***Citrus tristeza virus* p23: Determinants for Nucleolar Localization and Their Influence on Suppression of RNA Silencing and Pathogenesis**

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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.

Citrus tristeza virus (CTV)—family *Closteroviridae*, 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 agroinocu-

lated, 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

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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.

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 (dpi) 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 (Fig. 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 (Fig. 2), thus substantiating the accumulation of p23 in these subnuclear compartments. This perfect match and the lack of any apparent cellular aber-

ration 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 Fig. S1).

On the other hand, p23 was also detected in agroinfiltrated leaves in punctuated structures at the cell wall that could represent plasmodesmata (Fig. 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 (Fig. 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). 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) (Fig. 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);

Fig. 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.

p23 Δ 125-209 (only deleting the third motif); and p23 Δ 158-209 (deleting the C-terminal 51 amino acids without affecting any of the three regions or motifs). 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 p23 Δ 106-114 and p23 Δ 158-209 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 (Fig. 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. 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 p23 Δ 125-209 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, p23 Δ 143-209 (including the third motif rich in basic amino acids delimited by positions 143 to 155) which, in contrast with mutant p23 Δ 158-209, did

Fig. 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.

Fig 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.

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 p23 Δ 158-209 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 (Fig. 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 (Fig. 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 (Fig. 5). 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).

Fig 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.

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 (Fig. 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 dis-

played 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) (Fig. 6). Both RSS (p23 and HcPro) displayed very similar effects on the inversely correlated levels of GFP-mRNA and GFP-siRNAs (Fig. 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

Fig 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.

behaved as the deletion mutants: the fluorescence became almost undetectable at 6 to 7 dpi. 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.

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 (Fig. 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) (Fig. 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).

Fig 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.

Fig 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.

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 p23 Δ 158-209 deletion and the p23H75 substitution mutants incited symptoms (severe necrosis resulting in plant death) similar to those of the wt p23 (Fig. 7 and Supplementary Fig. 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 p23 Δ 158-209 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. 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 p23 Δ 158-209 (Fig. 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

Fig. 8. A, *Citrus tristeza virus* (CTV)-like foliar symptoms expressed by seven transgenic lines of Mexican lime transformed with the p23 Δ 158-209 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 p23 Δ 158-209 deletion mutant. Controls are the same as in the previous panel. **C,** Accumulation of p23 and its deletion p23 Δ 158-209 mutant as revealed by Western blot analysis with a specific antibody following electrophoresis in sodium dodecyl sulfate polyacrylamide (12%) gels.

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 (Fig. 8; Supplementary Fig. 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 (Fig. 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.

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 (Taliany 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 (Taliany et al. 2010). On the other hand, whereas the interactions detected in planta 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 CTV-p23 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 GVB-p14, remains an unanswered and challenging question.

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.

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. macropophylla*. 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°C and 8 h of darkness at 22°C), 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 organelles 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, U.S.A.), 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 *EagI* and *SalI* 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, U.S.A.), 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-methoxy-spiro [1,2-dioxetane-3,2'-(5'-chloro)tricyclo[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 *Hind*III and the cognate expression cassettes were subcloned into the vector pBin19-*sgfp*, between the *nos-pro/nptIII/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 buffer-saturated 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°C in 50% formamide. The radioactive *p23*-specific cDNA probe was prepared with Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.).

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, U.S.A.) (Bradford 1976). Aliquots

(50 µg) were electrophoresed in SDS-polyacrylamide gels (12%), electroblotted onto polyvinylidene difluoride 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°C. 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°C 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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