suggested the existence in p23 of a nuclear localization signal (NLS) and, more specifically, an NoLS, as well as of a plasmodesmatal localization signal (PLS).



Supplementary Figure 1 Confocal laser-scanning microscopy of *Citrus macrophylla* leaves co-agroinfiltrated with constructs expressing the protein fusions p23-GFP and fibrillarin-RFP. Overlay of the corresponding images confirms the predominant accumulation of p23-GFP in the nucleolus (No). Fluorescence was observed 2 days post agroinfiltration.

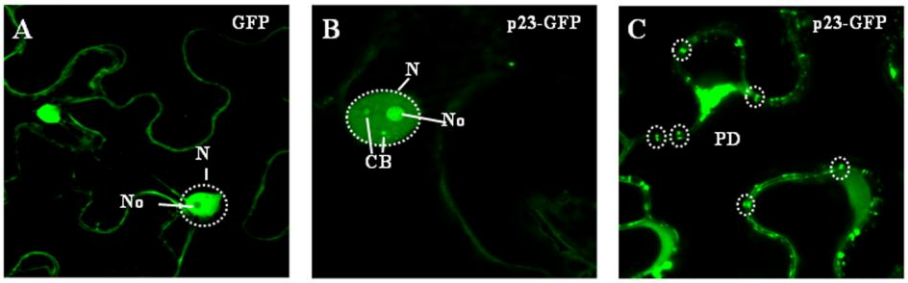


Figure 1 Confocal laser-scanning microscopy of *Nicotiana benthamiana* leaves agroinfiltrated with constructs expressing **A,** the green fluorescent protein (GFP) itself or **B** and **C,** fused to the C-terminus of the *Citrus tristeza virus* (CTV)-p23. Free GFP accumulates in the nucleus (N) and cytoplasm, being largely excluded from the nucleolus (No), while the fusion protein p23-GFP accumulates in the nucleolus, Cajal bodies (CB), and plasmodesmata (PD). Examinations were performed 2 days post agroinfiltration.

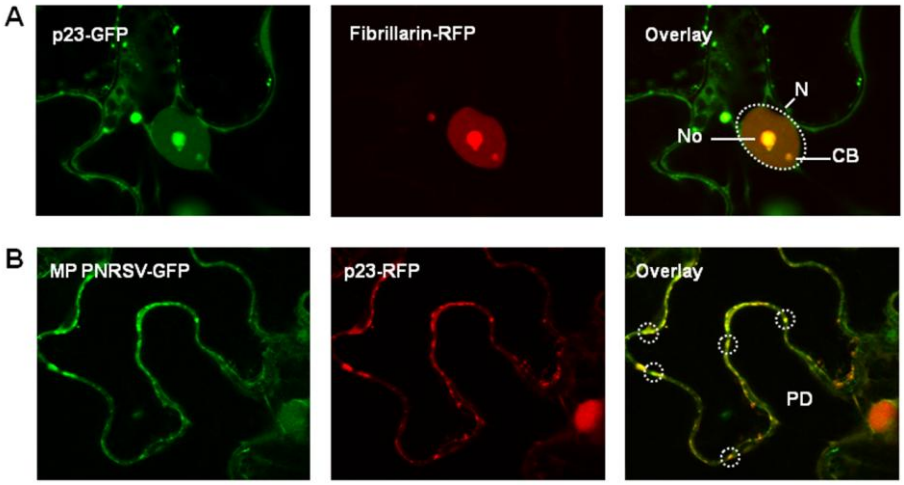


Figure 2 Confocal laser-scanning microscopy of *Nicotiana benthamiana* leaves co-agroinfiltrated with constructs expressing the **A,** protein fusions p23-green fluorescent protein (GFP) and fibrillarin-red fluorescent protein (RFP) and **B,** the protein fusions p23-RFP and the movement protein of *Prunus necrotic ringspot virus* fused to the yellow fluorescent protein (PNRSV-MP-YFP). Overlay of the corresponding images confirms the predominant accumulation of p23-GFP in the nucleolus (No) and Cajal bodies (CB), and in plasmodesmata (PD). Fluorescence was observed 2 days post agroinfiltration.

Because the motifs characteristically forming part of NLS and NoLS are relatively well known, we next examined their presence in p23.

Dissection of the p23 determinants of nucleolar localization

NLS and NoLS are formed by short motifs rich in basic amino acids that mediate the nuclear or nucleolar import of proteins, wherein they are contained, by binding to receptors known as importins (Hiscox 2007; Kosugi et al. 2009; Ryabov et al. 2004). Inspection of the amino acid sequence of p23 revealed three arginine- and lysine-rich regions or motifs (positions 51 to 86, 106 to 114, and 143 to 155) (Figure 3), the first including the putative Zn-finger domain (López et al. 2000). To examine whether one or more of these regions or motifs were part of the p23 NoLS, we constructed seven p23 mutants: p23Δ50-86, p23Δ50-66, and p23Δ67-86 (deleting the first region or portions thereof); p23Δ106-114 (deleting the second motif); p23Δ100-209 (deleting the second and third motifs); p23Δ125-209 (only deleting the third motif); and p23Δ158- 209 (deleting the C-terminal 51 amino acids without affecting any of the three regions or motifs).

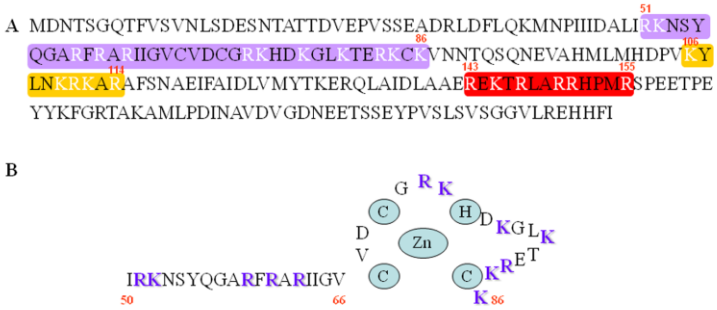


Figure 3 A, Amino acid sequence of p23 from *Citrus tristeza virus* (isolate T36) with the three arginine- and lysine-rich regions or motifs delimited by positions 51 to 86, 106 to 114, and 143 to 155 highlighted with different colors and **B,** the putative zinc-finger domain with the coordinating histidine and cysteines.

The 3′ termini of these mutated versions of *p23* were fused to the 5′terminus of the GFP gene and the resulting constructs were individually agroinfiltrated in *N. benthamiana* with the construct expressing fibrillarin- RFP.

Only p23Δ106-114 and p23Δ158-209 localized to the nucleolus and Cajal bodies like the full-length p23, whereas the remaining mutants were found in the nucleus but not in the nucleolus of transfected epidermal cells (Figure 4). Therefore, neither the second basic motif (positions 106 to 114) nor the last C-terminal 51 amino acids (positions 158 to 209) contribute to the NoLS.

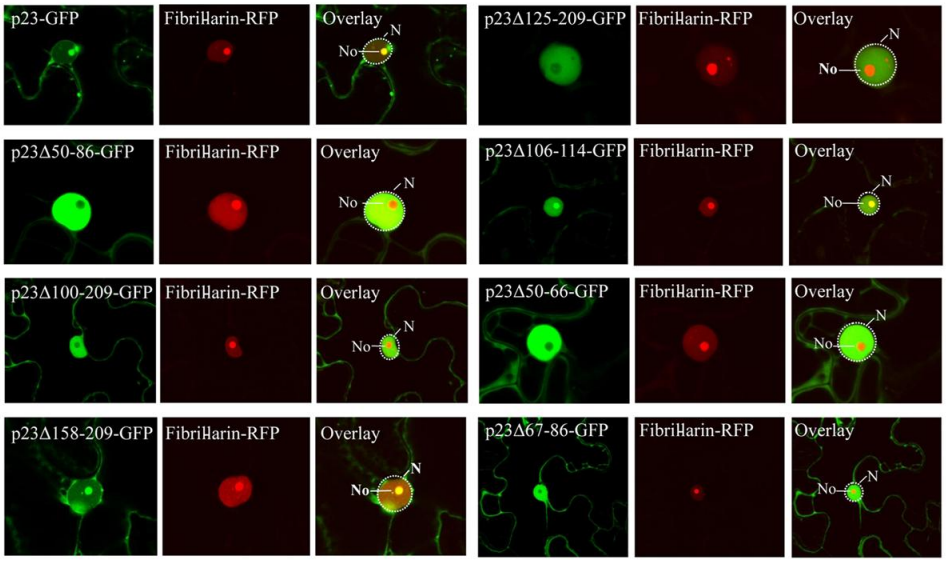


Figure 4 Confocal laser-scanning microscopy of *Nicotiana benthamiana* leaves, in which constructs expressing the protein fusion p23-green fluorescent protein (GFP) (and seven deletion mutants of p23 also fused to GFP) were individually co-agroinfiltrated with a construct expressing fibrillarin-red fluorescent protein (RFP). Overlays show that the nucleolar localization signal of p23 is bipartite and includes regions or motifs delimited by positions 50 to 86 and 143 to 155. GFP fluorescence was observed 2 days post agroinfiltration.

On the other hand, the region encompassing positions 50 to 86, as well as its segments delimited by positions 50 to 66 and 67 to 86, are critical for nucleolar targeting, as also is the region delimited by positions 125 to 157 (because p23Δ158-209 but not p23Δ125-209 is targeted to the nucleolus). Altogether, these results strongly support a bipartite NoLS (encompassing positions 50 to 86 and 125 to 157). To better map the NoLS existing within positions 125 to 157, we constructed a new deletion mutant, p23Δ143-209 (including the third motif rich in basic amino acids delimited by positions 143 to 155) which, in contrast with mutant p23Δ158-209, did not localize in the nucleolus (data not shown). Hence, amino acids 143 to 155 are also required for nucleolar import of p23. Incidentally, although a detailed analysis including co-agroinfiltration of the seven p23 mutants with the PNRSV-MP-GFP marker was not performed, we only observed plasmodesmata localization for p23Δ158-209 and not for the remaining mutants (data not shown).

Fine dissection of the bipartite NoLS of CTV-p23

To exclude the possibility that deleting large fragments of p23 could influence its global molecular structure and, indirectly, its subcellular localization, we additionally constructed 10 alanine-substitution mutants of p23: p23R51/K52 and p23R59/R61/R63 (basic amino acids preceding the Zn-finger domain); p23C68/C71/H75/C85, p23C71, and p23H75 (the four or just two individual amino acids potentially coordinating the Zn ion); p23R73/K74 and

p23K77/K80/R83/K84/K86 (basic amino acids within the Zn-finger domain); and p23R143/K145/R147, p23R150/R151/R155, and p23R143/K145/R147/R150/R151/ R155 (basic amino acids of the second motif required for nucleolar import of p23). The 3′ termini of these point-mutated versions of *p23* were fused to the 5′ terminus of the GFP gene and the resulting constructs were individually agroinfiltrated in *N. benthamiana* with the construct expressing fibrillarin-RFP. The seven mutants with substitutions mapping within the region delimited by positions 50 to 86 (the Zn-finger domain and preceding basic amino acids) lost their nucleolar localization, except p23H75, that behaved essentially like the wild-type (wt) p23 (Figure 5). Intriguingly, the quadruple mutant affecting the three cysteines and the histidine potentially binding the Zn ion (positions 68, 71, 75, and 85) induced a redistribution of the fibrillarin which, in addition to the nucleolus and Cajal bodies, was also observed in other subnuclear bodies of a size similar or even higher than the nucleolus. The mutant p23 accumulated in the subnuclear bodies, which should not be genuine but, most likely, resulted from this protein attracting some fibrillarin, and also in others not labeled by fibrillarin as revealed by the overlay (Figure 5).

The remaining three mutants with substitutions affecting the region delimited by positions 143 to 155 (the second motif forming part of the p23 NoLS) also lost their nucleolar localization. However, two of them behaved like the quadruple mutant affecting the three cysteines and the histidine potentially binding the Zn ion (discussed above), and induced a redistribution of the fibrillarin into one or

several subnuclear bodies (again, most likely not genuine) wherein the p23 mutants accumulated (Figure 5).

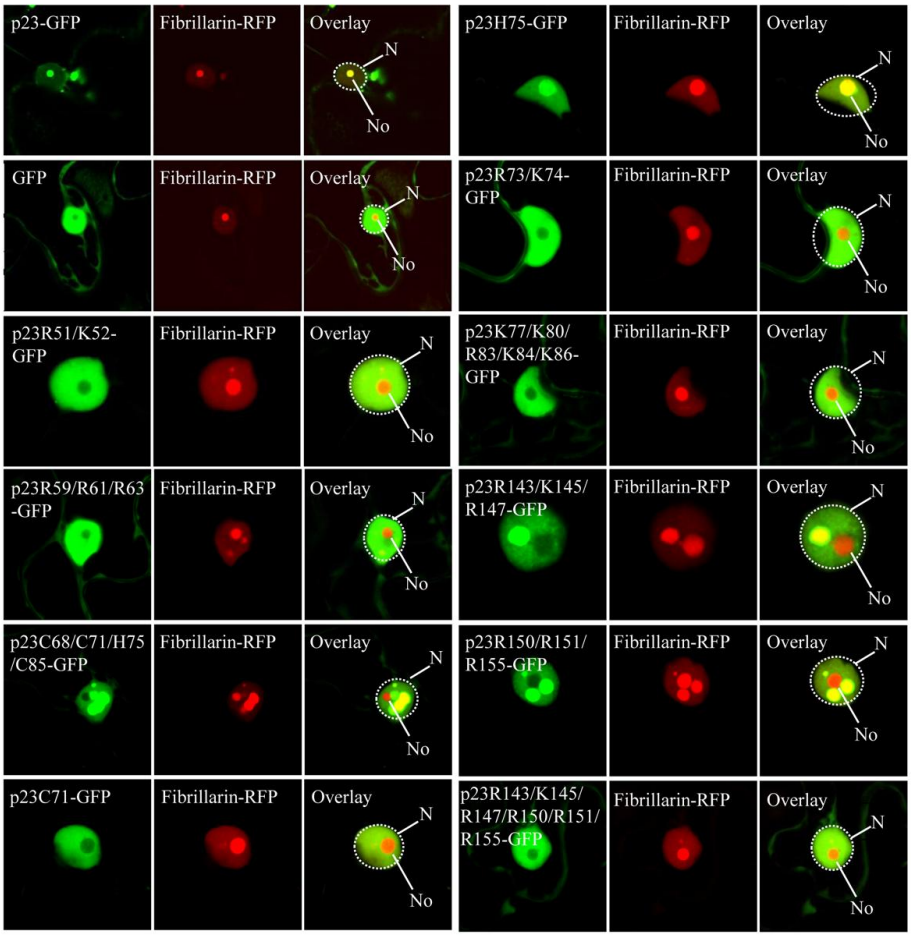


Figure 5 Confocal laser-scanning microscopy of *Nicotiana benthamiana* leaves in which constructs expressing the protein fusion p23-green fluorescent protein (GFP) (and 10 alanine-substitution mutants of p23 also fused to GFP) were individually co-agroinfiltrated with a construct expressing fibrillarin-red fluorescent protein (RFP). Overlays confirm that the nucleolar localization signal of p23 is bipartite and has two different components: short basic motifs and a zinc-finger domain. GFP fluorescence was observed 2 days post agroinfiltration.

Collectively, the data obtained with the substitution mutants confirmed and refined the conclusion inferred with the deletion

mutants: the NoLS of p23 is bipartite and has two different components, short basic motifs and a Zn-finger domain.

CTV-p23 has strict requirements to act as an RNA silencing suppressor

As indicated above, p23 has RSS activity when co-expressed by agroinfiltration with GFP in the transgenic line of *N. benthamiana* 16c constitutively expressing GFP (Lu et al. 2004). To further validate this result and to determine whether specific regions or motifs of p23 are necessary for suppressing RNA silencing, the wt form and its seven deletion mutants used previously in the subcellular localization experiments were individually co-agroinfiltrated with a plasmid expressing GFP (35S-GFP) into leaves of the same transgenic line 16c (Brigneti et al. 1998). Controls for this experiments included leaves of the transgenic line co-agroinfiltrated with plasmid 35S-GFP and either the empty plasmid or plasmid 35S-HcPro expressing the RSS of *Tobacco etch virus* (Kasschau and Carrington 1998).

At 2 to 3 dpai, GFP fluorescence was observed in leaves infiltrated with plasmid 35S-GFP alone or co-infiltrated with any of the other constructs. However, whereas leaves co-infiltrated with plasmids 35S-GFP and 35S-p23 or 35S-HcPro still emitted a strong fluorescence at 6 to 7 dpai, the fluorescence declined significantly with time in leaves infiltrated with plasmid 35S-GFP alone or co-infiltrated with the empty plasmid or with any of the seven plasmids expressing the individual p23 deletion mutants, becoming almost undetectable at 6 to 7 dpai (Figure 6). In line with these results,

Northern blot hybridizations with a GFP-specific riboprobe of RNAs extracted at 6 dpai showed a clear increase of GFP-mRNA accumulation in leaves co-agroinfiltrated with plasmids 35S-GFP and either 35S-p23 or 35S-HcPro, with respect to leaves agroinfiltrated with just plasmid 35S-GFP or co-agroinfiltrated with plasmids 35S-GFP and the empty plasmid or any of the seven p23 deletion mutants, all of which displayed a low GFP-mRNA accumulation similar to that resulting from the stably integrated GFP transgene. Concomitantly, strong bands with the mobility expected for GFP-specific small interfering RNAs (siRNAs) of 24, 22, and 21 nucleotides (nt) were detected in leaves expressing low GFP-mRNA levels whereas, in leaves expressing high GFP-mRNA levels, the intensity of the GFP-siRNA bands became very much attenuated (those of 24 and 22 nt) or undetectable (that of 21 nt) (Figure 6). Both RSS (p23 and HcPro) displayed very similar effects on the inversely correlated levels of GFP-mRNA and GFP-siRNAs (Figure 6). Leaving apart minor discrepancies regarding the optimal time for observing the RSS activity of p23, which may result from differences in the expression vector or growing conditions, our data essentially agree with those reported previously (Lu et al. 2004).

Regarding the effect on parallel co-agroinoculation experiments of the 10 substitution mutants of p23, all except p23H75 behaved as the deletion mutants: the fluorescence became almost undetectable at 6 to 7 dpai. As anticipated from these results, all substitution mutants induced low GFP-mRNA and high GFP-siRNAs,

with the exception of p23H75, in which the reverse situation was observed (data not shown).

Altogether, these results showed that the RSS activity of p23 was abolished by all deletions mutants as well as by all substitution mutants except p23H75. Therefore, the RSS activity of p23 involves most regions of this protein.

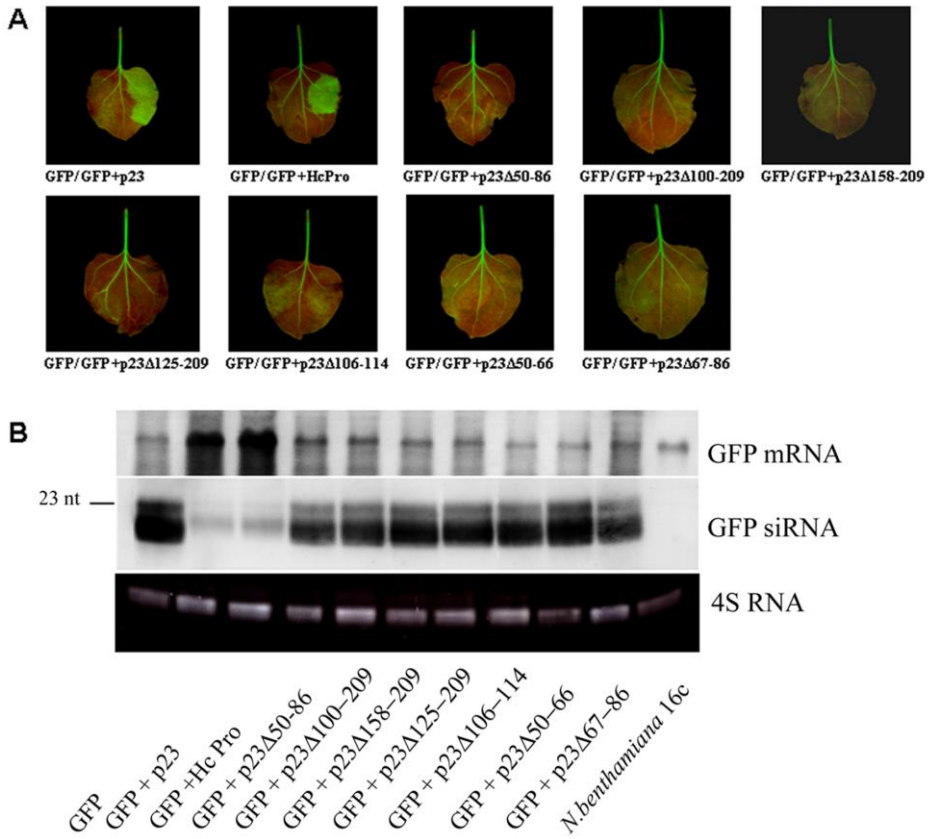


Figure 6 A, Confocal laser-scanning microscopy of *Nicotiana benthamiana* 16c leaves that were co-agroinfiltrated with the empty vector and a construct for expressing green fluorescent protein (GFP) (left half-leaf), or with constructs for expressing GFP and p23, Hc-Pro, or seven deletion mutants of p23 (right half-leaf). GFP fluorescence was observed 6 days post agroinfiltration. **B,** Accumulation of GFP mRNA and GFP-small interfering RNAs (siRNAs) as revealed by Northern blot hybridization with a specific riboprobe following electrophoresis in denaturing agarose (0.8%) and polyacrylamide (17%) gels, respectively. The 4S RNAs stained with ethidium bromide are shown as a loading control.

CTV-p23 is a pathogenic determinant in *N. benthamiana* when expressed from the heterologous vector PVX

In contrast to transgenic plants of different citrus species in which ectopic expression of p23 (wt) is accompanied by leaf symptoms sometimes resembling those incited by CTV in nontransgenic plants, ectopic expression of p23 in the CTV non-natural host *N. benthamiana* and in *N. tabacum* occurs without phenotypic aberrations (Fagoaga et al. 2005; Ghorbel et al. 2001), despite i) CTV T36 inducing symptoms in leaves of *N. benthamiana* (Ambrós et al. 2011), ii) p23 acting as an RSS suppressor in these two species (Lu et al. 2004), and iii) p23 accumulating at similar levels in citrus and *Nicotiana* spp. (Fagoaga et al. 2005). To investigate whether p23 could induce symptoms in *N. benthamiana* in a different context, this protein was expressed as a sgRNA of PVX (Voinnet et al. 1999). Although leaves mechanically inoculated with the wt PVX or its recombinant version (PVX-p23) did not exhibit visible alterations, the upper noninoculated leaves displayed vein clearing and a mild chlorotic mosaic at 7 days postinoculation (dpi) with either of the two infectious sources. However, at 10 dpi, PVX-p23 caused stunting and necrotic mottling in systemically infected leaves and stems and, at 15 dpi, the death of the plants, with no symptom accentuation being observed in PVX-infected plants (Figure 7). Therefore, p23, like other RSS (Pruss et al. 1997; Voinnet et al. 1999), is a pathogenic determinant in *N. benthamiana* when launched from PVX. Moreover, when launched from PVX, p23-GFP also accumulated preferentially

in the nucleolus of bundle sheath cells (and in the phloem but not in the xylem) (Figure 7A) (thus confirming its subcellular localization with an alternative expression vector) and induced symptoms in *N. benthamiana* similar to those caused by the expression of p23 (data not shown).

To know whether the severe symptoms incited by p23 might result from suppression of virus-induced gene silencing, we examined the effects of the seven deletion and 10 substitution mutants of p23 when expressed from the corresponding PVX sgRNAs: only the p23Δ158-209 deletion and the p23H75 substitution mutants incited symptoms (severe necrosis resulting in plant death) similar to those of the wt p23 (Figure 7 and Supplementary Figure S2, respectively). These results show that i) the pathogenic determinant of p23 resides in the first N-terminal 157 amino acids, and that the putative Zn-finger domain and flanking basic motifs form part of this determinant; and ii) the RSS activity and the ability to induce phenotypic aberrations are independent functions in p23 because p23Δ158-209 without the first activity retains the second ability.

To further examine the role that the interaction between PVX and p23 might have in exacerbating symptoms, we performed Northern blot analyses of RNA preparations from plants inoculated with PVX, as well as with PVX expressing p23 (wt) and its seven deletion mutants at 10 dpi.

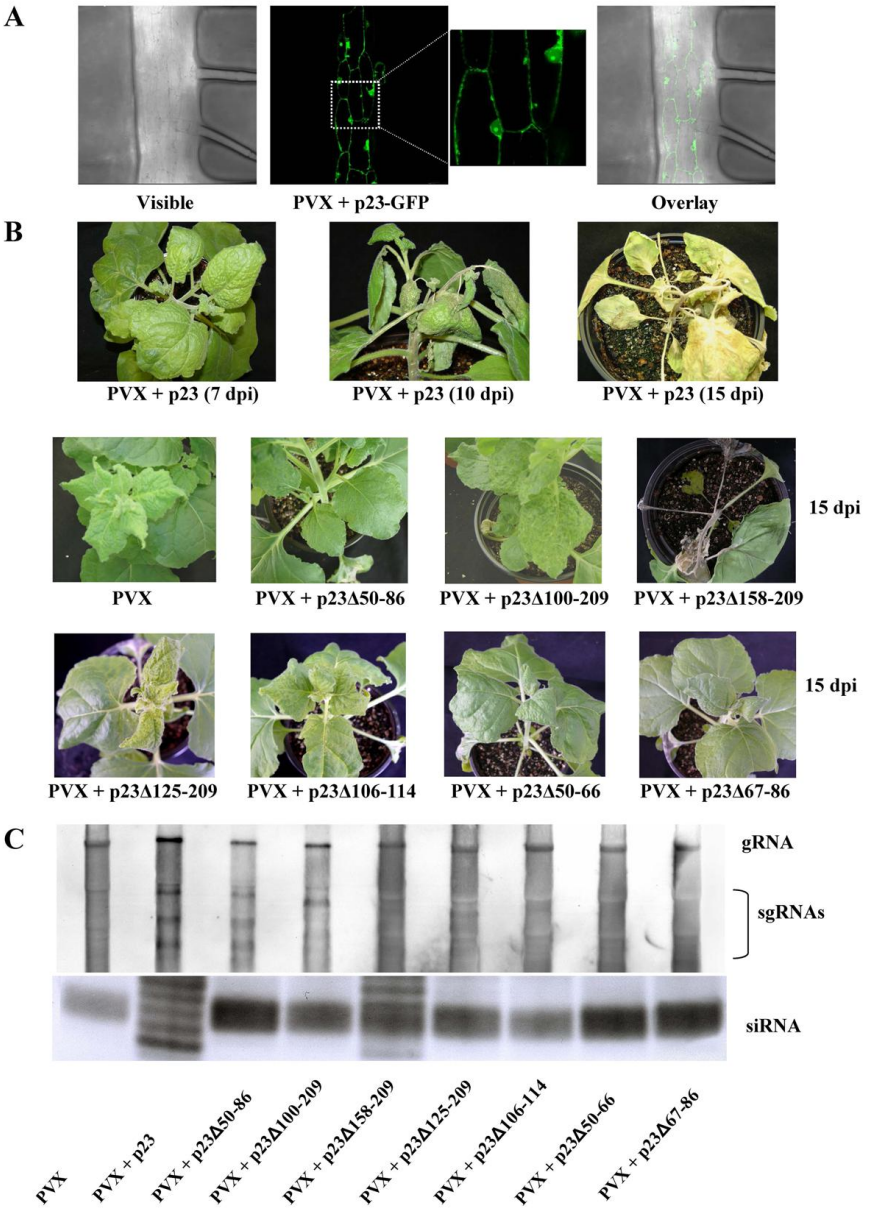
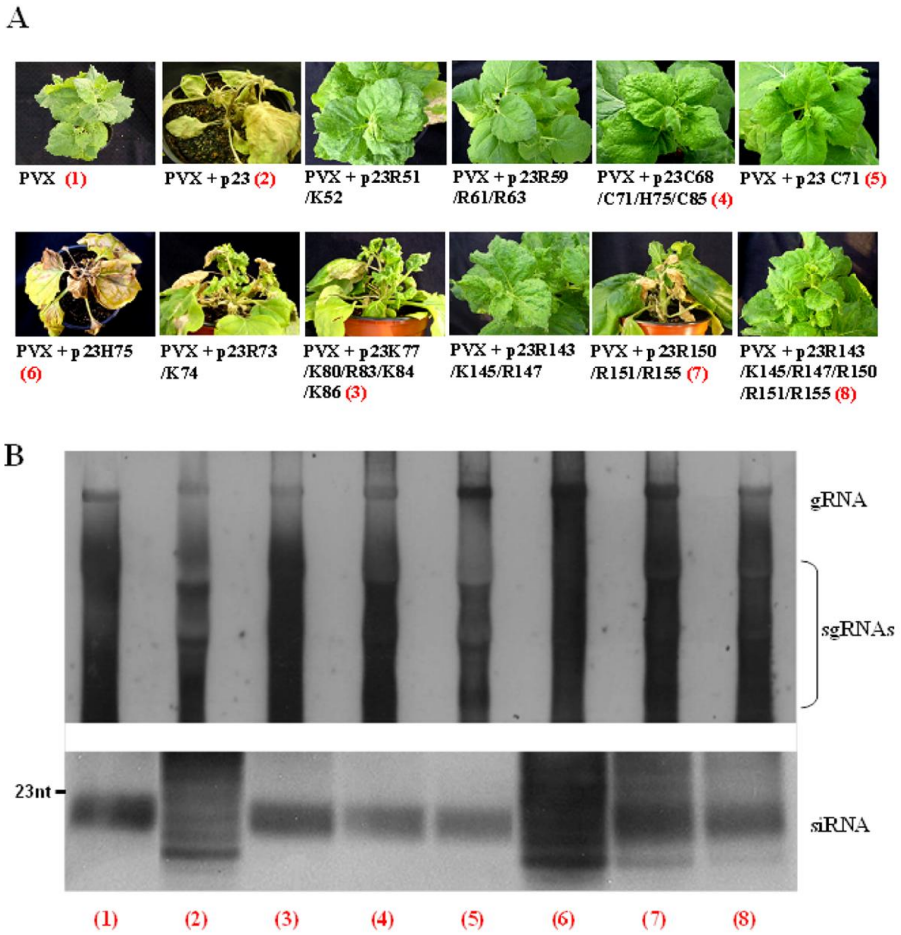


Figure 7 A, Confocal laser-scanning microscopy of vascular tissue from *Nicotiana benthamiana* infected by *Potato virus X* (PVX) expressing p23-green fluorescent protein (GFP) as a viral subgenomic (sg)RNA. **B,** Symptoms observed in upper noninoculated leaves of *N. benthamiana* in which p23 (first row) and seven deletion mutants thereof (second and third rows) were individually expressed as PVX sgRNAs. **C,** Accumulation of PVX genomic (g)RNA and sgRNAs, and of PVX-small interfering (si)RNAs, as revealed by Northern blot hybridization with a specific riboprobe for the PVX coat protein gene following electrophoresis in denaturing agarose (0.8%) and polyacrylamide (17%) gels, respectively.



Supplementary Figure 2 (A) Symptoms observed in upper noninoculated leaves of *Nicotiana benthamiana* in which p23 and ten substitution mutants were individually expressed as PVX sgRNAs. **(B)** Accumulation of *Potato virus X* (PVX) gRNA and sgRNAs, and of PVX siRNAs as revealed by Northern-blot hybridization with a specific riboprobe for the PVX CP gene following electrophoresis in denaturing agarose (0.8%) and polyacrylamide (17%) gels, respectively. Results are shown for only some representative mutants denoted with red numbers.

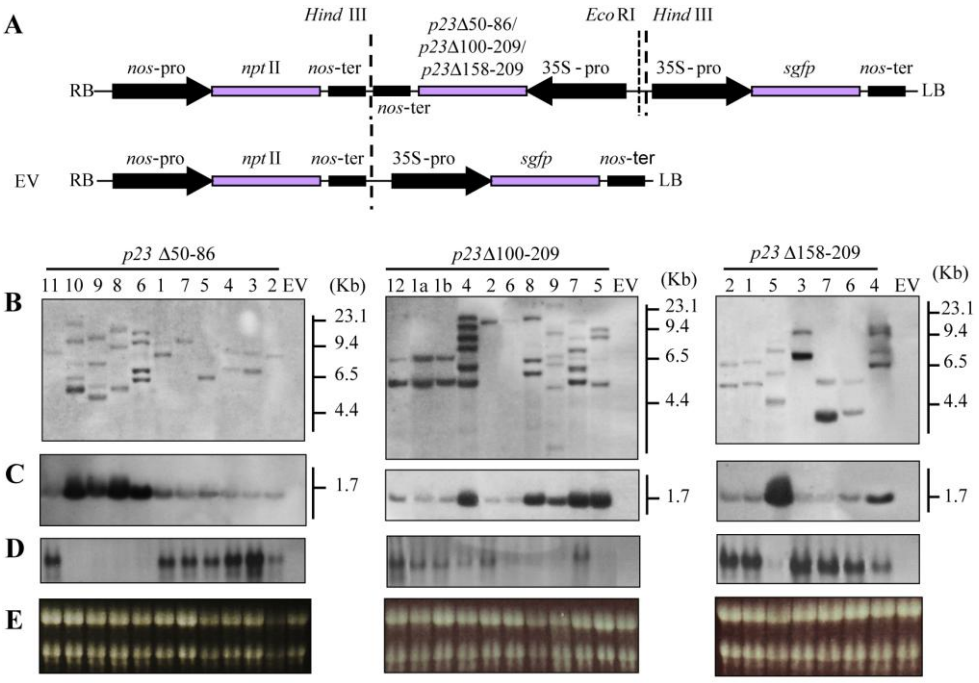
Using a riboprobe derived from the coat protein gene, no major differences were observed in the accumulation patterns of the g- and sgRNAs between the wild PVX and the different recombinants, indicating that symptom severity is not correlated with increased virus

titer. However, although this riboprobe detected PVX-specific small RNAs of approximately 21 and 22 nt in plants infected by PVX and by PVX expressing six of the seven p23 deletion mutants, these two prominent bands were replaced by several bands in the range of 18 to 25 nt in plants infected by PVX expressing p23 (wt) and, to a lesser extent, its deletion mutant p23Δ158-209 (Figure 7). Because these two constructs were the only inciting necrotic symptoms, it is possible that the observed pattern could result from increased exonuclease activity in damaged tissues trimming the protruding 3′ termini of the vsRNAs. Consistent with these observations, a similar pattern of vsRNAs was observed with p23H75, the only substitution mutant inducing necrosis similar to p23 (wt).

Ectopic expression in Mexican lime of CTV-p23 and some deletion mutants delimits a similar pathogenic determinant in the natural host

Given that p23 induces developmental aberrations resembling CTV symptoms when expressed ectopically in several citrus species. including Mexican lime (Fagoaga et al. 2005; Ghorbel et al. 2001), we examined whether similar p23 regions are involved in pathogenesis in this citrus host and in the non-natural host *N. benthamiana.* This question is important because effects observed in natural hosts (especially in woody species) and in model plants, such as *N. benthamiana* and *Arabidopsis thaliana,* may not be necessarily identical. To this end, the *p23* gene from CTV T36 and three truncated

versions thereof under the control of the CaMV 35S promoter were used to transform Mexican lime. Examination by Southern, Northern, and Western blot analyses identified several transgenic lines for each construct expressing p23 and its deletion mutants (Figure 8; Supplementary Figure S3). Although expression of a fragment comprising only the N-terminal 99 amino acids (p23Δ100-209) did not incite morphological aberrations in transgenic plants, expression of the fragment comprising the N-terminal 157 amino acids (p23Δ158-209) elicited CTV-like leaf symptoms and stem pitting similar to, albeit milder than, those resulting from expressing the complete p23 protein (Figure 8). Moreover, deletion of the fragment delimited by amino acids 50 and 86 (p23Δ50-86) disabled induction of developmental aberrations (data not shown), thus demarcating the region responsible for pathogenesis of p23 in citrus to a 157-amino-acid fragment that includes the Zn-finger domain and flanking basic motifs. Altogether, these data support the idea that similar regions of p23 may determine pathogenesis in Mexican lime and *N. benthamiana*, and that results obtained with this experimentally more tractable herbaceous plant may serve, at least in part, to anticipate results with transgenic citrus plants, the generation of which demand much more effort and time.



Supplementary Figure 3 (A) Diagram of the T-DNA from the binary vector pBin19-sgfp engineered to express three truncated versions of p23 from CTV T36 (p23Δ50-86, p23Δ100-209 and p23Δ158-209) under the control of the double-enhanced CaMV 35S promoter and the *nos* terminator (*nos*-ter). The expression cassettes are flanked by gene *npt II* between the *nos* promoter (*nos*-pro) and the *nos*-ter, and by the synthetic *green fluorescent protein* gene (*sgfp*) between the 35S promoter and the *nos*-ter. The empty vector (EV) is designed to express only the genes *npt II* and *sgfp*. Arrows indicate transcription orientation and vertical discontinuous lines restriction sites *Hind*III and *Eco*RI. **(B-E)** Southern- and Northern-blot hybridization of nucleic acid preparations from Mexican lime plants transformed with the vectors expressing p23Δ50-86, p23Δ100-209 and p23Δ158-209, and with the empty vector pBin19-sgfp (EV). DNA was digested with *Eco*RI, which cuts once the T-DNA **(B)**, or with *Hind*III, which excises the expression cassette **(C)**, and the membranes were probed with a digoxigenin-labeled fragment of the p23-coding region. Total RNA preparations from transgenic plants were separated by electrophoresis on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized with a ^{32}P-labeled p23-specific probe **(D)**, with ethidium bromide staining of the same gels showing equivalent amounts of loaded RNAs **(E)**.

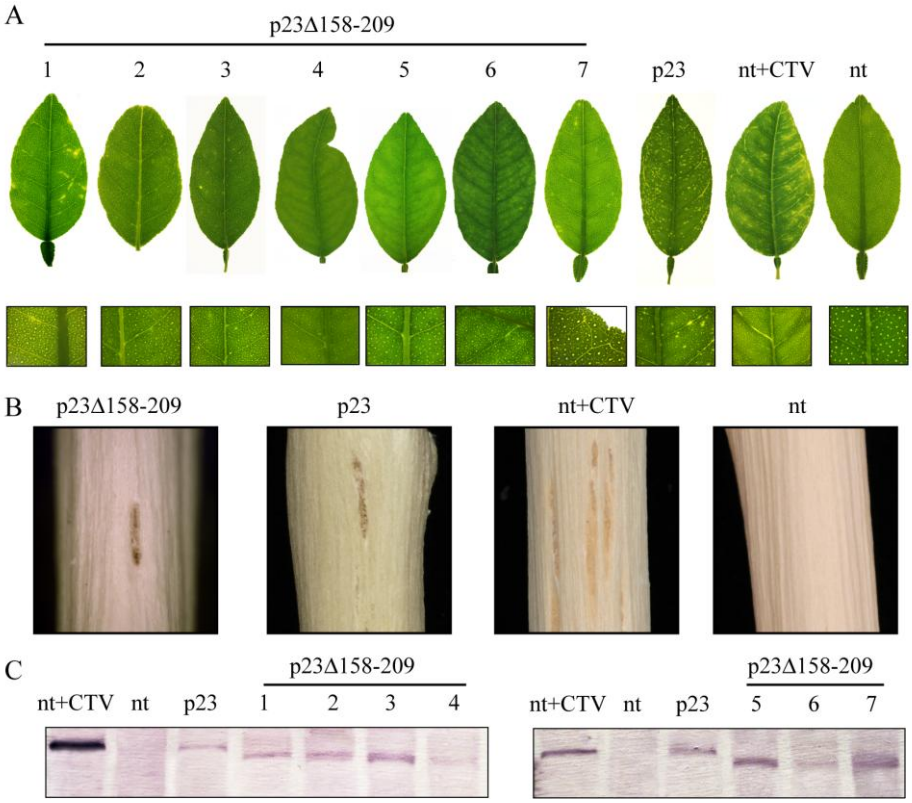


Figure 8 A, *Citrus tristeza virus* (CTV)-like foliar symptoms expressed by seven transgenic lines of Mexican lime transformed with the p23Δ158-209 deletion mutant and subsequently grafted on a vigorous rootstock. Controls include a noninoculated p23-expressing transgenic plant (p23), a nontransgenic plant inoculated with a severe CTV isolate (nt + CTV), and a noninoculated nontransgenic plant (nt). To better illustrate the phenotypic alteration (vein clearing), a magnification is shown below each leaf. **B,** Stem pitting expressed by one representative transgenic line of Mexican lime transformed with the p23Δ158-209 deletion mutant. Controls are the same as in the previous panel. **C,** Accumulation of p23 and its deletion p23Δ158-209 mutant as revealed by Western blot analysis with a specific antibody following electrophoresis in sodium dodecyl sulfate polyacrylamide (12%) gels.

3.2.3. Discussion

Discerning the subcellular localization of a protein is crucial for understanding its biological role. Within the genus *Closterovirus*, CTV is unique in encoding a protein, p23, with different functions (in replication or accumulation, pathogenesis, suppression of RNA

silencing, and, possibly, movement) which may be partially interdependent. From a structural perspective, p23 contains a putative Zn-finger domain flanked by motifs rich in basic amino acids required for RNA binding *in vitro* and for asymmetrical accumulation of plus and minus strands during RNA replication (López et al. 2000; Satyanarayana et al. 2002). The three cysteines and the histidine presumably coordinating the Zn ion are strictly conserved in essentially all CTV isolates deposited in databases, as also are most of the flanking basic amino acids between positions 50 and 86, and some additional motifs rich in basic amino acids (Sambade et al. 2003). Here, we report on another remarkable feature of p23: although there are examples of nucleolar proteins encoded by some DNA and RNA plant viruses (Taliansky et al. 2010), p23 is the first closterovirus protein with preferential localization in the nucleolus and Cajal bodies independently of other viral factors. This peculiar subcellular localization of p23 most likely determines some of its functions, while others may be related to p23 accumulating additionally in plasmodesmata (discussed below).

Our deletion and substitution analyses indicate that the NoLS of p23 is bipartite and includes regions or motifs delimited by positions 50 to 86 and 143 to 155, both rich in basic amino acids and the first also encompassing a Zn-finger domain. At least three other proteins encoded by ssRNA (+) viruses have, like p23, bipartite NoLS with characteristic basic amino acid stretches: the umbravirus *Groundnut rosette virus* (GRV) ORF3 protein (Kim et al. 2007; Ryabov et al. 2004), the potyvirus *Potato virus A* (PVA) NIa (VPg)

(Carrington et al. 1991; Schaad et al. 1996; Rajamäki and Valkonen 2009), and the cucumovirus *Cucumber mosaic virus* (CMV) 2b (Duan et al. 2012; González et al. 2010). In GRV ORF3 protein, mutations that block its nucleolar localization (or nuclear export) concurrently hamper assembly of cytoplasmic viral ribonucleoprotein particles and their long-distance movement, a process in which the nucleolar protein fibrillarin is also involved (Canetta et al. 2008; Kim et al. 2007). PVA VPg protein, which has an RSS activity dependent on its localization to the nucleolus and Cajal bodies, also interacts with fibrillarin but, although depletion of the latter reduces accumulation of PVA, it does not affect its long-distance movement (Rajamäki and Valkonen 2009), suggesting a different role for fibrillarin in GVR and PVA (Taliansky et al. 2010). On the other hand, whereas the interactions detected in plant between the CMV RSS 2b and AGO1, AGO4, and AGO6 also require nucleolar targeting of 2b (Duan et al. 20012; González et al. 2010; Hamera et al. 2012; Zhang et al. 2006), the interactions between the CMV 2b protein with fibrillarin have not been examined. However, in contrast to p23, neither GRV ORF3, PVA VPg, nor CMV 2b have a predicted Zn-finger forming part of the NoLS. It is unlikely that the nucleolar localization of p23 may be directly related to its involvement in the replication of CTV, because this process occurs in the cytoplasm. In contrast, our finding that p23 additionally targets plasmodesmata is consistent with this protein facilitating CTV cell-to-cell (and indirectly systemic) movement, in agreement with data that support a role for p23 in enhancing systemic infection and virus accumulation as well as in escaping from the

phloem in some natural hosts (Fagoaga et al. 2011). Whether these functions are mediated by interactions of p23 with fibrillarin or with one or more members of the AGO family (which, incidentally, might account for the RSS activity of p23) remains to be determined.

Most regions of p23 appear involved in its RSS activity because the seven deletion mutants and 9 of the 10 substitution mutants examined lost their RSS activity, concordant with observations showing that the RSS activity of other proteins from distinct RNA viruses is also very sensitive to sequence changes, including single amino acid substitutions (Martínez-Touriño and Hernández 2009; Qiu et al. 2002). Because vsRNAs accumulate to high levels in CTV-infected citrus species (Fagoaga et al. 2006; Ruiz-Ruiz et al. 2011), it is unlikely that p23 may act by binding dsRNA precursors and blocking their subsequent DCL-mediated processing; alternatively, vsRNA sequestration or inactivation of one or more AGO appear to be more feasible mechanisms, especially considering that accumulation in Mexican lime of siRNAs derived from a CTV transgene is not sufficient for conferring protection against the virus (López et al. 2010; Soler et al. 2012). Additionally, p23 might facilitate host epigenetic modifications through the transport of short interfering RNA to the nucleus, as has been recently proposed for the CMV 2b protein (Kanazawa et al. 2011). Nucleolar localization of p23 appears related to suppression of RNA silencing because i) the only mutant (p23H75) of the 17 tested that retained the RSS activity was targeted to the nucleolus and ii) none of the mutants that lost the nucleolar localization (14 of 17) kept the RSS activity. Because the

remaining two mutants (p23Δ106-114 and p23Δ158-209) localized to the nucleolus but did not display RSS activity, these results together indicate that RSS activity demands nucleolar localization while nucleolar localization is not sufficient for RSS activity.

The ability of p23 to incite symptoms (when expressed in *N. benthamiana* from a PVX sgRNA or in Mexican lime from a transgene) and its RSS activity (estimated by transitory agroexpression in *N. benthamiana*) are not strictly related, as revealed by the p23Δ158-209 mutant, which induces symptoms similar to p23 but lacks detectable RSS activity. Therefore, these results support the view that symptoms incited by viruses are not necessarily a consequence of their RSS (Díaz-Pendón and Ding 2008; Jay et al. 2011). Moreover, induction of phenotypic aberrations by p23 appears associated to its nucleolar localization, given that p23Δ158-209 and p23H75, the only two mutants inciting symptoms, are targeted to the nucleolus. However, this subnuclear localization is not sufficient by itself because mutant p23Δ106-114, also targeted to the nucleolus, does not incite phenotypic aberrations. This latter result also indicates that the 106-114 motif harbors a pathogenic determinant.

On the other hand, the pathogenic determinants of p23 involved in symptoms resulting from its expression via a PVX sgRNA in *N. benthamiana* and from its transgenic expression in citrus are restricted to the N-terminal 157-amino-acid fragment that includes the Zn-finger domain and flanking basic motifs, suggesting that p23 affects similar pathways in both hosts. However, transgenic expression of p23 is toxic to citrus, particularly to sweet and sour

orange, in which high levels of the transcript but not of the protein were observed (Fagoaga et al. 2005). In contrast, transgenic expression of this protein in *N. benthamiana* does not result in a visible phenotype (Fagoaga et al. 2005), indicating that this non-natural host species is more tolerant and that symptom induction demands considerably higher levels of p23, or that p23 might induce different epigenetic modifications in both hosts.

Finally, it is interesting to note that our finding that CTVp23 has a predominant nucleolar localization, to a good extent determined by basic amino acid motifs and a Zn-finger domain, poses the question of whether other proteins encoded in the 3′-proximal region of the genomic RNA of distinct filamentous viruses in which these two elements are conserved might also have similar subcellular localization. In such a case, the number of viral-encoded proteins with NoLS would expand significantly, considering that these viruses include *Grapevine virus A*, *Grapevine virus B* (GVB), and *Heracleum latent virus* (genus *Vitivirus*); *Garlic common latent virus, Potato virus S, Potato virus M,* and *Blueberry scorch virus* (genus *Carlavirus*) (Chiba et al. 2006); and, more recently, *Beet necrotic yellow vein virus* (BNYVV) (genus *Benyvirus*) (Chiba et al. 2013). Indeed, subsequent work has involved the Zn-finger domain and basic motifs of the small protein p12 (encoded by *Chrysanthemum virus B* [CVB], another member of genus *Carlavirus*) in its predominant accumulation in the nucleus (but not specifically in the nucleolus), as well as in nucleic acid binding and induction of a hypersensitive response in *N. benthamiana* when expressed from PVX

(Lukhovitskaya et al. 2009). Moreover, results with p14 of BNYVV and p14 of GVB show that they also accumulate preferentially in the nucleolus (Chiba et al. 2013) and the nucleus (S. W. Davino, S. Ruiz-Ruiz, and R. Flores, *unpublished data*), respectively. Therefore, why some proteins with basic amino acid motifs and a Zn-finger domain are targeted to the nucleolus, like CTV-p23 and BNYVV-p14, or to the nucleus, like CVB-p12 and GVBp14, remains an unanswered and challenging question.

3.2.4. Materials and methods

Recombinant plasmids for expressing p23 and its truncated and point-mutated versions

The CTV gene coding for p23 and its fragments coding for seven deletion mutants (p23Δ50-86, p23Δ50-66, p23Δ67-86, p23Δ106-114, p23Δ100-209, p23Δ125-209, and p23Δ158-209) and 10 alanine-substituted versions (p23R51/K52, p23R59/R61/R63, p23C68/C71/ H75/C85, p23C71, p23H75, p23R73/K74, p23K77/K80/R83/K84/ K86, p23R143/K145/R147, p23R150/R151/R155, and p23R143/ K145/R147/R150/R151/R155), were polymerase chain reaction (PCR) amplified with appropriate primers (Supplementary Table S1) from a complete cDNA clone of the CTV genotype T36 (Satyanarayana et al. 2001) or from cDNA clones of p23 with specific deletions (López et al. 2000). Then, the DNA coding for the GFP or RFP was fused in frame to generate by translation the recombinant p23-GFP or p23-RFP, as well as the

deleted or point-substituted versions of p23-GFP. All the constructs, flanked by the 35S promoter of CaMV and the NOS-t, were inserted in the pMOG binary vector (Knoester et al. 1998) and electroporated into *Agrobacterium tumefaciens* strains C58 or EHA105.

Supplementary Table 1. Oligonucleotides used for polymerase chain reaction amplification of the *Citrus tristeza virus* (CTV) gene coding for *p23* and seven deletion mutants.

Primer	Nucleotide sequence 3´-5'	Position in CTV T36
p23F *a*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23R *a*	GATGAAGTGGTGTTCG	19005-19020
p23Δ50-86F *b*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23Δ50-86R *b*	GATGAAGTGGTGTTCG	19005-19020
p23Δ50-66F *b*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23Δ50-66R *b*	GATGAAGTGGTGTTCG	19005-19020
p23Δ67-86F *b*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23Δ67-86R *b*	GATGAAGTGGTGTTCG	19005-19020
p23Δ100-209F *a*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23Δ100-209R *a*	CATATGCGCCACCTCGTTCTGAG	18691-18713
p23Δ106-114F *c*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23Δ106-114IR *c*	AACGGGGTCGTGCATTAACATATGC	18684-18708
p23Δ106-114IF *c*	GCCTTTTCTAATGCGGAGATATT	18736-18758
p23Δ106-114R *c*	GATGAAGTGGTGTTCG	19005-19020
p23Δ125-209F *a*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23Δ125-209R *a*	CGCAAATATCTCCGCATTAGAAAAGGC	18765-18791
p23Δ158-209F *a*	ATGGATAATACTAGCGGACAAACTTT	18394-19419
p23Δ158-209R *a*	CGGAGAACGCATCGGGTGTCTACG	18865-18887

a p23, p23Δ100-209, p23Δ125-209 and p23Δ158-209 were amplified from a complete cDNA clone of the CTV genotype T36. F and R refer to forward and reverse orientations.
b p23Δ50-86, p23Δ50-66 and p23Δ67-86 were amplified from cDNA clones of *p23* with specific deletions (40).
c p23Δ106-114 was amplified from a complete CTV-cDNA clone by two consecutive PCR amplifications. IF and IR refer to primers internal with respect to their F and R counterparts.

Confocal laser-scanning microscopy

The bacterial cultures, resuspended in infiltration buffer (10 mM MES, pH 5.6; 10 mM MgCl2; and 150 µM acetosyringone) to a final optical density at 600 nm of 0.5, were incubated for 3 h at room temperature, then infiltrated in leaves of *N. benthamiana* and *C. macrophylla.* In co-infiltration assays, equal volumes of the bacterial suspensions were mixed before infiltration. Plants were kept in growth chambers (16 h of light at 25 ºC and 8 h of darkness at 22 ºC), and the agroinfiltrated leaves were examined 2 days later with a confocal laser-scanning microscope (Leica TCS-SL, Heidelberg, Germany) equipped with appropriate emission sources and filters. Markers for different cell organella used in co-localization experiments included fibrillarin fused to the RFP, which specifically marks the nucleolus and Cajal bodies, and the TMV-MPs fused to RFP and of PNRSV-MP-YFP, which specifically mark plasmodesmata.

Suppression of RNA silencing assays

Lower leaves of the transgenic line 16c of *N. benthamiana*, constitutively expressing GFP (provided by D. C. Baulcombe) (Ruiz et al. 1998), were co-infiltrated with *A. tumefaciens* C58 cultures harboring pMOG binary plasmids for expressing GFP and p23 or its deletion and point-substitution mutants as indicated above. Following irradiation with a UV lamp (Black Ray model B-100AP, UVP, Upland, CA, USA.), the fluorescence emitted by GFP in the infiltrated patches was recorded with a Nikon D-200 digital camera equipped

with a yellow filter. Binary plasmids, either empty or expressing the silencing suppressor HcPro of *Tobacco etch virus* (Kasschau and Carrington 1998), were used as negative and positive controls, respectively.

Expression of p23 from a PVX vector

The CTV gene *p23* and its seven deletion and 10 point-substitution mutants were cloned into the PVX-derived plasmid pPVX202 (Sablowski et al. 1995), using *Eag*I and *Sal*I restriction sites incorporated in the corresponding primers. The resulting constructs were mechanically inoculated into three leaves (15 µg of DNA per leaf) of *N. benthamiana* plants, and symptoms were observed at 7, 10, and 15 dpi and photographed. Total nucleic acid preparations were obtained at these time intervals from 100 mg of infected leaf tissue using a standard protocol (Ancillo et al. 2007).

RNA fractionation and Northern blot analysis

Aliquots of total nucleic acid preparations from *N. benthamiana* were fractionated by electrophoresis in denaturing 0.9% agarose/formaldehyde gels (for PVX gRNA and sgRNAs), in 5% agarose/formaldehyde gels (for GFP mRNA), or in 17% polyacrylamide/urea gels (for sRNAs), stained with ethidium bromide, and transferred to Hybond-N+ nylon membranes (Roche Diagnostics, Mannheim, Germany). Digoxigenin-labeled riboprobes specific for GFP mRNA and the coat protein gene of PVX were generated by *in*

vitro transcription. After overnight hybridization at 68°C (for GFP mRNA and for PVX gRNA and sgRNAs) or 42°C (for sRNAs) in the ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA.), the membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) for 10 min at room temperature, twice with 0.1× SSC plus 0.1% SDS for 15 min at 42°C, and revealed with the chemiluminescent substrate disodium 3-(4-methoxyspiro {1,2- dioxetane-3,2'-(5'-chloro)tricycle [3.3.1.1]decan}-4-yl) phenyl phosphate (CSPD) (Roche Diagnostics) and exposure to X-ray film. Equal loading was assessed by UV spectrophotometry and by the intensity of 4S RNA bands after electrophoresis in 5% polyacrylamide/urea gels.

Citrus transformation and analysis

Recombinant plasmids pMOG-p23Δ50-86, pMOG-p23Δ100-209, and pMOG-p23Δ158-209 were digested with *Hin*dIII and the cognate expression cassettes were subcloned into the vector pBin19-*sgfp*, between the *nos*-pro/*nptII*/*nos*-ter and 35S-pro/*sgfp*/*nos*-ter cassettes. The empty vector and its three derivatives were electroporated into *A. tumefaciens* EHA105 and used to transform by co-cultivation internodal stem segments of Mexican lime. Selection and PCR analysis of transformants were as previously reported (Ghorbel et al. 2001). Buds from Mexican lime transformed with each of the deleted versions of gene *p23* and with the empty vector, and buds from two lines of Mexican lime transformed with the wt *p23* obtained previously (Ghorbel et al. 2001), were propagated by

grafting onto seedlings of Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*) and observed weekly for 3 years. Leaf details were observed with a Leica MZ 16 stereomicroscope and photographed with a Leica DFC490 camera (Leica Microsystems).

To analyze the integrity and copy number of the transgene cassettes in Mexican lime, aliquots (15 µg) of leaf DNA, extracted as described previously (Dellaporta et al. 1983), were digested with *Eco*RI, which cuts the T-DNA once, or with *Hind* III, which excises the cassette. After electrophoresis in 1% agarose gels and staining with ethidium bromide, DNA fragments were blotted onto a nylon membrane, fixed by UV irradiation, hybridized with a digoxigenin-labeled fragment of gene *p23* according to the manufacturer's instructions (Boehringer, Mannheim, Germany), and detected by chemiluminescence with the CSPD substrate.

To assess transcription of the transgene, total leaf nucleic acid preparations from Mexican lime were obtained with buffersaturated phenol and fractionated with 2 M LiCl (Carpenter and Simon 1998). Aliquots (20 µg) of the insoluble RNAs were electrophoresed in 1% agarose gels with formaldehyde, stained with ethidium bromide, blotted onto a nylon membrane, and fixed by UV irradiation. Prehybridization, hybridization, and washing of the membranes were as reported previously (Flores 1986), except that hybridization was at 50 ºC in 50% formamide. The radioactive *p23*-specific cDNA probe was prepared with Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA.).

Finally, to determine the accumulation of p23 and its deleted mutants, total leaf protein preparations from Mexican lime were extracted with 100 mM Tris-HCl, pH 6.8 (containing 0.3% β-mercaptoethanol and 1 mM phenyl-methyl-sulfonyl fluoride), and quantified with the Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA.) (Bradford 1976). Aliquots (50 µg) were electrophoresed in SDS-polyacrylamide gels (12%), electroblotted onto polyvinylidene diflouride membranes, and probed with a polyclonal antibody (1 µg/ml) against p23Δ50-86. To prepare this antibody, p23Δ50-86 fused to the maltose binding protein was expressed in bacterial cells, purified, and injected into a rabbit as reported previously (López et al. 2000). The rabbit was bled 5 days after the last immunization and the serum was recovered by centrifugation, titrated, and kept at -20 ºC. To avoid unspecific binding, the polyclonal antiserum was purified through a HiTrap Protein G HP column (Amersham Pharmacia Biotech Inc.), and a diluted antibody solution (15 µg/ml) was applied to an enzyme-linked immunosorbent assay plate coated with a crude extract of Mexican lime prepared at a 1:10 ratio (tissue weight to carbonate buffer volume) and kept at 4 ºC for 16 h. The antibody preparation was recovered and the treatment was repeated eight times. Binding of the antibody to proteins transferred to membranes was detected with an anti-rabbit immunoglobulin G (whole molecule) antibody from goat conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis), and visualized with 5- bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich).

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3.3. CHAPTER 3

Symptoms induced by transgenic expression of p23 from *Citrus tristeza virus* in phloem-associated cells of Mexican lime mimic virus infection and do not include aberrations accompanying constitutive expression.

Sumitted for publication.

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Summary

Citrus tristeza virus (CTV) is phloem-restricted in natural citrus hosts. The 23 kDa protein (p23) encoded by the virus is an RNA silencing suppressor and a pathogenicity determinant. Expression of p23, or its N-terminal 157 amino acid fragment comprising the zinc-finger and flanking basic motifs, driven by the constitutive 35S promoter incites CTV-like symptoms and other aberrations in transgenic citrus. To better define the role of p23 in CTV pathogenesis, we compared the phenotypes of Mexican limes transformed with p23-derived transgenes from the severe T36 or the mild T317 CTV strains under the control of the phloem-specific promoter from *Commelina yellow mottle virus* (CoYMV) or the 35S promoter. Expression of the constructs restricted to the phloem incited aberrations resembling CTV-specific symptoms (vein clearing and necrosis, and stem pitting), but not the non-specific symptoms (like mature leaf epinasty and yellow pinpoints, growth cease and apical necrosis) observed when p23 was ectopically expressed. Furthermore, vein necrosis and stem pitting in Mexican lime appeared specifically associated with p23 from T36. Phloem-specific accumulation of the p23Δ158-209(T36) fragment was sufficient to incite the same anomalies, indicating that the region comprising the N-terminal 157 amino acids of p23 is responsible (at least in part) for the vein clearing, stem pitting and possibly vein corking in this host.

3.3.1. Introduction

Citrus tristeza virus (CTV) is the causal agent of devastating epidemics that have changed the course of the citrus industry, provoking the worldwide loss of almost 100 million tress of sweet orange (*Citrus sinensis* (L.) Osb.), mandarin (*C. reticulata* Blanco), grapefruit (*C. paradisi* Macf.) and lime (*C. aurantifolia* (Christ.) Swing.) propagated on sour orange (*C. aurantium* L.) (Moreno et al., 2008). CTV is a member of the genus *Closterovirus*, family *Closteroviridae*, and it only infects naturally phloem-associated tissues of species of the genera *Citrus* and *Fortunella* within the family *Rutaceae*, subfamily *Aurantoideae*. The virus is readily transmitted with infected buds and spread locally by several aphid species in a semi-persistent mode (Bar-Joseph et al., 1989).

CTV has a plus-strand single-stranded genomic (g)RNA of approximately 19.3 kb organized in 12 open reading frames (ORFs), potentially encoding at least 17 protein products, delimited by 5' and 3' untranslated regions (UTRs) (Karasev et al., 1995). The two 5'-proximal ORFs encode components of the replicase complex (Karasev et al., 1995) and are translated directly from the gRNA (Hilf et al., 1995). The 10 ORFs located in the 3' moiety of the gRNA are expressed through a set of 3' co-terminal subgenomic mRNAs (Hilf et al., 1995) encoding proteins p33, p6, p65, p61, p27, p25, p18, p13, p20 and p23 (Karasev et al., 1995; Pappu et al., 1994). The small hydrophobic p6 is proposed to act as a transmembrane anchor, and p25 and p27 are the major and minor coat proteins, respectively.

About 97% of the gRNA is encapsidated by p25 and the 5'-terminal 650 nucleotides by p27 (Febres et al., 1996; Satyanarayana et al., 2004). These two proteins, together with p65 and p61, are required for virus assembly (Satyanarayana et al., 2000). While p33, p13 and p18 are dispensable for systemic infection of some citrus hosts but required for others (Tatineni et al., 2008; Tatineni et al., 2011), p20, a protein accumulating in amorphous inclusion bodies of CTV-infected cells (Gowda et al., 2000), and p23, are indispensable for invasion of all hosts (Tatineni et al., 2008). Additionally, p33 is needed for superinfection exclusion (Folimonova, 2012).

Unique to CTV is p23, with no homologs found in other closteroviruses (Dolja et al., 2006). It is expressed in early stages of cell infection (Navas-Castillo et al., 1997) and accumulates in infected plants at moderate levels compared to other viral proteins (Pappu et al., 1997). Dolja et al. (1994) showed the presence of a cluster of positively-charged amino acids in p23, and López et al. (1998) further characterized this conserved region that has a core with three cysteines and one histidine forming a putative zinc-finger domain. The presence of this domain suggested a regulatory function for p23, a view supported by the finding that p23 binds RNA *in vitro* in a sequence non-specific manner, and that mutations affecting the cysteine and histidine residues increase the dissociation constant of the p23-RNA complex (López et al., 2000). Moreover, p23 is involved in regulating the balance of plus and minus viral strands during replication, with the zinc finger domain and an adjacent basic region being indispensable for asymmetrical accumulation of the plus strand (Satyanarayana et

al., 2002). Together with p20 and p25, p23 acts as an RNA silencing suppressor (RSS) in *Nicotiana tabacum* and *N. benthamiana*, with p25 acting intercellularly, p23 intracellularly, and p20 at both levels (Lu et al., 2004). In addition, p23 is a viral pathogenicity determinant when expressed ectopically in citrus (see below). Moreover, the seedling yellows syndrome, induced by some CTV strains in sour orange and grapefruit, has been mapped at the p23-3′UTR region (Albiach-Martí et al., 2010).

The viral region coding for p23 is a hotspot for RNA silencing because small (s)RNAs from this region accumulate to high levels in CTV-infected Mexican lime and sweet orange (Ruiz-Ruiz et al., 2011). Ectopic expression of p23 enhances systemic infection and virus accumulation in transgenic sour orange and facilitates CTV escaping from the phloem of transgenic sweet and sour orange. Therefore, constraints to CTV movement in some citrus hosts, particularly in sour orange, may at least in part result from RNA silencing (Fagoaga et al., 2011). Moreover, recent data indicate that p23 accumulates preferentially in the nucleolus and Cajal bodies, as well as in plasmodesmata, being some basic motifs and the zinc-finger domain essential for nucleolar localization (Ruiz-Ruiz et al., 2013). The same motifs/domain are sufficient for inducing necrosis in *N. benthamiana* when p23 is expressed from *Potato virus X* and for inciting CTV-like aberrations in transgenic Mexican lime plants, thus linking pathogenicity of p23 to its nucleolar localization. In contrast, most p23 regions are needed for RSS activity in *N. benthamiana* (Ruiz-Ruiz et al., 2013).

The use of transgenic plants has been instrumental in identifying viral RSS and pathogenicity determinants, though most of this work has been restricted to *Nicotiana spp*. and *Arabidopsis thaliana* (Díaz-Pendón and Ding, 2008). However, transgenic expression of RSS in non-natural hosts does not necessarily reflect the effects of viral infection, because in their natural context these proteins are often expressed only in infected cells and tissues, unlike the constitutively expressed transgenes (Csorba et al., 2009; Díaz-Pendón and Ding, 2008). Alternatively, mutant viruses expressing dysfunctional proteins have been used to assess their role as pathogenicity determinants (Hsieh et al., 2009; Yambao et al., 2008; Ziebell and Carr, 2009). Unfortunately, this approach can neither be extended to p23 because it is essential for CTV replication (Satyanarayana et al., 1999), nor be replaced by a homolog from a related virus because p23 is unique among closteroviruses. Symptoms induced by certain CTV strains in citrus, such as vein clearing and stem pitting, are specific of this pathosystem and are recapitulated by some of the aberrations associated with the constitutive expression of p23 in Mexican lime. However, other non-specific symptoms accompanying the constituive expression of p23, like mature leaf epinasty and yellow pinpoints, growth cease and apical necrosis (Fagoaga et al., 2005; Ghorbel et al., 2001), are rarely seen in non-transgenic CTV-infected Mexican lime and other citrus species. These latter aberrations most likely result from the ectopic expression of p23 in cells other than those associated with the phloem, the only tissue infected by CTV.

To better define the role of p23 in CTV pathogenesis, we have now restricted the transgenic expression of p23 to phloem-associated cells of Mexican lime. For this purpose, constructions carrying different versions of p23 or fragments thereof, have been put under the control of the phloem-specific promoter from *Commelina yellow mottle virus* (CoYMV) (Medberry et al., 1992). We show here that: 1) aberrations associated with phloem-specific expression and accumulation of p23 are essentially identical to symptoms caused by CTV infection in Mexican lime, 2) some of these CTV-like symptoms induced by p23 from the severe strain T36 were not observed when using p23 from the mild strain T317, thus mimicking the effects of natural infections by both CTV strains, and 3) similar restricted expression of the fragment comprising the zinc-finger and flanking basic motifs of p23 is sufficient to induce the CTV-like aberrations, confirming that the N-terminal region of 157-amino acids determines, at least in part, CTV pathogenesis in Mexican lime.

3.3.2. Results and discussion

Vein clearing in Mexican lime transformed with CoYMV-*p23* constructs resembles that induced by CTV in non-transformed plants and correlates in intensity with p23 accumulation

Based on transgene integrity of *p23* and on locus/loci patterns, as determined by enzyme restriction and Southern-blot hybridization, at least ten independent Mexican lime lines were generated for each of the three constructs, CoYMV-*p23*(T36), CoYMV-*p23Δ158-209*(T36)

and CoYMV-*p23*(T317), as well as for the empty vector (EV) (Figure 1a). The selected transgenic lines contained at least one intact copy of the CoYMV-driven expression cassette (Figure 1c), and an estimated number of transgene loci ranging between one and four (Figure 1b). Moreover, Northern-blot analysis showed variable transgene expression depending on the line, with an inverse but not strict correlation between transgene loci number and transcript expression being observed (Figure 1d and 1e). Five propagations were prepared from each of the three selected CoYMV-*p23* and EV transgenic lines, as well as from selected 35S-*p23*-transgenic lines of Mexican lime generated and characterized in previous studies (Fagoaga et al., 2005; Ghorbel et al., 2001; Ruiz-Ruiz et al, 2013). CTV-infected non-transformed plants were also propagated in parallel and used as controls.

About 3-6 months after propagation in the greenhouse, transgenic Mexican limes harboring the CoYMV-*p23*(T36), CoYMV-*p23Δ158-209*(T36) or CoYMV-*p23*(T317) cassettes, displayed progressively vein clearing in developing leaves, in contrast with the asymptomatic phenotype exhibited by similar leaves from plants transformed with the EV cassette (Figure 2). This phenotypic anomaly was essentially identical to the vein clearing incited by CTV T36 in non-transgenic plants of this host (Figure 2; C+CTV lanes and corresponding leaf samples).

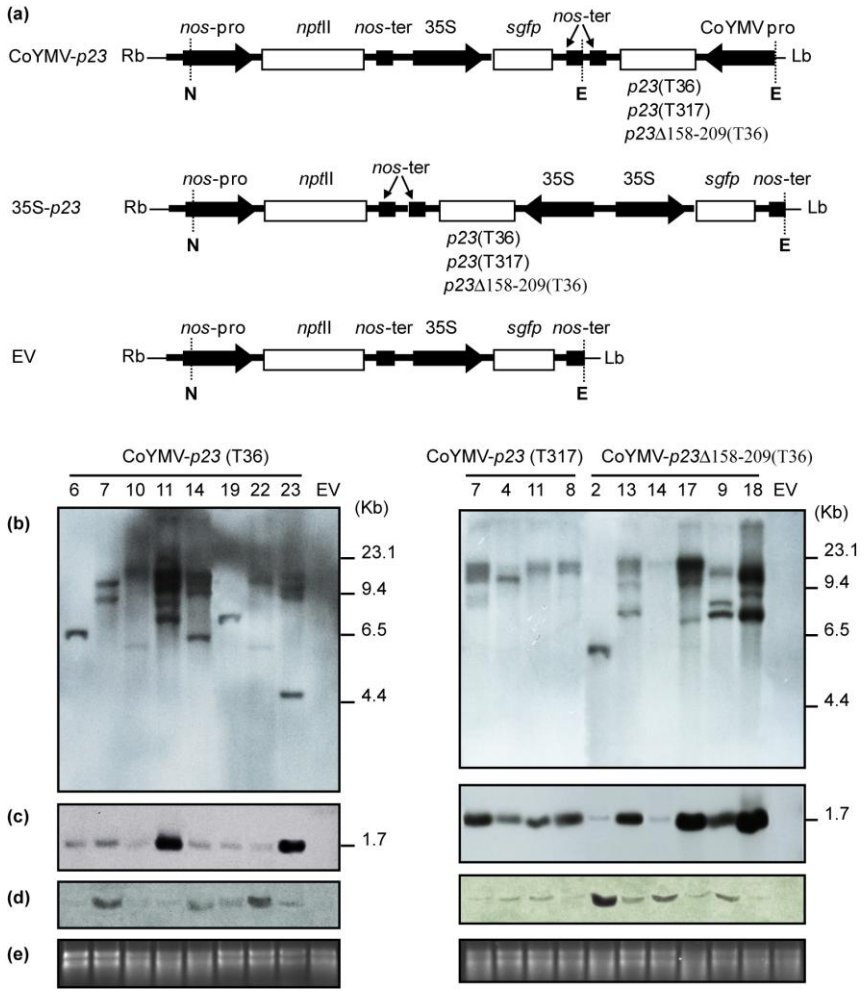

Figure 1 Diagram of the T-DNA from the binary vectors CoYMV-*p23* and 35S-*p23*, and Southern/Northern blot analyses from transgenic Mexican limes. (a) Diagram of the T-DNA from the binary vectors CoYMV-*p23* and 35S-*p23* carrying *p23*(T36), *p23*(T317) or *p23Δ158-209*(T36) cassettes under the control of the phloem-specific promoter from *Commelina yellow mottle virus* (CoYMV-pro) and the *nopaline synthase* terminator (*nos*-ter), and the constitutive 35S promoter from the *Cawliflower mosaic virus* (35S-pro) and the *nos*-ter, respectively. These cassettes were flanked by the *neomycin phosphotransferase* II gene (*npt*II) between the *nos* promoter (*nos*-pro) and *nos*-ter, and by the *green fluorescent protein* gene (s*gfp*) between 35S-pro and *nos*-ter. The binary vector pBin19-s*gfp* was used as empty vector (EV) control. Transcription orientation for each cassette is indicated by arrows. N and E denote *Nhe*I and *Eco*RI restriction sites, respectively. (continued)

Figure 1 (continued) (b, c, d and e) Southern and Northern blot hybridization of nucleic acid preparations from lime plants transformed with the CoYMV-*p23*(T36) construct (lines 6, 7, 10, 11, 14, 19, 22 and 23), the CoYMV-*p23*(T317) construct (lines 7, 4, 11 and 8), the CoYMV-*p23Δ158-209*(T36) construct (lines 2, 13, 14, 17, 9 and 18) and with the empty vector (EV). DNA was digested with *Nhe*I (b), which cuts once the T-DNA, or with *Eco*RI (c), which excises the CoYMV-*p23* expression cassette. Size of DNA markers is indicated at the right. (d) Total RNA extracted from transgenic plants was separated by electrophoresis in a formaldehyde-containing agarose gel, and transferred to a nylon membrane. (e) Ethidium bromide staining of the RNA gel showing that equivalent amounts of RNA were loaded in the different lanes. (b, c, and d) Membranes were probed with a digoxigenin-labeled fragment of the *p23*-coding region.

All CoYMV-*p23* Mexican lime transformants accumulated detectable amounts of p23 or p23Δ158-209, unlike those plants carrying the EV construct, as revealed by Western-blot analysis (Figure 2). Moreover, accumulation of p23 from CTV T36 or T317, or p23Δ158-209 from T36 in CoYMV-*p23*(T36), CoYMV-*p23*(T317) and CoYMV-*p23Δ158-209*(T36) transgenic plants, respectively, correlated positively with the CTV-like vein clearing intensity. For example, lines CoYMV-*p23*(T36)-7 and -22 (Figure 2a), lines CoYMV-*p23*(T317)-8 and -11 (Figure 2b), and lines CoYMV-*p23Δ158-209* (T36)-2 and -9 (Figure 2c), displayed pronounced vein clearing and high p23 accumulation, while lines CoYMV-*p23*(T36)-23 and -6 (Figure 2a), lines CoYMV-*p23*(T317)-4 and -7 (Figure 2b) and lines CoYMV-*p23Δ158-209*(T36)-14 and -17 (Figure 2c) showed milder vein clearing and accumulated low to moderate levels of p23 (Figure 2). Therefore, the phloem-specific expression of p23 from the mild strain T317 induced in Mexican lime vein clearing similar to that incited by the severe strain T36, with the intensity being correlated with p23 accumulation irrespective of the source strain, as previously reported for the constitutive expression of p23 from both strains (Fagoaga et al., 2005).

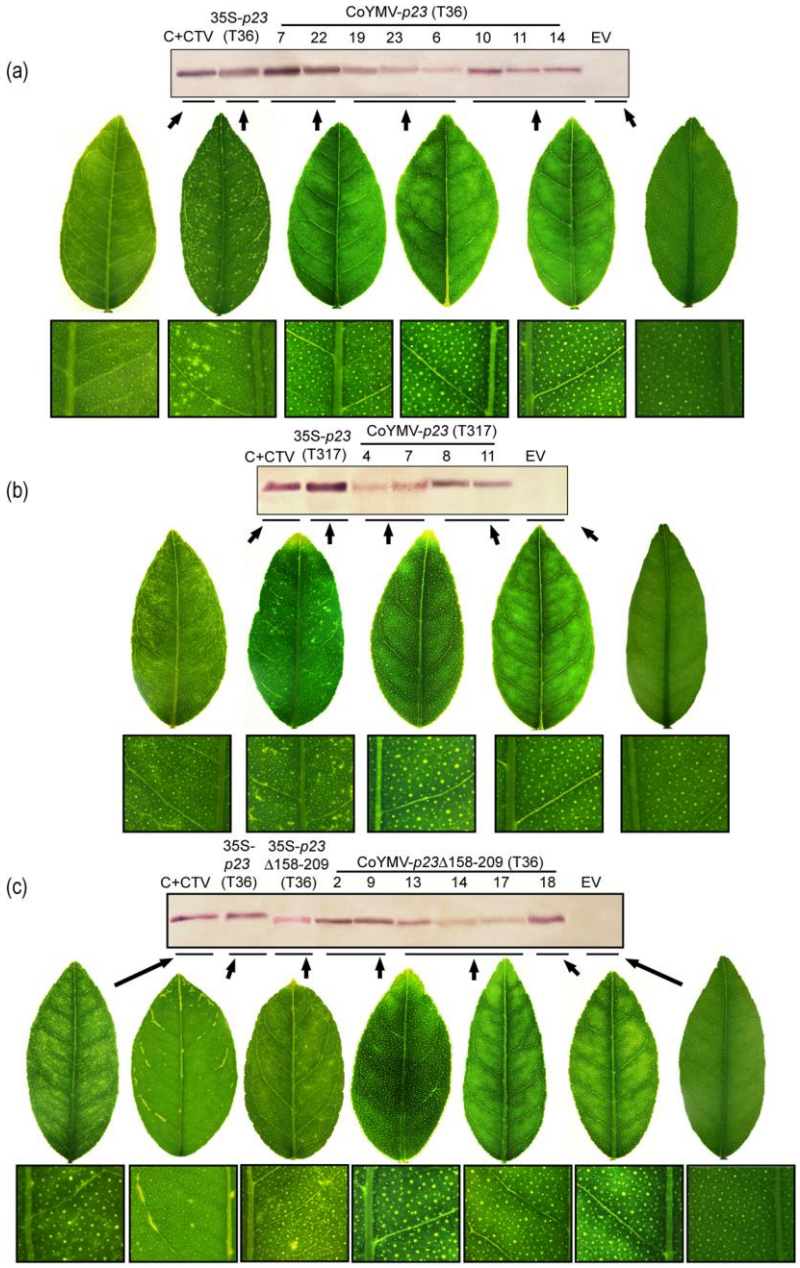

Figure 2 Accumulation of the p23 protein and vein clearing symptoms in developing leaves from transgenic Mexican limes. Western-blot analysis of protein preparations separated by SDS-PAGE (12%) and probed with a specific antibody of: (a) p23 from CTV T36, (b) p23 from CTV T317, and (c) p23Δ158-209 from CTV T36. Vein clearing shown by leaves of Mexican lime transformed with: (a) CoYMV-*p23*(T36), (b) CoYMV-*p23*(T317), and (c) CoYMV-*p23Δ158-209*(T36), Controls include non-transgenic Mexican limes infected with CTV T36 (C+CTV), and 35S-*p23* and empty vector (EV) transgenic plants. To better illustrate vein clearing, a magnification is shown below each leaf.

Mexican lime transformed with CoYMV-*p23* constructs develops stem pitting and vein necrosis similar to those induced by the severe CTV strain T36 in non-transformed plants

Six to twelve months after being propagated in the greenhouse, transgenic plants CoYMV-*p23*(T36) and CoYMV-*p23Δ158-209*(T36) exhibited stem pitting similar to that of transgenic plants 35S-*p23*(T36) (Ghorbel et al., 2001) and 35S-*p23Δ158-209*(T36) (Ruiz-Ruiz et al., 2013) (Figure 3a). This phenotypic aberration was also very similar to the stem pitting induced by CTV T36 in non-transgenic Mexican lime, though pitting was more pronounced and extended in this latter case (Figure 3a). The stem pitting intensity was comparable in all transgenic plants expressing the different versions of p23 and p23Δ158-209, irrespective of their accumulation levels (data not shown). This lack of correlation is possibly associated with weak stem pitting incited by both p23(T36) versions in transgenic Mexican limes (Figure 3a). Conversely, CoYMV-*p23*(T317) transgenic plants accumulating p23 did not show stem pitting at any developmental stage, behaving as the EV transgenic controls (Figure 3a). As 35S-*p23*(T317) transgenic plants neither displayed stem pitting (Figure 3a; Fagoaga et al., 2005), our results indicate that this symptom is depends on the p23 source rather than on its accumulation level. In non-transformed Mexican lime, strain T317 causes only mild vein clearing (Moreno et al., 1993).

After one year in the greenhouse, transgenic plants CoYMV-*p23*(T36) and CoYMV-*p23Δ158-209*(T36) exhibited vein necrosis in

the lower surface of mature leaves, resembling vein corking incited in non-transformed Mexican lime by severe CTV strains, including T36, though such vein corking usually occurs in the upper leaf surface (Figure 3b). Moreover, the intensity of vein necrosis paralleled generally accumulation of p23. For example, while lines CoYMV-*p23*(T36)-7 and -11 and lines CoYMV-*p23Δ158-209*(T36)-2 and -9 displayed marked vein necrosis and accumulated high to moderate levels of p23, lines CoYMV-*p23*(T36)-6 and -23 and lines CoYMV-*p23Δ158-209*(T36)-14 and -17 showed mild vein necrosis and lower p23 accumulation (Figure 2a and c; Figure 3b; data not shown). In contrast, vein necrosis was absent in transgenic plants CoYMV-*p23*(T317), as well as in transgenic plants 35S-*p23*(T36), 35S-*p23Δ158-209*(T36), 35S-*p23*(T317) and EV (Figure 3b), indicating that this aberration is exclusively associated to phloem-specific expression of p23 from T36. Therefore, the most severe phenotypic effects in Mexican lime transformants (stem pitting and vein necrosis) seem related to the source of p23 and, more specifically, to its N-terminal fragment of 157 amino acids.

Comparison of the predicted amino acid sequence of p23 from 18 CTV isolates of different pathogenicity showed three regions (demarcated by positions 24-29, 50-54 and 78-80), in which mild CTV isolates, but not other isolates, have the same sequence (Sambade et al., 2003). Interestingly, these regions include most amino acid differences between p23 from T317 and T36 (or its Δ158-209 version).

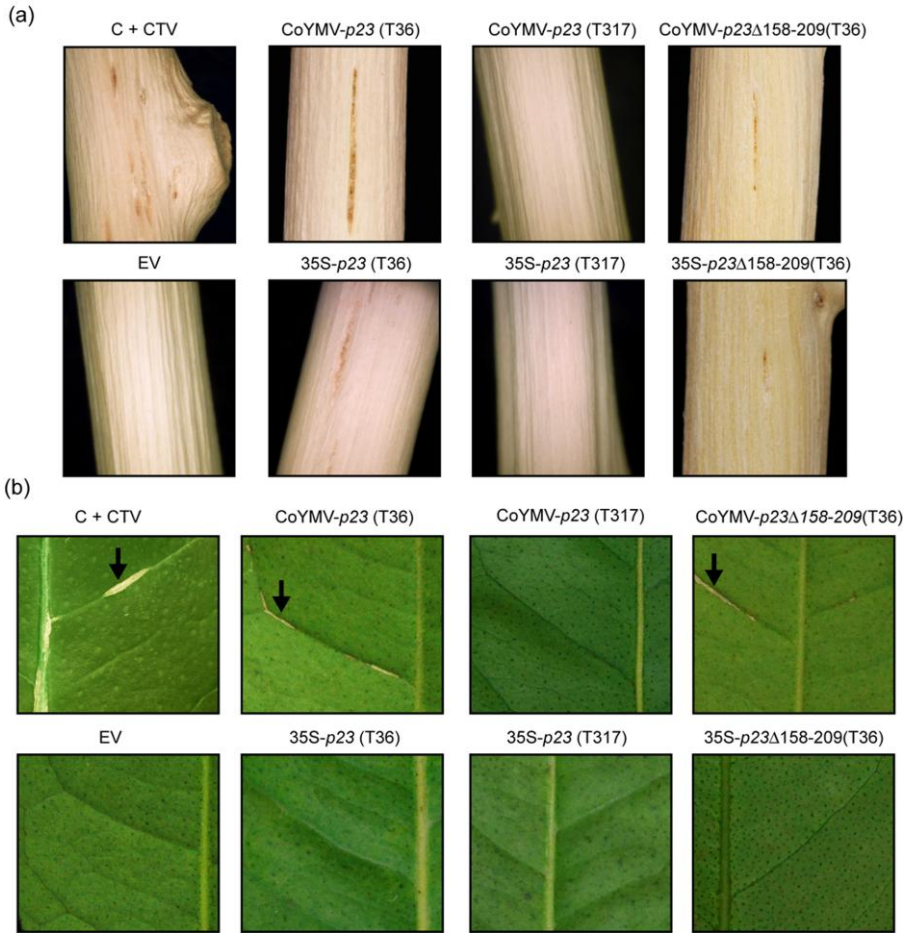

Figure 3 Stem pitting and vein necrosis CTV-like symptoms exhibited by CoYMV-*p23*(T36)- and CoYMV-*p23Δ158-209*(T36)-transgenic Mexican limes. Stem pitting (a) and vein necrosis (b) CTV-like symptoms exhibited by CoYMV-*p23*(T36)- and CoYMV-*p23Δ158-209*(T36)-transgenic Mexican limes. Neither stem pitting nor vein necrosis was detected in CoYMV-*p23*(T317)-transgenic plants (a, b). Controls include non-transgenic Mexican limes inoculated with CTV T36 (C+CTV), and 35S-*p23*(T36)-, 35S-*p23Δ158-209*(T36)- and 35S-*p23*(T317)- and EV-transgenic plants.

Regions 50-54 and 78-80 form part of two domains that include several basic residues (positions 50-67) and a putative zinc finger motif (positions 68-86), which are crucial for the RNA-binding

activity of p23 (López et al., 1998) and for its nucleolar localization (Ruiz-Ruiz et al., 2013). Altogether these results support the involvement of the p23 fragment encompassing the N-terminal 157 amino acids in the induction of the CTV-like stem pitting and vein necrosis.

Aberrations induced by phloem-specific expression of p23 or its N-terminal fragment of 157 amino acids in Mexican lime are histologically similar to those incited by CTV in non-transformed plants

To corroborate that vein clearing induced by the phloem-specific expression of p23 from T317, and vein clearing and necrosis induced by the phloem-specific expression of p23 and p23Δ158-209 from CTV T36 in Mexican lime mimic symptoms incited by the corresponding CTV strain in non-transformed plants, we looked at cross sections of leaf veins from: i) transgenic plants CoYMV-*p23*(T36), CoYMV-*p23*(T317) and CoYMV-*p23Δ158-209*(T36), ii) transgenic plants 35S-*p23*(T36), 35S-*p23*(T317), 35S-*p23Δ158-209*(T36) and EV, and iii) non-transformed controls infected with CTV T36 (C+CTV).

Examination by light microscopy showed that the xylem cap, phloem, and phloem fibers were fully differentiated in EV control veins, with air spaces on each side of the vein (Figure 4a, i). However, vein clearing areas in CTV-infected plants (C+CTV) showed hypertrophied cells developed from the primary phloem fibers, which

occluded air spaces found normally around veins (Figure 4b). Differentiation failed to occur, so veins lacked the caps of primary phloem fibers and sheath cells (Schneider, 1959). Vein clearing areas from CoYMV-*p23*(T36), CoYMV-*p23*(T317), CoYMV-*p23Δ158-209*(T36) (Figure 4f, 4g and 4h, respectively) and 35S-*p23*(T36), 35S-*p23*(T317), 35S-*p23Δ158-209*(T36) (Figure 4c, 4d and 4e, respectively) leaves displayed obliterated cells in the phloem cap and hypertrophied cells occluding part of the air spaces found normally around veins. Therefore, vein clearing shown by CoYMV-*p23* plants was histologically undistinguishable from that exhibited by 35S-*p23* plants and similar to, albeit less intense than, vein clearing incited by CTV T36 in the non-transformed counterparts. Cross sections from necrotic veins in CoYMV-*p23*(T36) (Figure 4k) and CoYMV-*p23Δ158-209*(T36) plants (Figure 4l) displayed excessive phloem formation displacing phloem fibers, and the phloem cap showed obliterated cells as well as collapsed and necrotic areas. Corking areas from C+CTV leaves exhibited a disorganized tissue, also with phloem overformation, obliterated cells, collapsed and necrotic areas, and lack of phloem fibers (Figure 4j). These results indicate that vein necrosis in CoYMV-*p23*(T36) and CoYMV-*p23Δ158-209*(T36) transgenic limes strongly resembles vein corking incited by CTV in this host, but with more pronounced tissue disorganization and necrosis in the second case (Figure 4j, k, l).

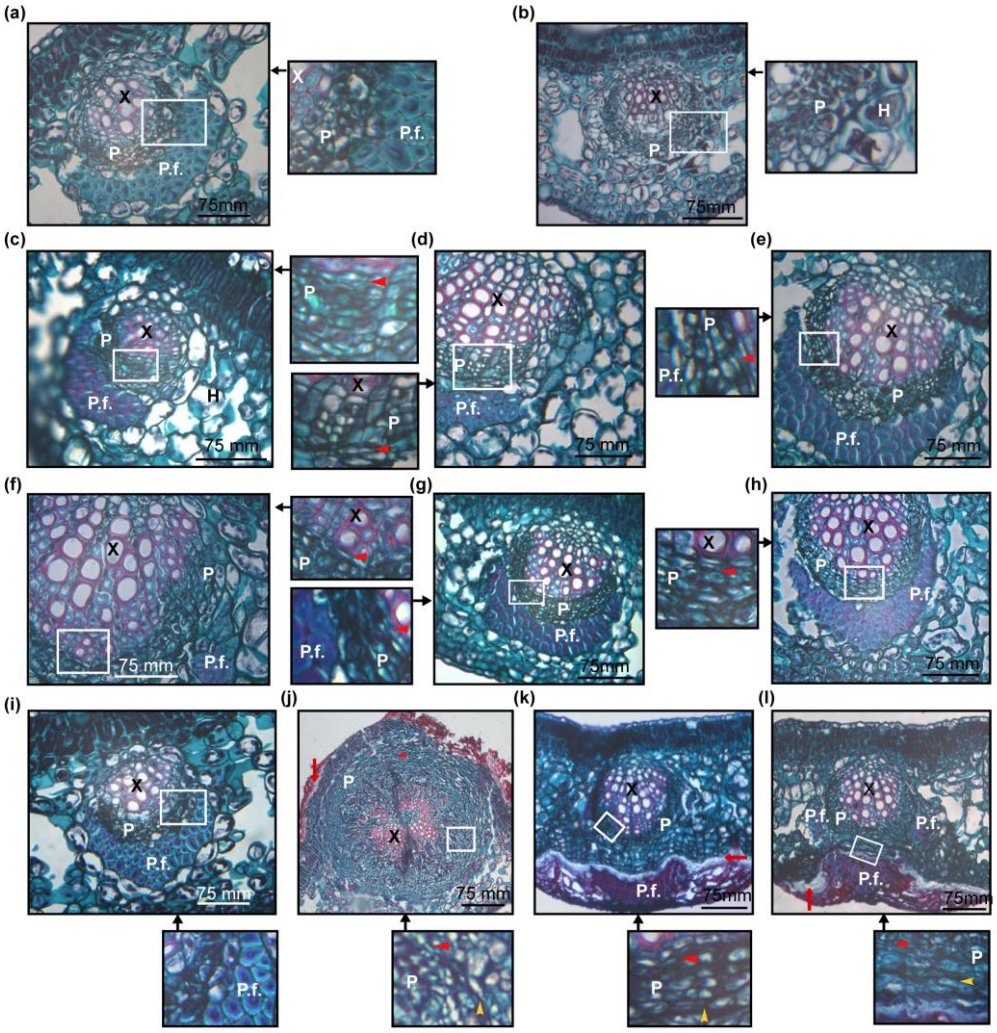

Figure 4 Cross sections of representative leaf veins from CoYMV-*p23*(T36)-, CoYMV-*p23Δ158-209*(T36)-, 35S-*p23*(T36)-, 35S-*p23*(T317), 35S-*p23Δ158-209*(T36)-, empty vector (EV)-transgenic limes and non-transgenic limes infected with CTV T36 (C+CTV), stained with a combination of safranin O and Fast Green FCI. (a, i) Healthy leaves from non-inoculated 6 and 12 month-old EV transgenic plants, respectively. (b) Vein clearing area from a non-transgenic lime infected with CTV T36 (C+CTV). (c-e) Vein clearing area from 35S-*p23*(T36)- (c), 35S-*p23*(T317)- (d) and 35S-*p23Δ158-209*(T36)- (e) transgenic plants. (f-h) Vein clearing area from CoYMV-*p23*(T36)- (f), CoYMV-*p23*(T317)- (g) and CoYMV-*p23Δ158-209*(T36)- (h) transgenic plants. (j) Vein corking area from a non-transgenic lime infected with CTV T36 (C+CTV). (k) Necrotic vein from a CoYMV-*p23*(T36)-transgenic plant. (l) Necrotic vein from a CoYMV-*p23Δ158-209*(T36)-transgenic plant. The white rectangles in (a) to (l) are shown at higher magnification in adjacent panels indicated by black arrows. Symbols: (X) xylem; (P) phloem; (P.f.) phloem fiber; (H) hypertrophic cells; obliterated cells, collapsed areas, and necrotic areas are indicated by small red, yellow, and long red arrows, respectively.

How phloem-specific expression of p23 incites CTV-like symptoms in Mexican lime?

Transgenic expression of p23 in Mexican lime controlled by a phloem-specific promoter mimics virus-induced symptoms more accurately than when expressed from a constitutive promoter. Particularly, phloem-specific expression of p23(T36) and p23Δ158-209(T36) in Mexican lime induced, in addition to vein clearing, vein necrosis and stem pitting very similar to those induced by T36 in non-transformed plants. Similarly, phloem-specific expression of p23(T317) induced vein clearing but not vein necrosis or stem pitting, recapitulating symptoms incited by T317 in non-transformed plants. In contrast, constitutive expression of p23 (from T36 or T317) failed to induce vein necrosis, but resulted in chlorotic pinpoints, apical necrosis and mature leaf epinasty (not observed in natural CTV infections) (Fagoaga et al., 2005; Ghorbel et al., 2001). Therefore, these latter aberrations are most likely associated to the ectopic accumulation of this protein in non-phloem cells, wherein CTV does not replicate and accumulate.

We have previously suggested that it is unlikely that p23 might interfere with synthesis of virus-derived small RNAs (vsRNAs) because they accumulate at very high levels in Mexican lime and some other CTV-infected citrus hosts (Fagoaga et al., 2006; Ruiz-Ruiz et al., 2011; Ruiz-Ruiz et al., 2013), with those derived from the *p23* ORF being the most abundant (Ruiz-Ruiz et al., 2011), and because accumulation of small RNAs derived from *p23* transgenes in different

configurations is insufficient to confer full resistance against CTV challenge in Mexican lime (López et al., 2010; Soler et al., 2012). p23 has characteristics in common with other viral proteins like 2b from cucumoviruses and P0 from poleroviruses, which are pathogenicity determinants that incite developmental aberrations when expressed in transgenic plants (Bortolamiol et al., 2007; Ghorbel et al., 2001; Lewsey et al., 2007; Ruiz-Ruiz et al., 2013; this work), show nuclear/nucleolar localization (Fusaro et al., 2012; González et al., 2010; Ruiz-Ruiz et al., 2013), and function as RNA silencing suppressors in *Nicotiana spp*. (Lu et al., 2004; Ruiz-Ruiz et al., 2013; Voinnet et al., 1999). Like 2b and P0, p23 might cause symptoms by targeting ARGONAUTE (AGO) proteins for degradation (Bortolamiol et al., 2007), by preventing *de novo* assembly of the RNA-induced silencing complex (RISC) (Baumberger et al., 2007; Duan et al., 2012; Zhang et al., 2006), or by promoting host epigenetic modifications via the transport of small RNA to the nucleus (Kanazawa et al., 2011). Since the region comprising the N-terminal 157 amino acids of p23 is responsible (at least in part) for symptoms in Mexican lime and, since p23Δ158-209 lacks the ability of RSS in *N. benthamiana* (Ruiz-Ruiz et al., 2013), the pathogenicity of p23 seems independent of its RSS activity (although the situation may not be the same when p23 is expressed in its natural virus-host context), and possibly linked to its subcellular localization (as well as that of p23Δ158-209) in the nucleolus and/or plasmodesmata (Ruiz-Ruiz et al., 2013). Alternatively, p23 might interact with host factors unrelated to RNA silencing pathways, as illustrated by the interactions of the

replicase protein from *Tobacco mosaic virus* with the Aux/IAA protein PAP1/IAA26 (Padmanabhan et al., 2005), the CMV 2b with catalase 3 (Inaba et al., 2011), and the p12 from a carlavirus with the promotor of a transcription factor that causes hyperplasia (Lukhovitskaya et al., 2013).

3.3.3. Experimental procedures

Recombinant vectors

The CoYMV promoter and the binary vector pGPTV harboring the CoYMV promoter were kindly provided by Dr. Neil Olszewski (University of Minnesota, USA) and Dr. Biao Ding (Ohio State University, Columbus, Ohio, USA), respectively. The DNA fragment containing the CoYMV promoter and the *nopaline synthase* terminator was excised with *Sal*I and *Eco*RI from plasmid pGPTV and then inserted into the respective restriction sites of plasmid pTZ57R (Fermentas GmbH, St. Leon-Rot, Germany) to generate the intermediate plasmid pTZ57R-CoYMV. PCR amplification of the *p23* gene from CTV-T36 (or a deletion mutant thereof) and CTV-T317 from the respective pMOG-*p23*-T36 (Ghorbel et al., 2001), pMOG-*p23Δ158-209*(T36) (Ruiz-Ruiz et al., 2013) and pMOG-*p23*-T317 (Fagoaga et al., 2005) plasmids was performed with *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) using the sense and antisense primers RF-167 (5´-CTTggatccATGGATAATACTAGCGG-3´) and RF-168 (5´-CTTggatccTCAGATGAAGTGGTGTTC-3´), respectively, containing

a *Bam*HI restriction site (in lowercase letters) to facilitate cloning. The pMOG-*p23*-T36 plasmid was also used to generate the deletion mutant pMOG-*p23Δ158-209*(T36), in which the nucleotide guanine at position 472 was replaced by a thymine, resulting in a stop codon immediately after amino acid 157 (thus deleting the C-terminal 51 amino acids from p23 without affecting the zinc finger or any of the three flanking basic motifs). For this purpose, the pMOG-*p2*3-T36 plasmid was PCR-amplified with *Pfu* DNA polymerase using the pair of divergent primers of opposite polarity RF-353 (5'-CATCGGGTGTCTACGAGCCAGTC-3') and RF-354 (5'-CGTTCTCCGtAAGAAACTCCGG-3'), in which the lowercase letter indicates nucleotide substitution, to yield pMOG-*p23Δ158-209*(T36).

After *Bam*HI digestion, the PCR-amplified fragments were inserted between the CoYMV promoter and the *nopaline synthase* terminator (*nos*-ter) by ligation in *Bam*HI-digested pTZ57R-CoYMV plasmid, generating the intermediate plasmids pTZ57R-CoYMV-*p23*(T36); pTZ57R-CoYMV-*p23*(T317) and pTZ57R-CoYMV-*p23Δ158-209*(T36). *Eco*RI restriction sites were inserted at the 5' end of the CoYMV promoter and at the 3' end of the *nos*-ter, in the three constructions by PCR amplification using primers Phloem-D-Eco (5'-CTTgaattcGGTATCGATTTCTTAGGGGC-3') and Phloem-R-Eco (5'- CTTgaattcCCGATCTAGTAACATAGATG-3'), with the restriction site indicated in lowercase letters. The CoYMV-*p23* cassettes were digested with *Eco*RI and inserted into the unique *Eco*RI site of the binary vector pBin19-*sgfp* (Chiu et al., 1996), adjacent to the *nos*-pro/*nptII*/*nos*-ter and 35S-pro/*sgfp*/*nos*-ter cassettes (Figure

1a). The plasmid pBin19-s*gfp* was used as empty vector (EV) control. Construction of pBin19-35S-*p23*(T36), pBin19-35S-*p23*(T317) and pBin19-35S-*p23Δ158-209*(T36), with the *p23* transgenes controlled by the 35S promoter of the *Cauliflower mosaic virus* (CaMV), have been described in Ghorbel et al. (2001), Fagoaga et al. (2005) and Ruiz-Ruiz et al. (2013), respectively. Binary vectors were electroporated into the disarmed *Agrobacterium tumefaciens* strain EHA105, which was used to transform Mexican lime, as described previously (Ghorbel et al., 2001).

Transgenic plant generation

Selection of transformants was performed on a culture medium containing kanamycin (100 mg/L) and the regenerated shoots were examined under a Leica MZ 16 Stereomicroscope equipped with a GFP-Plus Fluorescence module (Leica Microsystems, Wetzlar, Germany). Shoots exhibiting bright green fluorescence were excised and grafted *in vitro* on Troyer citrange (*C. sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.) seedlings (Peña and Navarro, 1999). The integrity of the *p23* transgenes was assessed by PCR with appropriate primers. PCR-positive plantlets were grafted on vigorous 6-month-old Carrizo citrange seedlings and grown in a greenhouse at 24–26ºC/ 16–18ºC (day/night), 60%–80% relative humidity and natural light. Buds from transgenic Mexican lime lines harboring the EV, the CoYMV-*p23* or the CaMV 35S-*p23* cassettes (Ghorbel et al., 2001; Fagoaga et al., 2005; Ruiz-Ruiz et al, 2013), or from non-transgenic control plants

infected with CTV T36 or CTV T317 were propagated on vigorous Carrizo citrange rootstocks in parallel. Plants were grown in individual 2.5 L pots containing a mixture of 55% Sphagnum peat and 45% siliceous sand, and were fertilized weekly.

The growth and symptom expression of transgenic and non-transgenic CTV-infected Mexican lime plants was periodically observed for at least three years. Developmental aberrations in transgenic lines and CTV-induced symptoms in infected plants were photographed with a Nikon D80 camera, and specific details from leaves and peeled branches were photographed with a Leica MZ 16 Stereomicroscope equipped with a camera Leica DFC490 (Leica Microsystems).

Southern, Northern and Western blot analyses

To analyze the integrity and loci number of the CoYMV-*p23* expression cassettes in Mexican lime plants, Southern blot hybridization analysis was performed. DNA aliquots (15 μg) extracted from leaves (Dellaporta et al., 1983) were digested with *Eco* RI, which excises the expression cassettes (Figure 1a), or with *Nhe* I, which cuts once the T-DNA (Figure 1a). After agarose gel electrophoresis, the DNA was blotted on to positively charged nylon membranes, fixed by UV irradiation, probed with a digoxigenin (DIG)-labelled cDNA fragment of the *p23* coding region prepared by PCR according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany) and detected using the chemiluminescent CSPD

substrate (Roche, Diagnostics Corporation, Indianapolis, USA). For detection of transgene-derived transcripts, Northern blot hybridization analysis was perfomed. Total RNA from leaf midribs of the transgenic plants was extracted with buffer-saturated phenol and fractionated with 2 M LiCl (Carpenter and Simon, 1998). Aliquots (20 μg) of the insoluble RNA fraction were electrophoresed in 1% agarose gels containing formaldehyde, blotted on to nylon membranes, fixed by UV irradiation and probed with a digoxigenin-labelled cDNA fragment of the *p23* coding region, according to the manufacturer's instructions (Boehringer-Mannheim) and detected by chemiluminescence with the CSPD substrate (Roche).

The accumulation of p23 and p23Δ158-209 proteins in the transgenic Mexican lime plants was tested by Western blot analysis. Total protein was extracted from leaf midribs with 100 mM Tris-HCl, pH 6.8, containing 0.3% β-mercaptoethanol and 1 mM phenyl-methyl-sulfonyl fluoride, and quantified with the Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard (Bradford, 1976). Aliquots (50 μg) were electrophoresed in SDS-polyacrylamide gels (12%), electroblotted onto PVDF membranes, and probed with a polyclonal antibody (1 μg/ml) against p23Δ50-86 (Ruiz-Ruiz et al., 2013). Binding of the antibody was detected with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St Louis, MO, USA) and visualized with 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium SIGMA *FAST*™ BCIP/NBT (Sigma-Aldrich). Extracts from leaf midribs of

transgenic Mexican lime plants carrying the EV control construct and from non-transgenic Mexican limes infected with CTV (C+CTV) were used as negative and positive controls, respectively.

Histological analysis

Leaf pieces of approximately 1 x 0.5 cm, including minor veins and midribs, were collected from areas with vein clearing or vein necrosis aberrations from CoYMV-*p23*(T36)-, CoYMV-*p23Δ158-209*(T36)-, and CoYMV-*p23*(T317)-transgenic Mexican lime plants, from their 35S-*p23* counterparts, from symptomatic areas of non-transgenic CTV-infected Mexican limes, and from similar asymptomatic areas of non-infected EV control plants.

Leaf samples were fixed in FAA solution (0.5:9:0.5, vol/vol/vol of formaldehyde, ethanol, acetic acid) for 15 days and dehydrated through a series of ethanol/ tertiary butyl alcohol solutions (Jensen, 1962). After embedding them in histosec® pastilles (solidification point: 56 to 58°C) (Merck, One Merck Drive, Whitehouse Station, NJ, USA), ten micrometer thick cross sections of leaf veins were obtained with a rotary microtome (Jung, Heidelberg, Germany). Sections were stained with a combination of safranin O (Merck) (lignified cellular walls stain red) and Fast Green FCI (Sigma- Aldrich) (cellulose stains blue-green), mounted with Canada balsam (Merck) (Jensen, 1962; Román et al., 2004), and examined and photographed with a Leica DMLS microscope equipped with a Leica DFC490 digital camera.

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4. GENERAL DISCUSSION AND OUTLOOK

The origin of modern citriculture was marked by the use of the citrus genotype sour orange (*C. aurantium* L.) as a rootstock for most citrus varieties around the second half of the XIX century, due to its resistance to foot root caused by *Phytophthora* spp. and to its excellent agronomic characteristics. However, the generalized use of sour orange had a dramatic consequence around 50 years later. Most scion types (including sweet orange (*C. sinensis* L. Osbeck), grapefruit (*C. paradidi* Macf.) and mandarins (*C. reticulata* Blanco)) propagated on sour orange resulted affected by a new disease called tristeza, which since then has caused decline and death of about 100 million citrus trees grown on this rootstock (Moreno et al., 2008). It is a bud-union disease caused by the sensitivity of sour orange to *Citrus tristeza virus* (CTV), but it occurs only when this citrus type (or lemon (*C. limon* L. Burm.)) is used as rootstock. This situation forced a drastic change in most citrus industries, with the progressive replacement of sour orange by tristeza-resistant or tolerant rootstocks, which usually do not perform so well as sour orange.

The objectives of this work have been: 1) looking for a biotechnological strategy to generate transgenic full resistance against CTV in a model citrus type highly sensitive to the virus, with the aim of incorporating it later into sour orange to make this rootstock resistant to the tristeza decline, and 2) get more insight in the role of the p23 protein from CTV in pathogenesis through transgenic expression of the whole p23 or specific fragments under the control of constitutive or phloem specific promoters in sensitive citrus plants.

Intron-hairpin constructs containing virus sequences induce a strong antiviral reaction because the transcribed dsRNA triggers RNA silencing that ultimately results in transgene-derived siRNAs accumulation and inactivation of the cognate viral ssRNA (Smith et al., 2000). Based on this, Mexican lime (*C. aurantifolia* Christm. Swing.) was transformed with an intron-hairpin construct containing full-length, untranslatable versions of genes *p25*, *p20* and *p23* (from CTV T36 clonal strain) to silence concurrently the expression of the three silencing suppressors of CTV in infected cells. Graft-inoculation with the same viral strain, either in the scion or in the rootstock, revealed that three transgenic lines were completely resistant: all their propagations remained asymptomatic and virus-free, with the accumulation of transgene-derived siRNAs being necessary but insufficient for CTV resistance. However, resistance was only partial following inoculation with a severe stem pitting strain (T318A), with nucleotide identities with T36 of 91-92% for the three genes, thus showing the involvement of a sequence-dependent mechanism for resistance. Apart from representing a step ahead in the quest for developing full transgenic resistance to CTV, these results show that the simultaneous inactivation of the three viral silencing suppressors is crucial for this aim, although the participation of other concomitant unknown resistance mechanisms cannot be dismissed (Soler et al., 2012).

We have been working with the objective of generating transgenic resistance to CTV during the last fifteen years. In most cases, our strategies have been based on the incorporation of genes

and sequences derived from the viral genome into the citrus model Mexican lime, which is highly sensitive to the virus, and then potential resistance could be tested in the greenhouse within a reasonable time period (about one-two years) (Ghorbel et al., 2000, 2001; Domínguez et al., 2002ab; Fagoaga et al., 2005, 2006; López et al., 2010). Those constructs that provided better results were transferred also to sour orange. In Spain, this rootstock cannot be used anymore due to its susceptibility to tristeza, but it has a series of excellent agronomic attributes, as fruit yield and quality in grafted varieties, adaptation to calcareous and saline soils, rusticity, extended harvesting season, and tolerance to various pathogens and additional abiotic stresses, which undoubtedly made it maybe the best citrus rootstock.

After Soler et al. (2012), we have incorporated the intron-hairpin construct containing genes *p25*, *p20* and *p23* into sour orange, characterized several independent transgenic lines at the molecular level, and selected four of them showing intact copy/copies of the whole transgene for further analysis. We expect viral RNA will be targeted and degraded through very efficient RNA silencing soon after invading the first sour orange rootstock cells in contact with the graft union cells of the susceptible scion, thus providing resistance to tristeza decline. Resistance to decline will be tested in Concordia (Argentina) with the collaboration of Catalina Anderson and M.I. Plata (INTA). Concordia is an ideal place to perform such assay because very severe CTV strains and their most efficient insect vector, *Toxoptera citricida*, are predominant. More than twenty cutting from

each transgenic line are already there. Non-transgenic Valencia sweet orange is being grafted onto the sour orange rootstocks (10 replications per line). Recently we got permit from the National Biosafety Committee (Conabia), and the trees will be planted in the field. CTV natural infection and decline symptomathology will be followed periodically by immunoprinting and visual inspections, respectively. It is expected that 100% of control plants show CTV-induced decline within a period of 5 years. We already have a field trial there to test susceptibility to tristeza in transgenic sour orange, which was approved by the Conabia in 2009 (see the Introduction for further details). Genetic improvement of this rootstock to overcome its sensitivity to decline would be highly desirable, and it remains an important objective for the citrus industry worldwide.

The protein p23, an RNA-binding protein with a putative Zn-finger domain and some basic motifs, is unique to CTV because no homologues have been found in other closteroviruses. Consequently, p23 might have evolved for the specific interaction of CTV with its citrus hosts. From a functional perspective p23 has been involved in many roles: i) regulation of the asymmetrical accumulation of CTV RNA strands (Satyanarayana et al., 2002), ii) induction of the seedling yellows syndrome in sour orange and grapefruit (Albiach-Martí et al., 2010), iii) intracellular suppression of RNA silencing (Lu et al., 2004), iv) elicitation of CTV-like symptoms when expressed ectopically as a transgene in several *Citrus* spp. (Ghorbel et al., 2001; Fagoaga et al., 2005; Ruiz-Ruiz et al., 2013), v) enhancement of systemic infection (and virus accumulation) in sour orange and CTV release from the

phloem in *p23*-expressing transgenic sweet and sour orange (Fagoaga et al., 2011), and vi) accumulation preferentially in the nucleolus as well as in plasmodesmata (Ruiz-Ruiz et al., 2013). Moreover, transformation of Mexican lime with intron-hairpin constructs designed for the co-inactivation of p23 and the two other CTV silencing suppressors results in complete resistance against the homologous virus (Soler et al., 2012).

To gain more insight on the role of p23 in CTV pathogenesis, we have restricted the expression of *p23*-derived transgenes to phloem-associated cells in Mexican lime plants by means of using the phloem-specific promoter from *Commelina yellow mottle virus* (CoYMV) (Medberry et al., 1992). Constructions carrying the complete gene *p23* from either the severe T36 or the mild T317 CTV strains, or a fragment comprising the zinc-finger and flanking basic motifs from the former, either under the control of the CoYMV promoter or the constitutive 35S promoter have been used for genetic transformation of Mexican lime. Examination of the phenotypic aberrations resulting from the transgenic expression of p23 under the control of a phloem-specific promoter, should better mimic the symptoms incited by CTV, which is a phloem-confined virus. Indeed, restricting the expression of p23-derived transgenes to phloem-associated cells of Mexican lime resulted in phenotypes closely resembling symptoms induced by virus infection, with the other abnormalities observed with transgenic expression of p23 under the control of the 35S promoter being most likely pleiotropic effects derived from p23 accumulation in non-phloem cells (Soler et al.,

unpublished data). In support of this view, some of the CTV-like symptoms induced by the phloem-specific expression of p23 from the severe strain T36 were not induced by p23 from the mild strain T317, in contrast with the similar effects observed when both protein variants are expressed constitutively (Fagoaga et al., 2005). Additionally, phloem-specific transgenic expression and accumulation of p23Δ158-209(T36) was sufficient to incite the same anomalies as the complete p23 from the same isolate. Moreover, these phenotypic aberrations were almost identical to the symptoms induced by CTV T36 in Mexican lime, indicating that the region comprising the N-terminal 157 amino acids of p23 is responsible (at least in part) for the vein clearing, stem pitting and possibly vein corking in this host (Soler et al., unpublished data).

To investigate how p23 causes CTV-like symptoms in Mexican lime, we have next performed a large-scale gene expression analysis of p23-transgenic plants using a 20k citrus cDNA microarray (Martinez-Godoy, et al., 2008). Using developing leaves from new shoots of different plants, gene expression in the p23-35 line, carrying the whole *p23* gene from CTV T36 under the control of the 35S promoter and NOS terminator sequences (Ghorbel et al., 2001), was compared with that of independent EV control plants, disposing transgenic vs. control samples on the same slide. Microarray slides were scanned with a GenePix 4000B using GenePix 6.0 image acquisition software and the identification of differentially expressed genes was done by one-class SAM test (Tusher, et al., 2001). Although results are now just preliminary and need to be confirmed

through qRT-PCR analysis, several genes putatively involved in CTV pathogenesis were clearly either up- or down-regulated in p23-transgenic samples.

Among them, the gene with ID: IC0AAA35DD11 (ID: Cs7g17970 in the database of the *Citrus sinensis* Annotation Project (http://citrus.hzau.edu.cn/orange/index.php), which was identified as the citrus homolog of Argonaute protein (AGO1) (AT1G48410) in Arabidopsis databases) was 1.64 fold up-regulated in p23-transgenic leaves. The protein p23 has characteristics in common with other viral proteins as 2b from cucumoviruses and P0 from poleroviruses. All these three proteins are pathogenicity factors that incite developmental aberration when expressed in transgenic plants (Ghorbel et al., 2001; Fagoaga et al., 2005; Lewsey et al., 2007; Bortolamiol et al., 2007; Ruiz-Ruiz et al., 2013), they show nuclear localization (González et al., 2010; Fusaro et al., 2012; Ruiz-Ruiz et al., 2013), and function as RNA silencing suppressors in *Nicotiana* spp. (Voinnet et al., 1999; Lu et al., 2004; Ruiz-Ruiz et al., 2013). Transgenic *Arabidopsis* plants overexpressing CMV 2b showed enhanced accumulation of AGO 1 mRNA (Zhang et al., 2006) as well as those *Arabidopsis* transformants ectopically expressing P0 (Bortolamiol et al., 2007). Moreover, CMV 2b transformants showed upregulation of miR168, which control de expression of AGO1 in *Arabidopsis* (Zhang et al., 2006). Interestingly, miR168 was also up-regulated in Mexican lime, sweet orange and sour orange plants upon infection with CTV (Ruiz-Ruiz et al. 2011). Collectively, these results suggest that, as in the cases of 2b and P0, p23 may cause symptoms by targeting

ARGONAUTE (AGO) proteins for blocking its slicer activity or degradation, preventing de novo formation of siRNA/miRNA containing AGO and consequently RISC assembly and target RNA cleavage (Zhang et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007; Csorba et al., 2010; Duan et al., 2012).

Microarray experiments also revealed that several Auxin Response Factors (ARFs) resulted mis-regulated in p23-transgenic Mexican lime plants. The gene with ID KN0AAI3CG02 [ID: Cs6g16030.7 in the database of the *Citrus sinensis* Annotation Project; homolog of *Arabidopsis* AT5G37020 (Auxin-Responsive Factor (ARF8))], was 2.45-fold up-regulated. Moreover, the gene with ID IC0AAA94CC12 [similar to Auxin response factor 10 related cluster/ Cs6g11800.3 (*Citrus sinensis* database)/AT4G30080 (ARF16)], was 1.64-fold up-regulated, and the gene with ID C03006F12 [highly similar to Auxin response factor 4 related cluster/Cs2g09440.2/ AT1G30330 (ARF6)], was 1.6-fold up-regulated in p23-transgenic leaves. Developmental abnormalities such as stunting and leaf curling represent common symptoms associated with many virus diseases in different plant hosts, and it has been proposed that they are the result of virus-induced disruptions in plant hormone metabolism (Culver et al., 2007). The same hypothesis may be extended to explain virus-like developmental aberrations induced by overexpression of specific viral pathogenicity determinants in transgenic plants. Transgenic *Arabidopsis* plants overexpressing P1/HC-Pro accumulated ARF8 and ARF10 mRNAs in leaf tissues at substantially higher levels than controls, leading to prominent morphological and developmental

alterations (Kasschau et al., 2003). Furthermore, it has been demonstrated that the mis-regulation of a single transcript encoding ARF8, a target of miR167, underline most of the developmental abnormalities caused by the expression of three different viral silencing suppressors in *Arabidopsis*, both in transgenic and in an authentic infection context (Jay et al., 2011). Therefore, the up-regulation of Auxin Response Factors, especially ARF8, could explain, at least in part, the developmental abnormalities exhibited by p23-transgenic plants. Whether such ARF mis-regulation were caused by the action of p23 over miR167, or through another type of interactions, such as those incited by the replicase protein of *Tobacco mosaic virus* (TMV), which disrupts the localization and stability of interacting auxin/indole acetic acid (Aux/IAA) proteins in *Arabidopsis*, that function as repressors of auxin-responsive transcription factors (ARFs) (Padmanabhan et al., 2005, 2008) would require further investigation.

The gene with ID IC0AAA15AB06 (Cs5g23640.1 from sweet orange genome database; homolog of *Arabidopsis* AT3g23780, which encodes NRPD2 catalytic subunit of the nuclear DNA-dependent RNA polymerase IV) was 4.23-fold up-regulated. DNA-dependent RNA polymerase IV (Pol IV) and V (Pol V) are two plant-specific RNA polymerases involved, as well as AGO4 and several other proteins, in the process of regulating epigenetically silent states of repeated loci, transposons and heterochromatin regions through their associated 24-nucleotide (nt) siRNAs (Matzke et al., 2009). RNA polymerase IV (Pol IV)/Pol IVa and Pol V/Pol IVb and Pol IV are

involved in the production of 24-nt siRNAs that mediate DNA and histone methylation (Wang et al., 2011). Its up-regulation may affect transcriptional gene silencing processes and consequently plant development in p23-transformants.

The gene with ID C31503D10 [Cs3g27280.4 in the sweet orange genome database; homolog of *Arabidopsis* AT1G20620.5 (Catalase 3)] was 2.19-fold down-regulated in p23-transgenic leaves. It has been reported that CMV 2b interact physically with Catalase3 (CAT3) in infected tissues. As CAT3 is a key enzyme in the breakdown of toxic hydrogen peroxide, the interaction leads to the induction of a specific necrosis (Inaba et al., 2011). However, CAT3 mRNA levels were not altered by that protein/protein interaction. Nevertheless, *Arabidopsis cat2* and *cat3* mutants typically display patches of chlorosis and necrotic lesions (Contento and Bassham, 2010). It may be then possible that the vein clearing and chlorotic pinpoints and/or necrosis caused by p23 overexpression in Mexican lime tissues could be caused by CAT down-regulation, as in *cat3* mutants.

The gene with ID KN0AAP12YF23 [similar zinc finger (C3HC4-type RING finger) family protein; 1.1t03338.1 in the sweet orange genome database; homolog of *Arabidopsis* AT2G22010, which encodes a protein predicted to act as a RING E3 ubiquitin ligase] was 4.59-fold up-regulated in p23-transgenic leaves. Interestingly, an *Arabidopsis* RING-type E3 ligase RKP was induced by the C4 protein of *Beet severe curly top virus* (BSCTV) (Lai et al., 2009). RING-type E3 ligase RKP up-regulation trigger the degradation of ICK/KRPs and

enhance the activity of cell cycle-dependent kinases to accelerate G1/S cell-cycle transitions, which could provide a suitable environment for virus replication (Lai et al., 2009).

These (and other perhaps less important) host gene mis-regulations caused by p23 overexpression in Mexican lime leaves require verification. Moreover, it would be of major interest extending these results to other p23-transgenic lines and additionally studying whether the same genes are also up or down-regulated in 1) transgenic Mexican lime plants overexpressing the *p23* gene from the mild CTV strain T317, 2) transgenic Mexican lime plants with p23 overexpression restricted to the phloem, 3) transgenic Mexican lime plants overexpressing just the N-terminal region (delimited by amino acids 1 and 157) of the *p23* gene, 4) other CTV transgenic hosts as sweet and sour orange in which p23 has been also ectopically overexpressed (Fagoaga et al., 2005), and 5) CTV-infected plants of at least Mexican lime, sweet and sour orange. If mis-regulation of any of these genes is confirmed and extended to some of the experimental contexts proposed above, transgenic complementation of host genes under the control of biotic stress-inducible promoters could be attempted to try to alleviate at least in part the symptoms induced by CTV in different citrus types, perhaps including sour orange rootstocks.

5. CONCLUSIONS

1. *Citrus tristeza virus* (CTV) 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 virus-derived sequences displayed RNA-mediated partial resistance to CTV, with a fraction of the propagations from some transgenic lines remaining uninfected. Looking for a more efficient strategy, here we have transformed Mexican lime with an intron-hairpin vector carrying full-length, untranslatable versions of the genes *p25*, *p20* and *p23* (SIAS construct) to silence simultaneously the expression of these three critical genes that are additionally involved in viral encapsidation (*p25*), replication (*p23*) and pathogenicity (*p23* and *p20*).

2. Transient co-expression of the green fluorescent protein marker gene (*gfp*), the SIAS construct and either CTV p20 or p23 by agroinfiltration in *gfp*-transgenic *Nicotiana 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.

3. We have obtained for the first time complete resistance to CTV infection in transgenic citrus, with all propagations from immune transgenic Mexican lime lines remaining symptomless and virus-free in successive flushes after graft-inoculation of CTV in the non-transgenic rootstock. Moreover, CTV was unable to infect scion propagations of these lines even when graft-inoculated directly.

Although a few discrete CTV 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. The accumulation of transgene-derived siRNAs was necessary but not sufficient for CTV resistance.

4. 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 RNA interference-inducing transgene and the challenging virus genome. 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.

5. The constitutive expression of amino acids 1 through 157 of p23 (p23Δ158-209) elicited CTV-like symptoms in Mexican lime plants, similar to, albeit milder than, those incited by expressing the whole p23 protein (209 amino acids), thus delimiting the region responsible for p23 pathogenesis in citrus to a 157 amino acid fragment including the Zn finger and flanking basic motifs of the protein.

6. RNA silencing suppressor activity of p23 in *N. benthamiana* involves most regions of the protein, because these activity was abolished by all p23 mutants tested, therefore indicating that the p23 function as pathogenicity determinant seems to be separated from its role as viral suppressor.

7. The restricted transgene expression of p23 to phloem-associated cells in Mexican lime resulted in phenotypes closely resembling those induced by virus infection, with the other abnormalities observed with transgenic expression of p23 under the control of the constitutive 35S promoter being most likely pleiotropic effects derived from p23 accumulation in non-phloem cells.

8. The most notorious CTV-like phenotypic aberrations induced by phloem-specific expression of the p23 gene from a severe strain in Mexican lime were absent when the p23 gene from a mild strain was used, which positively correlates the aberrant phenotype with the aggressiveness of the source CTV strain used.

9. Expression of the p23 fragment comprising the zinc-finger domain and flanking basic motifs specifically in phloem tissues was sufficient to incite the same anomalies as the complete p23 from the same isolate, indicating that the region comprising the N-terminal 157 amino acids of p23 is responsible (at least in part) for the vein clearing, stem pitting and possibly vein corking in Mexican lime.

CONCLUSIONES

1. El virus de la tristeza de los cítricos (CTV) ha desarrollado tres proteínas supresoras de silenciamiento que actúan a nivel intra- (p23 y p20) y /o intercelular (p20 y p25) para superar la defensa antiviral del huésped. Anteriormente, mostramos que plantas de lima Mexicana transformadas con secuencias derivadas del virus presentaban resistencia parcial mediada por RNA frente a la inoculación con CTV, manteniéndose sin infectar una parte de las propagaciones de algunas líneas transgénicas. Buscando una estrategia más eficaz, en este trabajo hemos transformado lima Mexicana con un vector intrón-horquilla portando las secuencias completas, en versiones no traducibles, de los genes *p25*, *p20* y *p23* (construcción SIAS) para silenciar simultáneamente la expresión de estos tres genes críticos que están además involucrados en la encapsidación (*p25*), replicación (*p23*) y patogenicidad viral (*p23* y *p20*).

2. La expresión transitoria por agroinfiltración del gen marcador de la proteína de fluorescencia verde de la medusa (*gfp)*, la construcción SIAS y bien p20 o bien p23 de CTV, en hojas de *Nicotiana benthamiana* transgénica *gfp* 16c, revertía la supresión de silenciamiento que producía tanto p23 como p20 cuando eran co-expresadas solo junto a GFP, indicando que la construcción SIAS contrarrestaba la actividad de cada uno de estos supresores de silenciamiento intracelulares de CTV en células de hojas de *N. benthamiana.*

3. Se ha obtenido por primera vez resistencia completa a la infección por CTV en cítricos transgénicos, manteniéndose todas las propagaciones de las líneas transgénicas inmunes de lima Mexicana asintomáticas y libres de virus en sucesivas brotaciones tras inocular mediante injerto con CTV el portainjertos no transgénico. Además, CTV no consiguió infectar ninguna de las propagaciones de estas líneas incluso cuando se inoculó por injerto directamente la variedad transgénica. Aunque se observaron unos pocos foci de CTV en células asociadas al floema transgénicas en contacto directo con el trozo de corteza usado como inóculo, después de más de un año de realizada la inoculación, la infección no había progresado. La acumulación de siRNA derivados del transgén fue necesaria pero no suficiente para lograr resistencia frente a CTV.

4. Al inocular propagaciones de las líneas transgénicas inmunes con una cepa de CTV divergente, la resistencia fue parcialmente superada, lo cual apoya la idea de que la eficacia del silenciamiento de RNA depende de la identidad de secuencia entre el transgén que induce la interferencia de RNA y el genoma del virus desafiante. Una resistencia mayor o incluso inmunidad frente a CTV podría obtenerse fusionando en una construcción quimérica tipo intrón-horquilla secuencias de esos tres genes virales de diferentes cepas divergentes con el fin de maximizar la identidad de secuencia.

5. La expresión constitutiva de los aminoácidos del 1 al 157 de p23 (p23Δ158-209) indujo síntomas similares a los producidos por CTV en plantas de lima Mexicana, similares pero más suaves que los

inducidos por la expresión de la proteína p23 entera (209 aminoácidos), permitiendo delimitar la región responsable de la patogénesis de p23 en cítricos a un fragmento de 157 aminoácidos que incluye el dedo de zinc y los motivos básicos flanqueantes de la proteína.

6. La actividad de p23 como supresor de silenciamiento de RNA en *N. benthamiana* implica a la mayoría de las regiones de la proteína, puesto que dicha actividad se perdía en todos los mutantes de p23 probados, lo cual indica que la función de p23 como determinante patogénico parece estar separada de su función como supresor viral.

7. La expresión de transgenes derivados de p23 restringida a células asociadas al floema de lima Mexicana dio lugar a fenotipos muy parecidos a los inducidos por la infección viral, permitiendo además asignar otras anormalidades observadas con la expresión transgénica de p23 bajo el control del promotor constitutivo 35S probablemente a efectos pleiotrópicos derivados de la acumulación de p23 en células no floemáticas.

8. Las aberraciones fenotípicas más notorias similares a síntomas inducidos por CTV generadas por la expresión específica en floema del gen *p23* de una cepa agresiva del virus en lima Mexicana no se produjeron cuando se utilizó el gen *p23* de una cepa suave, lo cual correlacionaba positivamente las aberraciones fenotípicas con la agresividad de la cepa de origen utilizada.

9. La expresión en tejidos floemáticos del fragmento de p23 que comprende el dominio de dedo de zinc y los motivos básicos

flanqueantes fue suficiente para inducir las mismas anomalías que la p23 completa del mismo aislado, indicando que la región N-terminal de 157 aminoácidos de p23 es responsable (al menos en parte) del aclaramiento de venas, acanaladuras en la madera y posiblemente del acorchamiento de venas en lima Mexicana.

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