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Double-stranded RNA interferes in a sequence-specific manner with infection of representative members of the two viroid families

Alberto Carbonell, Ángel-Emilio Martínez de Alba, Ricardo Flores* and Selma Gago

Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Campus Universidad Politécnica, Avenida de los Naranjos, s/n, 46022 Valencia, Spain

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*Corresponding author:

Dr. Ricardo Flores

Tel: (34)-96-3877861

Fax: (34)-96-3877859

E-mail: rflores@ibmcp.upv.es

Abstract

Infection by viroids, non-protein-coding circular RNAs, occurs with the accumulation of 21-24 nt viroid-derived small RNAs (vd-sRNAs) with characteristic properties of small interfering RNAs (siRNAs) associated to RNA silencing. The vd-sRNAs most likely derive from dicer-like (DCL) enzymes acting on viroid-specific dsRNA, the key elicitor of RNA silencing, or on the highly-structured genomic RNA. Previously, viral dsRNAs delivered mechanically or agroinoculated have been shown to interfere with virus infection in a sequence-specific manner. Here, we report similar results with members of the two families of nuclear- and chloroplast-replicating viroids. Moreover, homologous vd-sRNAs co-delivered mechanically also interfered with one of the viroids examined. The interference was sequence-specific, temperature-dependent and, in some cases, also dependent on the dose of the co-inoculated dsRNA or vd-sRNAs. The sequence-specific nature of these effects suggests the involvement of the RNA induced silencing complex (RISC), which provides sequence-specificity to RNA silencing machinery. Therefore, viroid titer in natural infections might be regulated by the concerted action of DCL and RISC. Viroids could have evolved their secondary structure as a compromise between resistance to DCL and RISC, which act preferentially against RNAs with compact and relaxed secondary structures, respectively. In addition, compartmentation, association with proteins or active replication, might also help viroids to elude their host RNA silencing machinery.

Introduction

RNA silencing, in addition to mediating a broad range of developmental events, also operates as a defense mechanism against invading plant, insect and mammalian viruses (Baulcombe, 2004; Ding et al., 2004; Dunoyer and Voinnet, 2005; Schutz and Sarnow, 2006). In the defense pathway, RNA silencing is triggered by: i) double-stranded (dsRNA) generated during viral replication, ii) single-stranded (ssRNA) that, due to their anomalous accumulation or peculiar structural features, is recognized by a host RNA-dependent RNA polymerase (RDRP) and converted into dsRNA, or iii) genomic or defective viral ssRNAs with extensive fold-back structure (Dalmay et al., 2000; Molnar et al., 2005; Mourrain et al., 2000; Szittya et al., 2002; Moissiard and Voinnet, 2006). The dsRNA or highly-structured ssRNA is processed by a bidentate RNase III-like enzyme —Dicer (Berstein et al., 2001), or Dicer-like (DCL) in plants— into 21-24 nt fragments (Hamilton and Baulcombe, 1999) called small interfering RNAs (siRNAs). These siRNAs are transferred to the RNA induced silencing complex (RISC) and guide its Argonaute component, an RNase H-like enzyme, for degrading the cognate ssRNA (Hammond et al., 2000; Omarov et al., 2007; Pantaleo et al., 2007). The presence of siRNAs homologous and complementary to the targeted ssRNA in all systems exhibiting antiviral RNA silencing, make them reliable markers for this phenomenon also known as post-transcriptional gene silencing (PTGS) in plants.

Viroids are a singular class of plant pathogens composed exclusively by a small (256-401 nt) circular ssRNA, with a compact secondary structure and without protein-coding capacity (Diener, 2003; Ding and Itaya 2007; Flores et al., 2000; Flores et al., 2005; Tabler and Tsagris 2004). They are able to replicate independently (without the assistance of a helper virus) in plants and, as an indirect effect of altering their gene expression, to incite in most cases specific diseases. Viroids, which replicate through an RNA-based rolling circle mechanism with the likely involvement of dsRNA intermediates (Branch et al., 1984; Daròs et al., 1994; Feldstein et al., 1998; Navarro et al., 1999), are grouped into two families: *Pospiviroidae,* type species *Potato spindle tuber viroid* (PSTVd) (Diener, 1972; Gross et al., 1978), and *Avsunviroidae,* type species

Avocado sunblotch viroid (ASBVd) (Hutchins et al., 1986). PSTVd and ASBVd replicate (and accumulate) in the nucleus and the chloroplast, respectively, as most likely also do the other members of both families. This classification scheme is supported by additional criteria that include the presence of conserved motifs in the family *Pospiviroidae,* and of hammerhead ribozymes in the family *Avsunviroidae* that catalyze self-cleavage of the oligomeric strands of both polarities resulting from their rolling-circle replication (Flores et al., 2005).

Recently, viroid-derived small RNAs (vd-sRNAs) with characteristic properties of the PTGS-associated siRNAs, have been detected in tissues infected by different viroids (Itaya et al., 2001; Markarian et al., 2004; Martínez de Alba et al., 2002; Papaefthimiou et al., 2001), strongly suggesting that these unconventional RNAs are also targeted by PTGS. The vd-sRNAs could result from the action of one or more DCL isoenzymes not only on the dsRNA replicative intermediates, but also on the genomic viroid RNA, which being unencapsidated and highly structured appears particularly vulnerable to enzymes of this class. Indeed, two recent reports on the vd-sRNAs from tomato infected by PSTVd, there referred to as small RNAs of PSTVd (srPSTVds) (Itaya et al., 2007), or by the closely related *Citrus exocortis viroid* (CEVd) (Martín et al., 2007), show that they are mostly of plus polarity, phosphorylated and methylated at their 5′ and 3′ termini respectively, and map predominantly to certain domains of the viroid rod-like secondary structure.

Because direct delivery of dsRNA by mechanical inoculation or by *Agrobacterium*-mediated transient-expression has been shown to interfere in a sequence-specific manner with infection by three different plant RNA viruses (Tenllado and Díaz-Ruiz, 2001), we examined whether these results could be extended to viroids, particularly considering that the PSTVd rod-like secondary structure has been proposed to play a key role in resistance to RISC-mediated cleavage (Itaya et al., 2007). Here, we report our results with PSTVd and CEVd (Semancik and Weathers, 1972) of the family *Pospiviroidae,* and with *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Navarro and Flores, 1997) of the family *Avsunviroidae.* These results support the notion that: i)

the highly-structured genomic viroid ssRNA or some dsRNA derivative thereof, operate as the primary elicitors of PTGS in natural viroid infections, paralleling the situation observed previously in natural virus infections (Ratcliff et al., 1997; Ratcliff et al., 1999), and ii) despite their compact secondary structure, viroid RNAs are sensitive to RISC-mediated degradation under certain conditions.

Results

Titration of inocula

Given that, in contrast to viruses, local lesion hosts have not been reported for most viroids and, when reported, they have very narrow growth conditions, viroid bioassays had to be performed with systemic hosts: tomato (PSTVd and CEVd), gynura (CEVd), and chrysanthemum (CChMVd). To study the effects on viroid infectivity of different co-delivered RNAs, it was first necessary to determine the minimal amount of each viroid that elicited symptoms in all the plants inoculated mechanically. Dilution assays showed that these minimal amounts were: 25-50 ng of circular CEVd RNA per gynura or tomato plant, and 5 ng of circular PSTVd RNA per tomato plant (obtained from infected gynura and tomato, respectively), and 10 ng of monomeric linear CChMVd RNA (resulting from *in vitro* self-cleavage of a dimeric head-to-tail transcript) per chrysanthemum plant. Results from bioassays were quantitatively expressed by the infectivity index (Semancik et al., 1976). This index, the total number of infected-plant-days over a certain period of time (20-50 days post-inoculation, depending on the experiment), combines in a single figure the number of infected plants and the time course of the onset of symptoms.

Co-inoculated homologous dsRNA interferes with representative viroids of both families

Because previous results have shown that direct delivery by mechanical inoculation of certain RNA viruses, together with a 5000 molar excess of their homologous dsRNAs, interferes with infection in a sequence-specific manner (Tenllado and Díaz-

Ruiz, 2001), we explored whether a similar effect could be observed with representative viroids of both families. With the CEVd-gynura system, co-inoculation of the viroid RNA with a 1250 molar excess of CEVd-dsRNA (inoculating a 5000 molar excess was technically difficult) significantly reduced the infectivity index with respect to inoculations with the viroid RNA alone. This reduction was very much influenced by the inability of the viroid, in the presence of its homologous dsRNA, to infect half of the plants in two independent experiments, as revealed by the lack of symptoms and hybridization signals in Northern-blot analysis after denaturing PAGE (with the signals generated by the infected plants being considerably weaker) (Fig. 1A, C and E). Moreover, the effect was sequence-specific because co-inoculation of CEVd with the same molar excess of dsRNA of similar size derived from the non-related CChMVd neither decreased the infectivity index, nor the hybridization signals (Fig. 1C and E), and parallel results were obtained with dsRNA from a bacteriophage (data not shown). A parallel outcome was observed when gynura was replaced by tomato: only the co-inoculated homologous CEVd-dsRNA afforded a protective effect against CEVd (Fig. 1B, D and F).

With the PSTVd-tomato system, the effect of co-inoculating the viroid RNA with a 5000 molar excess of PSTVd-dsRNA was not so pronounced because all plants eventually became infected, although the onset of symptoms was retarded and, as a consequence, they were less severe (Fig. 2A). This effect was again sequence-specific because substitution of the PSTVd-dsRNA by CChMVd-dsRNA did not essentially affect infectivity (Fig. 2B). Moreover, analysis by denaturing PAGE and Northern-blot hybridization showed a reduction in the accumulation of the circular and linear forms of PSTVd in plants co-inoculated with PSTVd plus PSTVd-dsRNA, with respect to those inoculated with PSTVd alone, or with PSTVd plus the non-related CChMVd-dsRNA (Fig. 2C). Therefore, a direct correlation could be established between PSTVd accumulation and the precocity and severity of symptoms.

Finally, with the CChMVd-chrysanthemum system, a 1250 molar excess of the homologous dsRNA also had a marked negative effect on infectivity when co-

inoculated with the viroid: half of the plants did not express symptoms and Northern-blot analysis failed to detect the viroid in these plants (Fig. 3A and C). The observed effect did not increase at a CChMVd-dsRNA molar excess of 5000 and disappeared when the molar excess was 250 or 50, indicating that it was dose-dependent within a certain interval. The sequence-specificity of the protection observed was confirmed in co-inoculations of CChMVd with the non-related PSTVd-dsRNA: the infectivity index remained basically unchanged when compared with that of the block of plants inoculated with the viroid only (Fig. 3B), and similar results were obtained in co-inoculations with the bacteriophage dsRNA (data not shown). Altogether, these results with four different viroid-host combinations, which included representative members of the two viroid families, extend the interfering effects of co-delivering homologous dsRNAs observed previously in virus infections to these subviral pathogens, and indicate that these effects most likely result from RNA silencing. More specifically, their sequence-specific nature suggests the involvement of RISC, which also acts in a sequence-specific manner.

Co-inoculated homologous vd-sRNAs also interfere with some viroids

Considering the interference afforded by dsRNAs when co-inoculated with their homologous viroids, we tested whether similar effects could be also provided by the primary products of DCL on dsRNAs (or highly-structured ssRNAs) and direct effectors of RNA silencing. The vd-sRNAs were obtained by *in vitro* digestion with the cloned RNase III from *E. coli,* which, acting on dsRNA, catalyzes the production of small RNAs with the same 3' di-nucleotide overhangs and with 5'-phosphate and 3'-hydroxyl termini as those generated by human Dicer (Nicholson, 1999; Yang et al., 2002). Because RNase III produces shorter cleavage products, digestion conditions were controlled to ensure that the resulting vd-sRNAs had a size that included the range of 21-24 nt, typical of DCL products (Fig. 4A). With the CEVd-gynura system, the presence of a 100 molar excess of the CEVd-sRNAs accompanying the infecting viroid decreased the infectivity index with respect to the inoculum containing only

the viroid RNA. This effect was the result of delaying the onset of symptoms in one of the experiments (data not shown) and reducing the number of infected plants in the other experiment (Fig. 4B), and was also reflected in the Northern-blot hybridization signals (Fig. 4C). Similar observations were made in one experiment with the CEVd-tomato system, in which co-inoculation of the CEVd-sRNAs resulted in a decrease in the number of infected plants to half (Fig. 4B). As previously found in the co-inoculations with dsRNAs, the effects of the vd-sRNAs were sequence-specific because co-inoculating heterologous vd-sRNAs did not significantly modify the infectivity index (Fig. 4B).

However, parallel attempts with the PSTVd-tomato system (using a 100 molar excess of PSTVd-sRNAs) and the CChMVd-chrysanthemum system (using 500 and 1250 molar excesses of CChMVd-sRNAs) failed to reveal any observable effects of co-inoculating the vd-sRNAs with their homologous viroids. At least with the first system, these negative results could be in part the consequence of PSTVd accumulating in tomato to levels significantly higher than CEVd (see below). In any case, our results show that in certain situations, mechanical co-delivery of homologous vd-sRNAs interferes with viroid infectivity, as predicted for *bona fide* siRNAs, suggesting that vd-sRNAs generated in natural infections may program RISC and exert a regulatory effect on the titer of mature viroid RNAs. Application of a 1000 molar excess of PSTVd-sRNAs induced rapid collapse of tomato plantlets (data not shown). Interestingly, over-saturation of cellular microRNA/short hairpin RNA pathways has also been associated with fatality in mice (Grimm et al., 2006).

Growing temperature influences the interfering effects of homologous dsRNAs

Because it is known that the accumulation of PSTVd and CEVd are unusually temperature-dependent within the interval between 25°C and 35°C (Sänger and Ramm, 1975), we examined whether the protective effects of the homologous dsRNAs observed previously under greenhouse conditions (20-22°C with thermal oscillations between 25°C and 15°C) were also temperature-dependent. In plants kept

in a growth chamber (30°C for 16 h with fluorescent light and 25°C for 8 h in darkness), either no significant protective effect (CEVd-gynura and CEVd-tomato systems), or just a minor effect (PSTVd-tomato system), was detected (data not shown).

To confirm the influence of temperature on viroid titer, blocks of PSTVd- CEVd- and CChMVd-inoculated tomato, gynura and chrysanthemum plants, respectively, were grown in parallel in the greenhouse and the growth chamber. Analysis by denaturing PAGE and Northern-blot hybridization showed a marked increase in the accumulation of the circular and linear forms of the three viroids in plants grown under the high temperature regime (Fig. 5). This increase most likely accounts for the reduction or lack of protective effects observed in co-inoculations with homologous dsRNAs using the high temperature regime. Moreover, quantification of viroid RNAs by gel staining with ethidium bromide revealed that with the low temperature regime the accumulation of CEVd in gynura and tomato was considerably lower than that of PSTVd in tomato, whereas the difference was attenuated with the high temperature regime (data not shown). These observations could explain why the protective effects of the homologous dsRNAs in co-inoculations under the low temperature regime were more clearly visible with CEVd. To further discard that other environmental variables, particularly light, could influence the observed differences, we performed a second experiment using two growth chambers set at the same light intensity and photoperiod, but differing in the temperature regime (30°C-25°C and 23°C-19°C). Results from this experiment confirmed those described above (Supplementary Fig. 1).

We also analyzed the influence of temperature on the accumulation of the vd-sRNAs: the titer increased with temperature, following a trend similar to their genomic RNAs. At least two populations were detected in all instances; in the nuclear viroids, the vd-sRNAs of smaller size (comigrating with the RNA marker of 21 nt) were more abundant in CEVd than in PSTVd, whereas in the chloroplastic viroid (CChMVd)

the vd-sRNAs of larger size (also comigrating with the RNA marker of 21 nt) were the most abundant (Fig. 5 and Supplementary Fig. 1).

Co-delivery of homologous dsRNA by agroinfiltration also affects PSTVd infection

To provide additional support for the previous results, the dsRNA was co-delivered by agroinfiltration instead of by mechanical inoculation. To this end, we used the experimental host *N. benthamiana* because it can be easily agroinfiltrated and sustains replication of PSTVd, albeit without symptoms. Given that the strong autocomplementarity of PSTVd RNA could pose some problems for proper folding of the dsRNA *in vivo,* we delivered a hairpin construct formed by an inverted repeat designed to comprise only the upper strand of the rod-like structure, separated by an intron, instead of a nearly full-length PSTVd sequence (Smith et al., 2000). Computer-based predictions indicated that this viroid RNA segment, however, can still adopt a structured folding that includes the so-called hairpin I conserved in all members of the family *Pospiviroidae* (Flores et al., 2005). Controls for the experiment included the corresponding direct repeat construct and the empty vector (Fig. 6A).

Northern-blot hybridization of RNAs from leaves agroinfiltrated with the plasmid expressing the hairpin construct showed high levels of PSTVd-sRNAs 3-4 days post infiltration (dpi), while these levels were significantly lower and undetectable in leaves agroinfiltrated with the plasmid expressing the direct repeat construct and with the empty vector, respectively (data not shown). When each of these three constructs were co-infiltrated with the recombinant plasmid expressing an infectious dimeric head-to-tail PSTVd (+) RNA, Northern-blot hybridization of RNAs extracted 4 dpi showed reduced levels of the dimeric transcript, as well as of the monomeric circular and linear PSTVd RNAs, in leaves co-infiltrated with the hairpin construct with respect to those co-infiltrated with the direct repeat construct and the empty vector (Fig. 6B). The effects of the three different constructs were attenuated, but still visible, in RNAs extracted 5 and 6 dpi (Fig. 6B). As expected, an inverse relationship could be

established with the vd-sRNAs, which reached the highest levels in the leaves co-infiltrated with the hairpin construct (Fig. 6B).

These results were extended to RNA preparations from the upper non-inoculated leaves. Northern-blot hybridizations revealed that co-infiltration of the plasmids expressing the dimeric PSTVd (+) RNA and the hairpin construct induced a decrease in the accumulation of the monomeric circular and linear viroid RNAs, with respect to parallel co-infiltrations with the direct repeat construct and the empty vector. The effect was clearly visible in leaves collected 10, 12 and 20 dpi, whereas no differences could be discerned at 30 dpi, presumably because by then the viroid had overcome the protective effect afforded by the transient expression of the hairpin construct (Fig. 6C). Altogether these results, which were reproduced in two independent experiments, support the view that dsRNA interferes in a sequence-specific manner with PSTVd infection through the generation of vd-sRNAs.

Discussion

Detection of vd-sRNAs with the typical properties of siRNAs accumulating *in vivo* in different viroid-host combinations indicates that viroids, like plant viruses, are inducers and targets of the RNA silencing machinery of their hosts. More specifically, these results implicate DCL in the genesis of the vd-sRNAs. At least formally, this enzymatic activity might be the only one responsible for degrading viroid RNAs and regulating viroid titer in natural infections. However, and also paralleling what has been recently shown for plant RNA viruses (Omarov et al., 2007; Pantaleo et al., 2007), vd-sRNAs might act as authentic siRNAs and program RISC for viroid RNA degradation. Supporting this view, some srPSTVds are active biologically in guiding RISC-mediated cleavage, as revealed with a system in which the green fluorescent protein (GFP) gene was fused to a sensor sequence complementary to a fragment of the PSTVd genomic RNA. When the GFP-sensor was introduced by agroinfiltration in PSTVd-infected tomato, the fluorescence was very much reduced (Itaya et al., 2007), in line with previous results showing that expression in tobacco and *N. benthamiana*

of non-infectious partial or full-length fragments of PSTVd or *Hop stunt viroid* (HSVd) —also of the family *Pospiviroidae*— fused to the GFP-coding region was suppressed upon viroid infection (Vogt et al., 2004; Gómez and Pallás 2007). However, replication of PSTVd proceeded normally when protoplasts were co-transfected with infectious full-length PSTVd (+) RNA and either srPSTVds or PSTVd-dsRNA, as did replication of transgenically expressed HSVd, suggesting that the viroid secondary structure plays a critical role in resistance to RISC-mediated cleavage (Itaya et al., 2007; Gómez and Pallás, 2007). We have here re-examined this question for several representative species of both viroid families with three different *in planta* approaches, two of which have been used previously with plant viruses (Tenllado and Díaz-Ruiz, 2001).

First, mechanical co-inoculation of the viroid RNA with an excess of homologous dsRNA induced significant reduction of the infectivity in CEVd-infected gynura, CEVd-infected tomato, PSTVd-infected tomato and CChMVd-infected chrysanthemum. At least in the CChMVd-chrysanthemum system, the effects were dose-dependent (lower amounts of the co-inoculated dsRNA induced less pronounced responses) and, most importantly, sequence-specific in the four viroid-host combinations examined (co-inoculations with similar amounts of unrelated dsRNA failed to provide any protection). The most direct interpretation for these results is that the co-delivered homologous dsRNAs are processed into vd-sRNAs that then prime RISC for degrading their cognate viroid genomic RNAs in the cytoplasm during intercellular movement, with the need of an excess of the dsRNA probably resulting from its inefficient delivery into cells, as presumably also occurs for viruses (Tenllado and Díaz-Ruiz, 2001). The alternative interpretation that RISC could target some viroid replicative intermediate appears less likely because of their dsRNA nature and because the vd-sRNAs should move into the nucleus or the chloroplast where replication occurs.

Second, additional support for this notion was obtained by mechanical co-inoculation of the viroid RNA with an excess of vd-sRNAs generated *in vitro* by bacterial RNase III: only the homologous vd-sRNAs significantly reduced the

infectivity in CEVd-infected gynura and CEVd-infected tomato. The effect was less pronounced than that induced by the homologous dsRNA, as revealed by the lack of protection observed in PSTVd-infected tomato and CChMVd-infected chrysanthemum. Although, as already indicated, this difference could result from the higher titer that PSTVd reaches in tomato, it is also possible that only a minor fraction of the vd-sRNAs generated *in vitro* are functional *in vivo.*

And third, co-agroinfiltration of *N. bentamiana* with recombinant plasmids expressing a dimeric head-to-tail PSTVd (+) RNA and a homologous PSTVd dsRNA (resulting from an inverted repeat construct separated by an intron) delayed viroid accumulation in the infiltrated areas and in the upper non-inoculated leaves. This effect was dependent on the formation of the dsRNA *in vivo* because it was not observed in co-infiltrations in which the hairpin construct was replaced by a direct repeat control. The inhibitory effect of the hairpin construct on PSTVd accumulation is presumably due to RISC-mediated cleavage of viroid RNA. However, considering that the hairpin and the dimeric PSTVd (+) RNAs were produced in the nucleus, we cannot exclude that the inhibitory effect could result from transcriptional interference of dimeric PSTVd (+) RNA production (Matzke and Birchler, 2005), although the same mechanism would be then expected to inhibit transcription of the hairpin construct and production of the hairpin-derived sRNAs. Transient co-expression of hairpin constructs may thus provide a rapid evaluation of the RNA silencing potential of viroid-derived sequences before attempting the generation of viroid-resistant transgenic plants. We believe that the inherent differences between the *in planta* systems used here and previously (Vogt et al., 2004; Itaya et al., 2007) and the protoplast system (Itaya et al., 2007), may explain the different results observed on the sensitivity/resistance of viroids to RISC-mediated cleavage. In particular, the molar excess of PSTVd-dsRNA used here was considerably higher than in the protoplast system and, of the three viroids here examined, PSTVd was the most resistant to inhibition by homologous dsRNA or vd-sRNAs. Moreover, the constructs used in the agroinfiltration experiments were also different.

We noticed that consistent reproduction of the protective effects demanded specific experimental conditions that included titration of the inocula and temperature. The importance of this latter environmental factor was revealed by the attenuation or lack of any effect afforded by mechanical co-delivery of homologous dsRNAs when the growing temperature was increased. In contrast to most plant RNA viruses, viroid levels increase between 25°C and 35°C and the genomic RNAs are more structured than their viral counterparts and, hence, more sensitive to DICER-mediated degradation. It is not thus surprising that raising the temperature not only induced higher accumulation of the genomic viroid RNA, but also of the corresponding vd-sRNAs, given that higher temperatures also increase the accumulation of the small RNAs derived from two plant viral RNAs (Kalantidis et al., 2002; Szittya et al., 2003). The RISC-mediated degradation that we propose here would be more clearly detectable when viroids are co-inoculated —mechanically or by agroinfiltration— with their homologous dsRNA or hairpin construct respectively, in plants grown under the low temperature regime. Under these conditions, the vd-sRNAs resulting from DICER-mediated degradation of the dsRNA would program RISC against the initial viroid progeny in the co-inoculated leaves, thus impeding or delaying systemic spread; the inverse relationship detected between the genomic viroid RNA and the vd-sRNAs is consistent with this view (Fig. 6B). When this barrier is overcome, the viroid replicates actively and most of the vd-sRNAs result from DICER-mediated degradation of the genomic RNA, thus accounting for the direct correlation between both RNAs observed in the infected non-inoculated leaves. On the other hand, this direct correlation does not allow us to discriminate which of the two RNA species is the primary effector of the more severe symptoms observed at high temperature. Therefore, although it is possible that some viroid-specific vd-sRNAs might act like endogenous microRNAs targeting host mRNAs for degradation (Papaefthimiou et al., 2001; Wang et al., 2004), the alternative implicating the genomic viroid RNA as the primary pathogenic effector remains open (Flores et al.,

2005). Pertinent to this context is the recent finding that the endogenous tomato miRNA pathway does not seem to be affected by CEVd infection (Martín et al., 2007).

The idea regarding RNA silencing as an important selection pressure shaping the evolution of the secondary structure of viroids has been advanced previously (Wang et al., 2004). In view of our present results indicating that viroids are not only targets of DCL but also of RISC, we propose that these RNAs could have evolved their typical secondary structure as a tradeoff to face degradation by DCL and RISC, which act preferentially against RNAs with compact and relaxed secondary structures, respectively. However, compartmentation in organella (nuclei or chloroplasts), association with proteins (Daròs and Flores, 2002; Martínez de Alba et al., 2003) or a very active replication, might also help viroids to elude the RNA silencing machinery of their hosts. In a different context, viroid sensitivity to RISC-mediated degradation at least under certain conditions, may also explain the sequence-specific cross-protection observed in viroids of both families (Niblett et al., 1978; De la Peña et al., 1999), which due to their lack of protein-coding ability must be necessarily RNA-based. Because RNA-mediated cross-protection in plant viruses is mechanistically equivalent to PTGS (Ratcliff *et al.* 1999), cross-protection between viroids may be interpreted by assuming that the vd-sRNAs resulting from the pre-inoculated mild strain load and guide RISC against the incoming RNA of the challenging severe strain. Confirmation of this hypothesis would provide an example of viroid sensitivity to RISC-mediated degradation under natural conditions.

Materials and methods

Viroids and hosts

PSTVd, CEVd and CChMVd variants were M16826 (intermediate), S67446 (severe) and AJ878085 (severe), respectively. Tomato (*Lycopersicon esculentum* L.) cv. 'Rutgers' and *Nicotiana benthamiana* plants were seedlings. Gynura (*Gynura aurantiaca* DC) and chrysanthemum (*Dendranthema grandiflora* Tzvelez) cv. 'Bonnie Jean', were propagated vegetatively from cuttings.

Preparation of dsRNAs and vd-sRNAs

Viroid-specific dsRNAs were prepared by annealing *in vitro* less-than-full-length or mutated (non-infectious) ssRNAs of both polarities obtained by *in vitro* transcription of recombinant plasmids. PSTVd and CEVd ssRNAs had a deletion of 6 nt affecting positions 354-359 (PSTVd) and 365-370 or 90-95 (CEVd), and CChMVd ssRNAs a mutation (CUGA to CUUA) in the conserved core of the hammerhead ribozymes of both polarities rendering them catalytically inactive. A 400 bp fragment of the phage lambda DNA was cloned in both orientations in an expression plasmid to obtain by *in vitro* transcription and annealing a non-viroid dsRNA control. Equal amounts of plus and minus ssRNAs were annealed by heating at 95ºC for 30 min (PSTVd and CEVd) or 10 min (CChMVd and phage lambda) and slow cooling to 25ºC, with annealing being confirmed by the change in electrophorectic mobility in non-denaturing 5% polyacrylamide gels. The vd-sRNAs were obtained by digesting *in vitro* the dsRNAs with RNAse III from *E. coli* (Silencer siRNA cocktail kit RNase III, Ambion). Digestions were controlled (0.1 or 1 U RNAse III/µg dsRNA at 37ºC for 1 h) to produce small 21-25 bp duplexes as revealed by non-denaturing PAGE in 15% gels.

Bioassays

RNAs were quantified by comparing the intensity of their electrophoretic bands stained with ethidium bromide with those generated by a preparation of 5S RNA quantified spectrophotometrically. Bioassays were performed under two conditions: in the greenhouse at 20-22ºC with a thermal oscillation between 25 and 15ºC (standard conditions for PSTVd and CEVd), or in a growth chamber at 30ºC for 16 h with fluorescent light and at 25ºC for 8 h in darkness (standard conditions for CChMVd, which under greenhouse conditions did not incite symptoms). In each bioassay (repeated at least once) blocks of four identical plants were inoculated mechanically by gentle rubbing two carborundum-dusted young leaves with: i) buffer, ii) viroid RNA, iii) viroid RNA and an excess of homologous dsRNA or vd-

sRNAs, and iv) viroid RNA and an excess of heterologous dsRNA or vd-sRNAs. Plants were examined for symptom expression (leaf curling in tomato and gynura, and chlorotic mottle in chrysanthemum), with the exact day of symptom appearance being recorded for each plant to estimate the infectivity index (total number of infected-plant-days over the bioassay length). Bioassays were concluded when all plants of the block co-inoculated with the heterologous dsRNA or vd-sRNAs showed symptoms.

Nucleic acids were extracted from upper non-inoculated leaves collected at the end of the bioassays, either with a protocol without organic solvents (Dellaporta et al., 1983), complemented with a fractionation with 2 M LiCl to enrich preparations in viroid RNAs, or with a phenol-based protocol (De la Peña et al., 1999). Viroid genomic RNAs were detected by denaturing PAGE in 5% gels containing 1X TBE and 8 M urea, and Northern-blot hybridization at 70ºC in the presence of 50% formamide (Daròs et al., 1994) with strand-specific ^{32}P-labeled riboprobes transcribed *in vitro* that were quantified by their acid-precipitable counts. Viroid-specific vd-sRNAs were detected by denaturing PAGE in 17% gels (acrylamide:bis-acrylamide ratio 19:1) containing 1X TBE and 8 M urea, and Northern-blot hybridization at 35ºC with the same riboprobes.

Agroinfiltration

A cDNA fragment corresponding to the upper strand of the PSTVd rod-like secondary structure (positions 1-179) was PCR-amplified from a recombinant plasmid. Two copies of this fragment, separated by the intron-2 of the *PDK* gene of *Flaveria* (Smith et al., 2000), were introduced in a modified version of pUC18 to obtain the direct repeat (drPSTVd) and the hairpin (hpPSTVd) constructs. The DNA fragments corresponding to drPSTVd, hpPSTVd and to a head-to-tail infectious dimeric PSTVd (dPSTVd), were subcloned into a modified version of the pMOG180 vector between a double copy of the 35S CaMV promoter and the Nos-terminator. The expression cassettes were again subcloned into the plant binary expression vector pBIN19sGFP by replacing the sGFP cassette to obtain pBINdrPSTVd, pBINhpPSTVd, pBINdPSTVd,

and pBINØ (the empty vector). These plasmids were introduced in *Agrobacterium tumefaciens* strain COR 308 by direct transformation, and the recombinant bacterial lines were grown overnight at 28ºC in tubes containing LB medium supplemented with 50 and 5 µg/ml of kanamycin and tetracyclin, respectively. Saturated cultures were diluted and used to inoculate fresh induction media (LB supplemented with 10 mM MES pH 5.6, 20 µM acetosyringone and 10 µg/ml tetracycline) that were then grown overnight at 28ºC. Cells were harvested by centrifugation, resuspended to OD_{600}=1 in infiltration medium (10 mM $MgCl_2$, 10 mM MES pH 5.6 and 150 µM acetosyringone) and incubated at room temperature for 5 h before infiltration (Tenllado and Díaz-Ruiz, 2001). Two or three leaves of young plants, grown in the greenhouse, were infiltrated in the lower side of the lamina with a 1-ml syringe without needle. For co-infiltration experiments, equal volumes of the two bacterial cultures were previously mixed. Nucleic acids from infiltrated and upper non-inoculated leaves were extracted as indicated before.

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Legends to figures

Fig. 1. Effects on CEVd infection of dsRNAs co-inoculated mechanically. **(A)** and **(B)** Gynura and tomato plants, respectively, co-inoculated with CEVd plus CEVd-dsRNA (symptomless, left), or CEVd plus CChMVd-dsRNA (symptomatic, right). The CEVd/dsRNA molar ratio was 1/1500. **(C)** and **(D)** Histograms representing the infectivity index of two independent bioassays in which blocks of four gynura and tomato plants respectively, were mock-inoculated, or inoculated with CEVd (black), CEVd plus CEVd-dsRNA (white), and CEVd plus CChMVd-dsRNA (gray). Infectivity index is expressed as the total number of infected-plant days over the bioassay length (with error bars indicating the standard deviation), and infectivity as the fraction of symptomatic plants at the end of the bioassay (40 days). **(E)** and **(F)** Analysis by denaturing PAGE and Northern-blot hybridization with a riboprobe for detecting (+) strands of the genomic circular (c) and linear (l) CEVd RNAs extracted from upper non-inoculated leaves (collected at the end of the bioassays) of blocks of four gynura (bioassay 1) and tomato (bioassay 2) plants, inoculated with CEVd plus CEVd-dsRNA, and CEVd plus CChMVd-dsRNA. RNAs from mock- and CEVd-inoculated control plants were also included. 5S RNA stained with ethidium bromide was used as loading control.

Fig. 2. Effects on PSTVd infection of dsRNAs co-inoculated mechanically. **(A)** Tomato plants co-inoculated with PSTVd plus PSTVd-dsRNA (symptomless, left), or PSTVd plus CChMVd-dsRNA (symptomatic, right). The PSTVd/dsRNA molar ratio was 1/5000. **(B)** Histograms representing the infectivity index of two independent bioassays in which blocks of four tomato plants were mock-inoculated, or inoculated with PSTVd (black), PSTVd plus PSTVd-dsRNA (white), and PSTVd plus CChMVd-dsRNA (gray), and examined for symptom expression along 30 days. **(C)** Analysis by denaturing PAGE and Northern-blot hybridization with a riboprobe for detecting (+) strands of the genomic circular (c) and linear (l) PSTVd RNAs extracted from upper non-

inoculated leaves of blocks of four tomato plants, inoculated with PSTVd plus PSTVd-dsRNA, and PSTVd plus CChMVd-dsRNA (bioassay 2). Other details as in the legend to Fig. 1.

Fig. 3. Effects on CChMVd infection of dsRNAs co-inoculated mechanically. **(A)** Chrysanthemum plants co-inoculated with CChMVd plus CChMVd-dsRNA (symptomless, left), or CChMVd plus PSTVd-dsRNA (symptomatic, right). The CChMVd/dsRNA molar ratio was 1/1250. **(B)** Histograms representing the infectivity index of two independent bioassays in which blocks of four chysanthemum plants were mock-inoculated, or inoculated with CChMVd (black), CChMVd plus CChMVd-dsRNA (white), and CChMVd plus PSTVd-dsRNA (gray), and examined for symptom expression along 20 days. **(C)** Analysis by denaturing PAGE and Northern-blot hybridization with a riboprobe for detecting (+) strands of the genomic linear (l) CChMVd RNAs (the circular forms were only visible after prolonged exposure, data not shown) extracted from upper non-inoculated leaves of blocks of four chrysanthemum plants, inoculated with CChMVd plus CChMVd-dsRNA, and CChMVd plus PSTVd-dsRNA (bioassay 2). Other details as in the legend to Fig. 1.

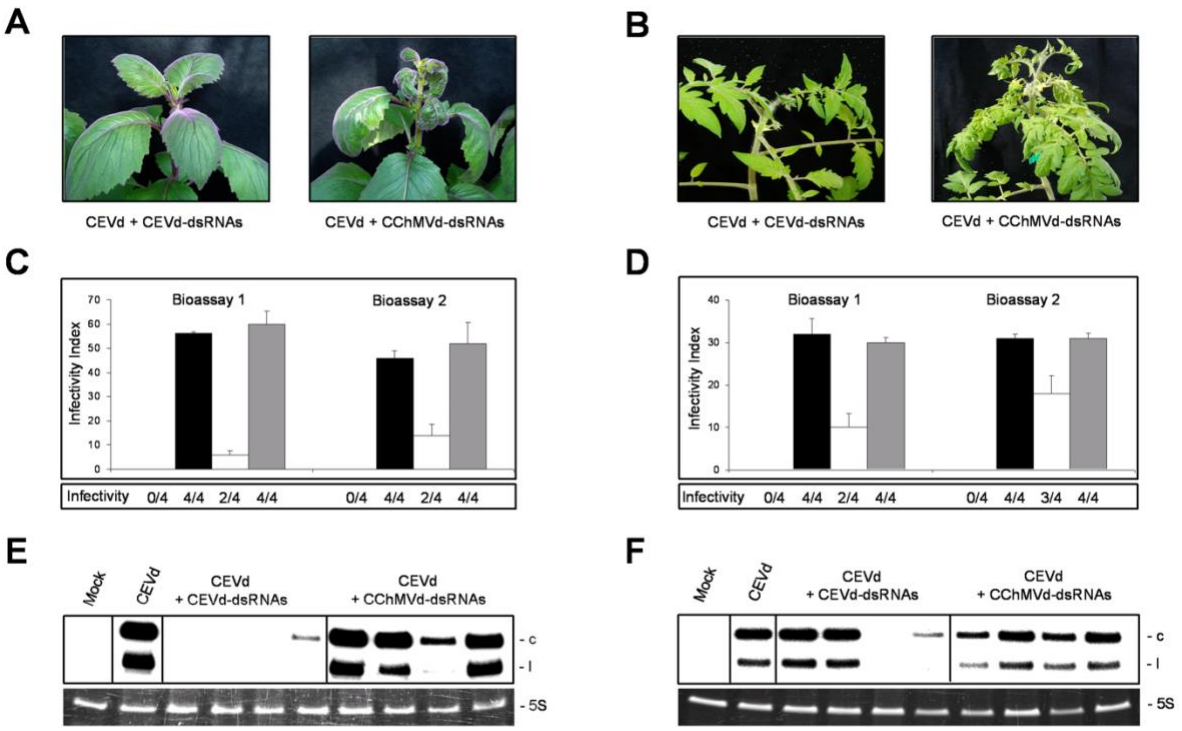
Fig. 4. Effects on CEVd infection of vd-sRNAs co-inoculated mechanically. **(A)** Analysis by non-denaturing PAGE of dsRNA untreated (lane 1), and treated with 0.1 or 1 U of *E. coli* RNase III per μg of dsRNA (lanes 2 and 3, respectively). Lane 4, molecular size markers. **(B)** Histograms representing the infectivity index of two bioassays in which blocks of four gynura (left) and tomato (right) plants were mock-inoculated, or inoculated with CEVd (black), CEVd plus CEVd-sRNAs (white), and CEVd plus CChMVd-sRNAs (gray), and examined for symptom expression along 50 days. The CEVd/sRNA molar ratio was 1/100. Other details as in the legend to Fig. 1. **(C)** Analysis by denaturing PAGE and Northern-blot hybridization with a riboprobe for detecting (+) strands of the genomic circular (c) and linear (l) CEVd RNAs extracted from upper non-inoculated leaves of blocks of four gynura plants, inoculated with

CEVd plus CEVd-sRNA, and CEVd plus CChMVd-sRNA. Other details as in the legend to Fig. 1.

Fig. 5. Effects of the growing temperature on the acumulation of viroid RNAs. Analysis by denaturing PAGE and Northern-blot hybridization with riboprobes for detecting viroid (+) strands in RNA preparations from three tomato, gynura and chrysanthemum plants inoculated with PSTVd **(A)**, CEVd **(B)**, and CChMVd **(C),** respectively, grown in the greenhouse (20-22ºC) or in a growth chamber (25-30ºC). Top panels show the signals generated by the monomeric circular (c) and linear (l) viroid RNAs (the CChMVd circular form was only visible after prolonged exposure), and lower panels the signals corresponding to the vd-sRNAs (with the position of an RNA marker of 21 nt indicated at the left). RNAs from mock-inoculated control plants were also included. 5S or tRNAs stained with ethidium bromide were used as loading controls.

Fig. 6. Local and systemic effects on PSTVd accumulation of different PSTVd constructs co-agroinfiltrated in *Nicotiana benthamiana*. **(A)** Schematic diagrams of the constructs: empty vector (Ø), dimeric PSTVd (+) [dPSTVd(+)], inverted repeat (hairpin) PSTVd (hpPSTVd), and direct repeat PSTVd (drPSTVd). **(B)** Denaturing PAGE and Northern-blot hybridization with a riboprobe for detecting PSTVd (+) strands of RNAs extracted from pools of three co-infiltrated leaves collected 4, 5 and 6 days post-infiltration. Leaves were co-infiltrated with the dPSTVd(+) construct and either the Ø, hpPSTVd or drPSTVd constructs. Positions of the dimeric primary transcript and unit-length circular (c) and linear (l) viroid RNAs (upper panels) and of the vd-sRNAs (lower panels) are indicated. 5S or tRNAs stained with ethidium bromide were used as loading controls. **(C)** Denaturing PAGE and Northern-blot hybridization with a riboprobe for detecting PSTVd (+) strands of RNAs extracted from upper non-infiltrated leaves of four individual plants collected 10, 12, 20 and 30 days after infiltrating young basal leaves. Other details as in panel **(B)**.

Supplementary Fig. 1. Effects of the growing temperature on the accumulation of viroid RNAs. Analysis by denaturing PAGE and Northern-blot hybridization with riboprobes for detecting viroid (+) strands in RNA preparations from two blocks of three tomato plants inoculated with PSTVd **(A)** and CEVd **(B)**, and three chrysanthemum plants inoculated with CChMVd **(C)**, grown in two growth chambers at the same light intensity and photoperiod but differing in the temperature regime (19°C-23°C and 25°C-30°C). Other details as in the legend to Figure 5.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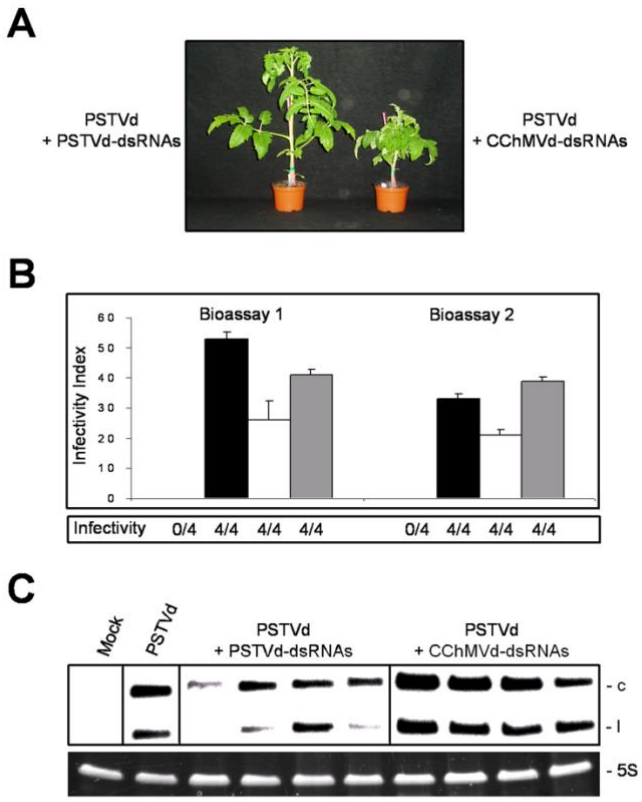
CEVd + CEVd-dsRNAs
CEVd + CChMVd-dsRNAs
B
CEVd + CEVd-dsRNAs
CEVd + CChMVd-dsRNAs
C
Bioassay 1
Bioassay 2
Infectivity Index
Infectivity 0/4 4/4 2/4 4/4 0/4 4/4 2/4 4/4
D
Bioassay 1
Bioassay 2
Infectivity Index
Infectivity 0/4 4/4 2/4 4/4 0/4 4/4 3/4 4/4
E
Mock
CEVd
CEVd + CEVd-dsRNAs
CEVd + CChMVd-dsRNAs
- c
- l
- 5S
F
Mock
CEVd
CEVd + CEVd-dsRNAs
CEVd + CChMVd-dsRNAs
- c
- l
- 5S

Fig. 1


A
PSTVd
+ PSTVd-dsRNAs
PSTVd
+ CChMVd-dsRNAs
B
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Bioassay 2
Infectivity Index
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50
40
30
20
10
0
Infectivity 0/4 4/4 4/4 4/4 0/4 4/4 4/4 4/4
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PSTVd
+ PSTVd-dsRNAs
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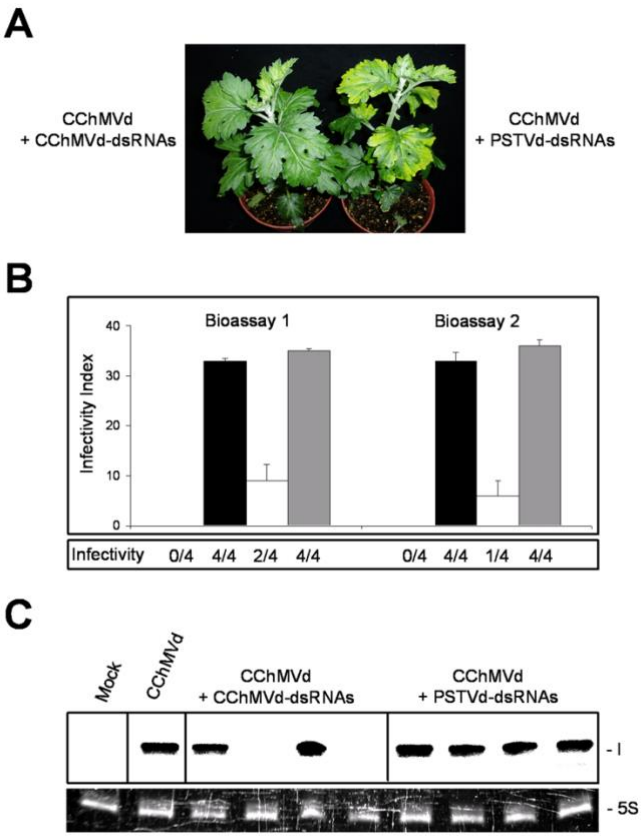
- c
- I
- 5S

Fig. 2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CChMVd
+ PSTVd-dsRNAs
B
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Bioassay 2
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20
10
0
Infectivity
0/4
4/4
2/4
4/4
0/4
4/4
1/4
4/4
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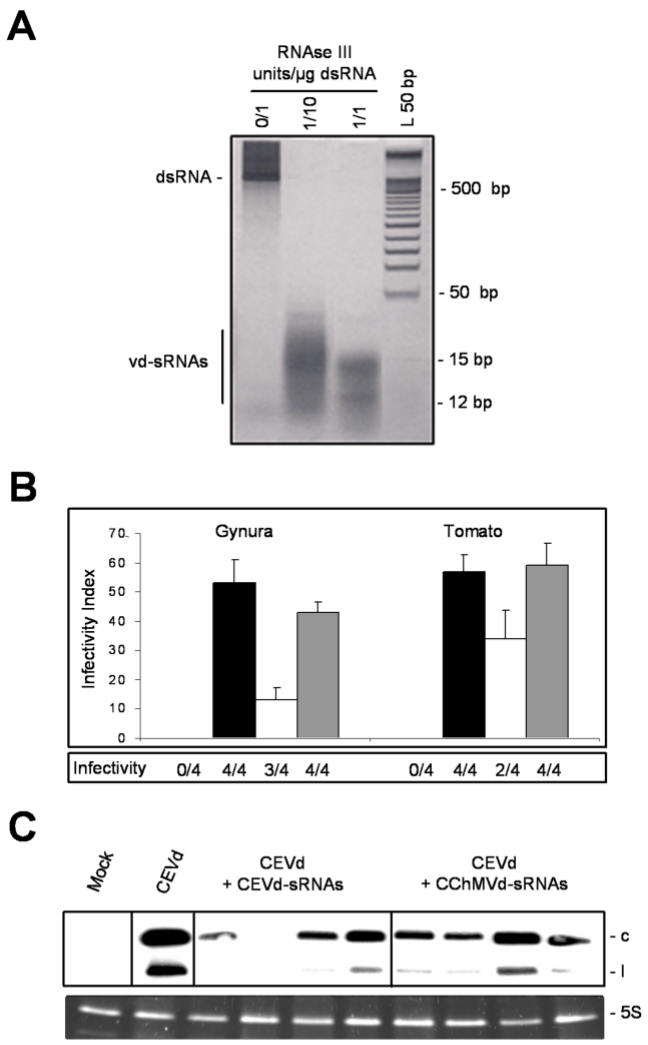
+ PSTVd-dsRNAs
- I
- 5S

Fig. 3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1/1
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dsRNA -
vd-sRNAs
- 500 bp
- 50 bp
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- 12 bp
B
Gynura
Tomato
Infectivity Index
70
60
50
40
30
20
10
0
Infectivity 0/4 4/4 3/4 4/4 0/4 4/4 2/4 4/4
C
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+ CEVd-sRNAs
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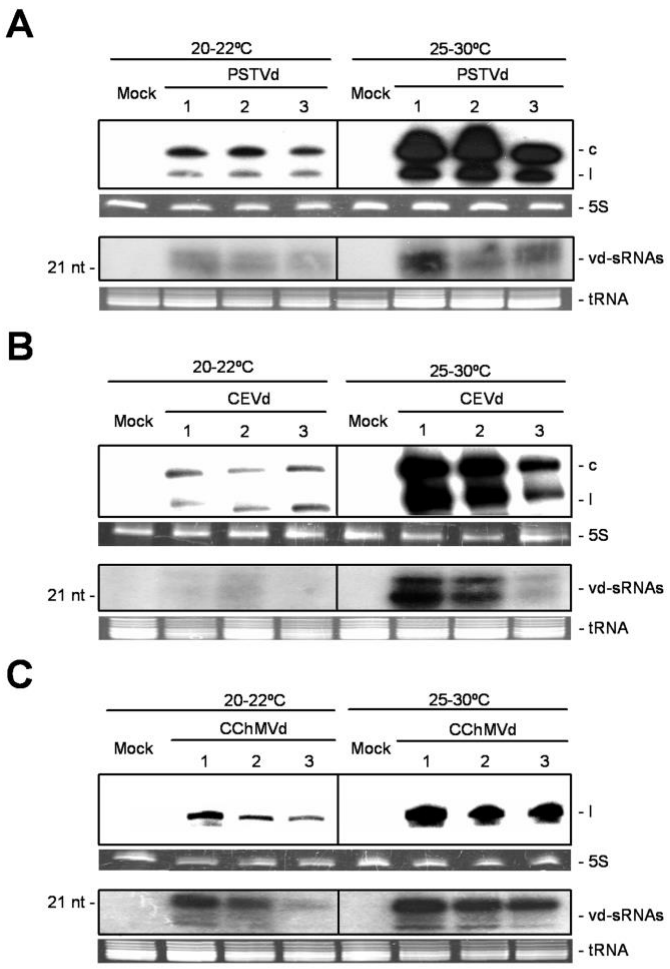
+ CChMVd-sRNAs
- c
- l
- 5S

Fig. 4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20-22ºC
25-30ºC
PSTVd
Mock
1
2
3
- c
- I
- 5S
21 nt -
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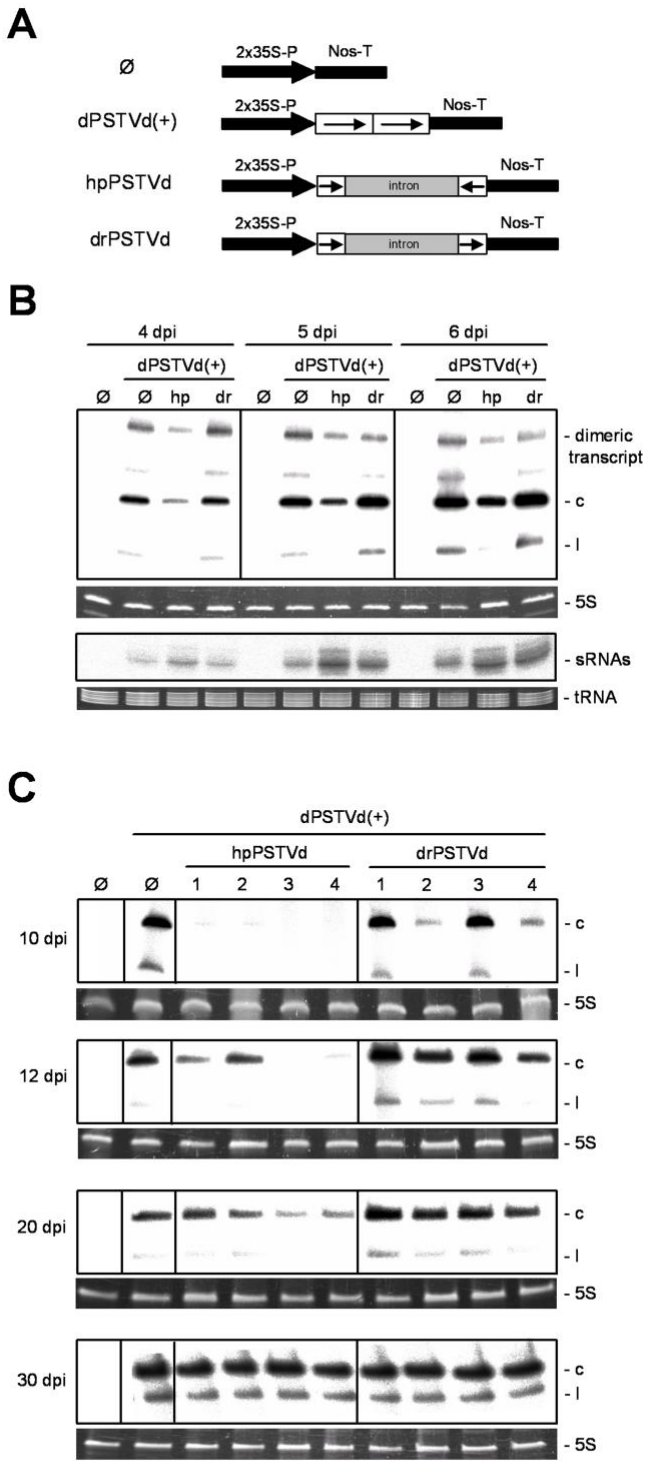
- tRNA
B
CEVd
C
CChMVd

Fig. 5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intron
drPSTVd
B
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6 dpi
dPSTVd(+)
Ø
hp
dr
- dimeric transcript
- c
- l
- 5S
- sRNAs
- tRNA
C
hpPSTVd
drPSTVd
1
2
3
4
10 dpi
12 dpi
20 dpi
30 dpi

Fig. 6