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# RNAi tools for controlling viroid diseases

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ABSTRACT

Viroids are small (250–400 nucleotides), single-stranded, circular RNAs without protein-coding capacity that infect a large number of ornamental and crop plant species, causing high economic losses worldwide. Strategies to control viroid diseases have included the use of naturally resistant cultivars in breeding programs, the superinfection exclusion with mild strains, the expression of ribonucleases, sense or antisense (catalytic) RNAs and, more recently, RNA interference (RNAi)-based tools. Here, I review the different RNAi strategies used to control viroid infections in plants, with particular focus on highly specific artificial small RNA (art-sRNA)-based tools such as artificial microRNAs and synthetic trans-acting small interfering RNAs. The advantages and future perspectives of art-sRNA-based RNAi for controlling viroid diseases are discussed.

## 1. Introduction

Viroids are composed by a small (250–400-nucleotide), non-protein-coding, circular, single-stranded RNA (ssRNA) with compact secondary structure (Flores et al., 2015; Navarro et al., 2021). They replicate through an RNA-based rolling-circle mechanism including double-stranded RNA (dsRNA) intermediates and viroid RNAs of (+) and (-) polarities (Branch and Robertson, 1984), and are classified into two families, *Pospiviroidae* and *Avsunviroidae*, whose members replicate and accumulate in the nucleus and the chloroplast of plant cells, respectively. Viroids exit the replication organelles, move cell to cell through plasmodesmata and access the vasculature for long-distance trafficking, and invade non-vascular distal tissues to restart the cycle (Ding and Itaya, 2007).

Like viruses, viroids are targeted by the plant RNA silencing defensive response of their hosts, as inferred by the observation that viroid-derived small interfering RNAs (vd-siRNAs) accumulate in plants infected by members of both families (Itaya et al., 2001; Martinez de Alba et al., 2002; Papaefthimiou et al., 2001). The presence of vd-siRNAs strongly supports that viroid RNAs, presumably dsRNA replicative intermediates and genomic structured RNAs, are processed by Dicer-like (DCL) RNAses of class III (Qi et al., 2005), with a proposed combined activity of DCL2 and DCL3 being crucial for antiviroid defense (Katsarou et al., 2016). Moreover, the observation that several ARGONAUTE (AGO) proteins bind vd-siRNAs and attenuate viroid titers *in vivo* (Minoia et al., 2014) suggests that viroid RNAs, as described for viruses (Carbonell and Carrington, 2015), may also be targeted by AGO/vd-siRNA complexes.

Viroids are the simplest infectious agents, able to infect a large range of ornamental and crop plant species and cause high economic losses worldwide. The most important viroid-induced losses occur in vegetatively propagated crops like potato, citrus, fruit trees, grapevine, hop or several ornamental plants. In these plants the primary control measure is using viroid-free certified propagation material to establish new plantations. When no viroid-free plant of the desired variety is available, sanitation can be achieved in some cases by *in vitro* culture of shoot tips (for example in potatoes) or shoot-tip grafting *in vitro* (for example in citrus). Viroids usually do not reach the meristem and close tissues due to lack of differentiated phloem vessels, therefore, plants regenerated from small shoot tips taken from infected plants are often viroid-free.

Diverse strategies to control viroid-induced losses have been examined and reported in the last decades such as breeding programs for resistance using naturally resistant cultivars, superinfection exclusion with mild strains, expression of ribonucleases, antisense (and sense) RNAs, hammerhead ribozymes, and RNA interference (RNAi)-based tools [reviewed in Dalakouras et al. 2015, Flores et al. 2017, Kovalskaya and Hammond 2014]. Next, i describe and discuss the different RNAi strategies used to control viroid infections in plants, that include: (i) the topical delivery of double-stranded RNAs (dsRNAs), (ii) the transgenic expression of hairpin RNAs (hpRNAs), and (iii) the transgenic expression of artificial sRNAs (art-sRNAs) such as artificial microRNAs (amiRNAs) and synthetic trans-acting small interfering RNAs (syn-tasiRNAs). A particular importance is given to highly specific art-sRNA-based RNAi (art-sRNAi) tools and their potential in viroid disease control.

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## 2. Classic dsRNA-based RNAi tools for controlling viroid infections

Classic antiviral RNAi tools such as the topical delivery of dsRNAs or the transgenic expression of hairpin RNA (hpRNA) constructs consist of introducing dsRNAs bearing sequences of the target virus to trigger the RNAi defensive response of the plant [reviewed recently in (Gaffar and Koch, 2019; Mitter et al., 2017)]. Antiviral dsRNAs of virus sequence are processed by plant DCLs into 21–24 nt small interfering RNA (siRNA) duplexes. One strand of each duplex can be incorporated into an AGO protein complex to target highly complementary viral RNAs and induce antiviral resistance. Such strategies have also been applied to interfere with viroid infections in a similar way (Fig. 1), as detailed next.

### 2.1. Topical delivery of antiviroid dsRNAs

The topical delivery of viroid-derived dsRNAs was shown to induce sequence-specific interference with the infection of both nuclear- and chloroplast-replicating viroids (Carbonell et al., 2008). *In vitro*-produced dsRNAs mechanically co-inoculated ogether with titrated inoculum of *Potato spindle tuber viroid* (PSTVd), *Citrus exocortis viroid* (CEVd) or *Chrystanthemum chlorotic mottle viroid* (CChMVd) induced a significant inhibition of viroid infection in tomato (*Solanum lycopersicum*), gynura (*Gynura aurantiaca*) and chrysanthemum (*Dendranthema grandiflora*), respectively. Importantly, the observed interference was dose-dependent and sequence-specific, as similar amounts of heterologous dsRNAs did not alter viroid infection. Interestingly, the protective effects were temperature-dependent and disappeared when growing temperature was increased from 20–22 to 25–30 °C, a temperature that favors viroid replication (Carbonell et al., 2008). These results, in contrast to previous reports (Itaya et al., 2007; Wang et al., 2004) demonstrated that viroids, like viruses, are susceptible to AGO-mediated degradation, at least in some experimental and environmental conditions.

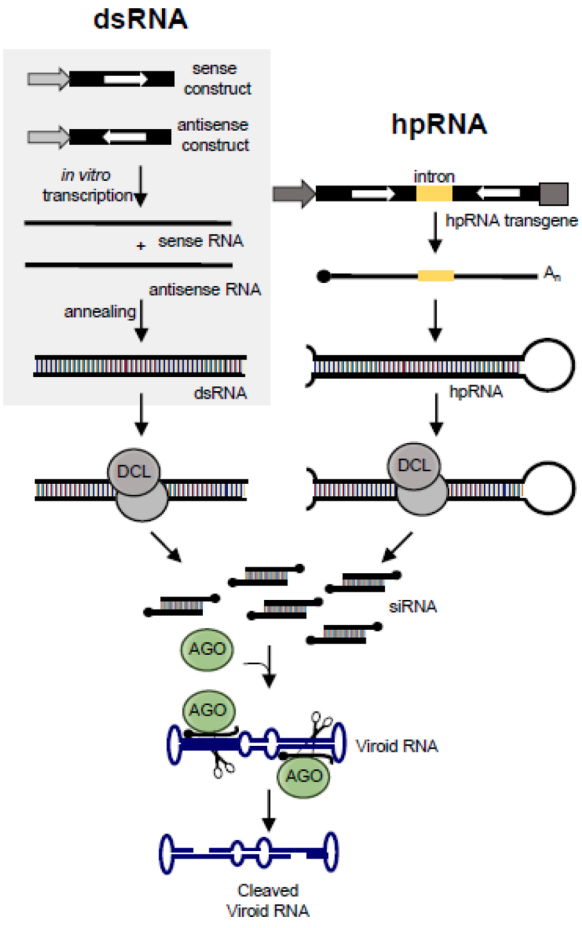


**Fig. 1.** Double-stranded RNA (dsRNA)-based strategies for controlling viroid infections. DsRNA (left) is produced by annealing sense and antisense RNAs of viroid sequence obtained by *in vitro* transcription, and delivered to the plant by mechanical inoculation of leaves. Hairpin RNA (hpRNA, right) is produced *in vivo* by expressing a transgene including sense and antisense viroid sequences separated by an intron. In both cases, dsRNA/hpRNA precursors are processed by plant DCLs to generate a population of different 21–24 nucleotides siRNA duplexes of viroid sequence. According to their size and 5' nucleotide among other factors, one strand of each duplex can be loaded into specific AGOs and target viroid RNAs at multiple sites to induce antiviroid resistance.

### 2.2. Transgenic expression of antiviroid dsRNAs

To extend previous results obtained with co-inoculated dsRNAs, PSTVd dsRNAs were also produced *in planta* through the agoinfiltration in *N. benthamiana* of a hpRNA construct comprising only the upper strand of the rod-like structure of PSTVd, separated by an intron (Carbonell et al., 2008). The co-expression of the hpRNA construct together with a construct expressing a dimeric head-to-tail PSTVd (+) RNA delayed viroid accumulation in both agroinfiltrated and upper non-agroinfiltrated leaves. A few years later, complete resistance to PSTVd was observed in two out of three transgenic tomato lines accumulating high levels of viroid-specific siRNAs derived from a hpRNA construct including near full-length PSTVd sequences (Schwind et al., 2009).

In a later study 21 *N. benthamiana* transgenic lines expressing seven truncated versions of PSTVd hpRNA under the control of constitutive or phloem-specific promoters were developed (Adkar-Purushothama et al., 2015). Truncated regions were selected according to the hotspots of both PSTVd vd-siRNAs and functional/structural domains of PSTVd. When challenged with PSTVd, five of these lines expressing high levels of hpRNA-derived siRNAs (hp-siRNAs) from the constitutive promoter accumulated reduced levels of viroid RNAs. In particular, hpRNAs as short as 26–49 nucleotides were processed into hp-siRNAs and inhibited viroid infection.

In a different study, transgenic *N. benthamiana* lines expressing a PSTVd hpRNA in companion cells were grafted with a wild-type scion which was subsequently inoculated with PSTVd (Kasai et al., 2013). Interestingly, PSTVd accumulation in the wild-type scion was attenuated compared with control plants (propagated on empty vector-transformed rootstock), likely due to the translocation of hp-siRNAs from the PSTVd-transgenic rootstock to the scion. This strategy shows that viroid infections can be controlled in non-genetically modified scions grafted onto transgenic stocks expressing hp-siRNAs of viroid sequence.

## 3. Art-sRNAi tools for controlling viroid infections

Despite the success of dsRNA-based RNAi tools in controlling viroid infections, these approaches may have a relatively high risk of inducing undesired off-target effects, as the populations of siRNA species produced from dsRNAs could lead to the accidental targeting of other cellular transcripts sharing high sequence complementarity with that of certain dsRNA-derived siRNAs. Pertinent to this context, tomato plants expressing a hpRNA construct against PSTVd and accumulating high levels of hp-siRNAs exhibited symptoms similar to those of natural PSTVd infections (Wang et al., 2004) most likely due to the unintended targeting of cellular RNAs which would cause developmental aberrations. However, the possible contribution of other factors to the observed phenotypes cannot be ruled out.

The limited specificity of classic dsRNA-based tools was overcome by a "second-generation" set of RNAi tools based on art-sRNAs, such as amiRNAs and syn-tasiRNAs (Fig. 2), which are computationally designed to silence the intended target with high specificity (no off-

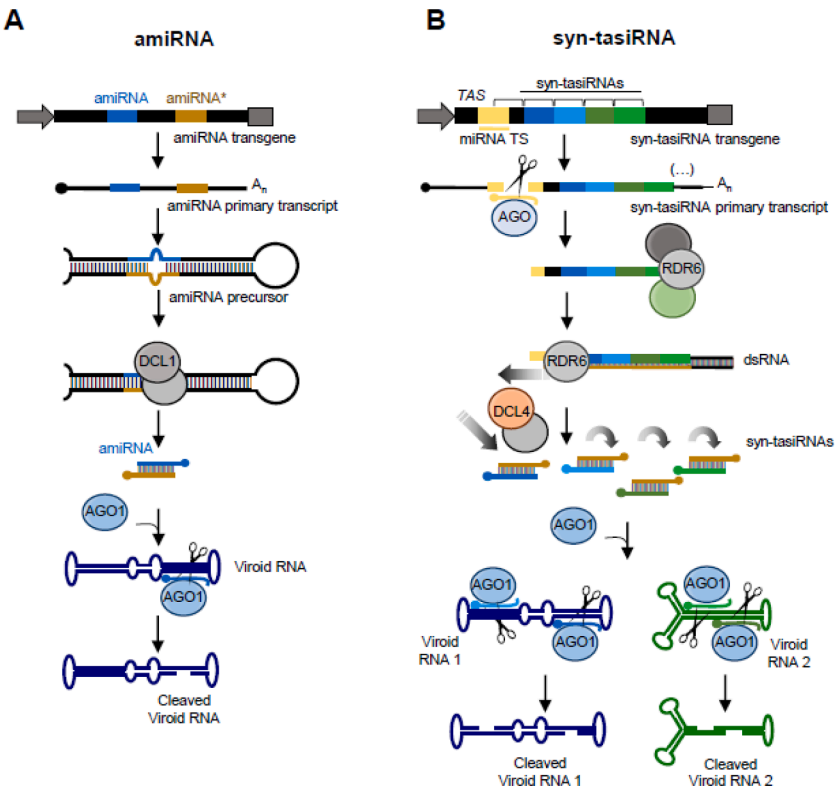


**Fig. 2.** Artificial small RNA (art-sRNA)-based strategies for controlling viroid infections. A, The artificial microRNA (amiRNA) pathway. An amiRNA transgene is introduced into plants to express a *MIRNA* precursor in which the endogenous miRNA guide and star sequences are substituted by the anti-viroid amiRNA guide and star sequences. The amiRNA primary transcript is sequentially processed by DCL1 into the amiRNA precursor and the amiRNA duplex. The 5'U containing amiRNA guide is selectively loaded into AGO1 to target and cleave a highly complementary site in viroid RNAs. B, The synthetic trans-acting small interfering RNA (syn-tasiRNA) pathway). A syn-tasiRNA transgene is introduced into plants to express a *TAS* precursor in which the endogenous tasiRNA sequences are substituted by the antiviroid syn-tasiRNA sequences. The syn-tasiRNA primary transcript is cleaved by an AGO/miRNA complex, and RDR6 synthesizes a dsRNA from one of the cleaved products. DCL4 processes the dsRNA into phased syn-tasiRNA duplexes in 21-nucleotide register with the miRNA cleavage site. The 5'U containing syn-tasiRNA guides are selectively loaded into AGO1 to target and cleave multiple highly complementary sites in the same or in different viroid RNAs.

targets). Art-sRNAs have been extensively used to confer plant resistance against a large number of viruses [for a recent review see (Cisneros and Carbonell, 2020)] and, more recently, have also been tested against viroids [see Section 3.4 (Carbonell and Daròs, 2017)].

### 3.1. AmiRNAs

AmiRNAs are produced *in planta* by expressing a transgene that includes a *MIRNA* gene in which endogenous miRNA and miRNA* sequences are substituted by computationally designed amiRNA and amiRNA* sequences (Fig. 2A). Importantly, nucleotide substitutions must preserve the secondary structure of the primary transcript for its accurate and sequential processing by DCL1 into the precursor amiRNA foldback and the amiRNA duplex. The amiRNA guide strand of the duplex is selectively incorporated into AGO1 to silence highly complementary viroid sequences.

### 3.2. Syn-tasiRNAs

Syn-tasiRNAs are produced *in planta* by expressing a transgene that includes a *TAS* gene in which various endogenous trans-acting siRNA (tasiRNA) sequences are substituted by multiple computationally designed antiviroid syn-tasiRNA sequences (Fig. 2B). The syn-tasiRNA primary transcript is cleaved by a miRNA/AGO complex, and RNA-dependent RNA polymerase 6 (RDR6)-mediated dsRNA synthesis from one of the cleavage products is triggered. Resulting dsRNA is sequentially processed by DCL4 into phased syn-tasiRNA duplexes in 21 nt register with the miRNA cleavage site. Syn-tasiRNA guide strands are selectively loaded into AGO1 to direct specific silencing of multiple sites in the same or in distinct viroid RNAs.

### 3.3. Design of antiviroid art-sRNAs

The major advantage of art-sRNA tools compared to dsRNA approaches is that art-sRNAs are computationally designed to (i) be selectively and efficiently loaded into AGO1, (ii) extensively base pair with its target RNA, and iii) be highly specific with no off-targets. Art-sRNAs can be computationally designed with several webtool platforms such as P-SAMS (from Plant Small RNA Maker Suite) (Fahlgren et al., 2016) and WMD3 (from Web MicroRNA Designer 3) (Ossowski et al., 2008). Webtool-based automated designs are optimized to select art-sRNAs with high specificity and efficacy. The specificity of the art-sRNA is assessed computationally through the analysis of all predicted base pairing interactions between the art-sRNA candidate and the plant transcriptome. The efficacy of the art-sRNA depends on several factors such as target site accessibility and stability, or the degree of base pairing between the art-sRNA and its target. This last factor is indeed considered during the art-sRNA design process by selecting art-sRNAs with extensive base paring with the target RNA and with limited or no mismatches near the cleavage site or in the 5' seed region.

Art-sRNA sequences start with an AGO1-preferred 5' U for selective and efficient AGO1 loading, and, in P-SAMS designs, contain a C at position 19 to produce a star strand with an AGO1 non-preferred 5'G. Also, in WMD3 designs, the hybridization energy of the amiRNA/target RNA interaction must be between -35 and -40 kcal/mole. For designing highly specific antiviroid art-sRNAs, webtools typically allow to introduce the target viroid RNA sequence in FASTA format and the use of the target specificity module.

### 3.4. AmiRNAs and syn-tasiRNAs interfere with viroid infection

The use of art-sRNAs to interfere with viroid diseases was recently explored by combining the use of high-throughput methods for art-sRNA construct generation and the *Potato spindle tuber viroid* (PSTVd)/*N. benthamiana* pathosystem (Carbonell and Daròs, 2017). P-SAMS was used to design 12 highly specific amiRNAs targeting sites included in bulged or stem regions and distributed along the five structural domains of PSTVd in RNAs of (+) or (-) polarity (Table 1), with no off-targets in *N. benthamiana*. AmiRNA sequences were cloned into *BsaI/ccd*B "B/c" vectors (Carbonell, 2019), and transiently co-expressed in

**Table 1**
Successful applications of RNAi tools to control viroid infections in plants.

| RNA class | sRNA produced | Precursor | Target viroid[1](size, accession) | Target coordinates (strand), domain(s)[2] | Effects | Delivery[3] | Plant species | Refs. |
|---|---|---|---|---|---|---|---|---|
| dsRNA | siRNA | dsRNA of viroid sequence | CEVd (370 nt, S67446) | 1-364 (+), all | Symptom and accumulation delay | MI | *G. aurantiaca* | Carbonell et al. (2008) |
| | | | ChCMVd-CM20 (399 nt, AJ878085) | 1-399 (+) | Symptom and accumulation delay | MI | *D. grandiflora* | " |
| | | | PSTVd-Int (359 nt, M16826) | 1-353 (+), all | Symptom and accumulation delay | MI | *S. lycopersicum* | " |
| | | hpRNA of viroid sequence | PSTVd-Int (359 nt, M16826) | 1-160 (+), all | Accumulation delay | TE | *N. benthamiana* | " |
| | | | PSTVd-Nb (359 nt, AJ634596) | 1-359 (+), all | Resistance | SE | *L. esculentum* | Schwind et al. (2009) |
| | | | PSTVd (359 nt, M16826 | 1-160 (+), all | Reduced accumulation | SE | *N. benthamiana* | Adkar-Purushothama et al. (2015) |
| | | | PSTVd-Int (359 nt, AY937179) | 6-352 (+), all | Reduced accumulation | TR | *N. benthamiana* | Kasai et al. (2013) |
| art-sRNA | amiRNA | *AtMIR390a* | PSTVd-RG1 (359 nt, U23058) | 130-150 (+), V | Accumulation delay | TE | *N. benthamiana* | Carbonell and Daròs (2017) |
| | | | | 199-219 (+), TR-V | Accumulation delay | TE | *N. benthamiana*, | " |
| | | | | 253-273 (+), C | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 349-10 (+), TL | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 78-98 (+), C | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 322-342 (+), TL | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 359-20 (-), TL | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 63-83 (-), P | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 95-115 (-), C | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 140-160 (-), V-TR | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 316-336 (-), P-TL | Accumulation delay | TE | *N. benthamiana* | " |
| | | | | 249-269 (-), C | Accumulation delay | TE | *N. benthamiana* | " |
| | Syn-tasiRNA | *AtTAS1c* | PSTVd-RG1 (359 nt, U23058) | 199-219 (+), TR-V<br>78-98 (+), C<br>349-10 (+), TL<br>316-336 (-), P-TL<br>95-115 (-), C | Accumulation delay | TE | *N. benthamiana* | " |

[1] CChMVd, *Chrystanthemum chlorotic mottle*; CEVd, *Citrus exocortis viroid*; PSTVd, *Potato spindle tuber viroid.*
[2] C, conserved domain; P, pathogenic domain; TL, terminal left domain; TR, terminal right domain; V, variable domain.
[3] MI, mechanical inoculation; SE, stable expression; TE, transient expression; TR, transgenic rootstock.

*N. benthamiana* plants together with a construct expressing a dimeric head-to-tail PSTVd (+) RNA of the severe RG1 strain. The antiviroid activity of each amiRNA construct was assessed in agroinfiltrated leaves by Northern blot analysis of viroid genomic RNAs of (+) polarity. Interestingly, the majority of amiRNAs were highly active although with different efficacies. Indeed, amiRNA efficacy could not be explained neither by the amiRNA expression level nor by the secondary structure of the target site, and thus other factors such as target site accessibility may also contribute to define the efficacy of each amiRNA. Unexpectedly, despite that during an infection viroid RNAs of (+) polarity are in excess compared with those of (-) polarity, amiRNAs against PSTVd (+) RNAs were more active than those against PSTVd (-) RNAs. It is possible that, while amiRNAs against PSTVd (-) RNAs may only target oligomeric (-) replicative intermediates, amiRNAs against PSTVd (+) RNAs might target primary dimeric PSTVd (+) transcripts and genomic circular PSTVd (+) RNAs besides oligomeric (+) replicative intermediates. Also, the different subcellular localization of viroid RNAs of both polarities could affect amiRNA efficacy. In any case, this approach may be also used to identify highly active amiRNAs against other viroids infecting *N. benthamiana* such as *Hop stunt viroid* (Gomez et al., 2008).

Similarly, the antiviroid effect of a syn-tasiRNA construct including the five most effective anti-PSTVd amiRNA sequences (determined in the previous amiRNA screening) was confirmed. A comparative analysis showed that the effects of the most effective amiRNA and of syn-tasiRNAs were similar in both agroinfiltrated and in upper non-agroinfiltrated leaves, where PSTVd accumulation was delayed. These results indicated that both classes of art-sRNAs induced similar protective effects. Complete resistance was not observed, most likely due to the transient expression of the art-sRNAs that were not expected to move systemically throughout the plant. Thus, it is reasonable to think that the stable expression of these art-sRNA constructs in transgenic plants could confer enhanced resistance against PSTVd. Still, it is also possible that target sites in viroid RNAs could mutate due to the selective pressure imposed by the art-sRNA, as has been previously observed during virus infections (Carbonell et al., 2019; Lafforgue et al., 2011). However, the syn-tasiRNA approach in which multiple viroid sites could be targeted simultaneously with several syn-tasiRNAs from a single policistronic precursor may circumvent this problem as observed before in tomato transgenic plants expressing high levels of syn-tasiRNAs against *Tomato spotted wilt virus* (Carbonell et al., 2019).

## 4. Conclusions and future perspectives

Over the last years, RNAi tools were employed for controlling viroid infections by directly targeting viroid RNAs. These approaches usually reduce the viroid titer or delay viroid infection but do not allow to recover viroid-free plants, thus limiting their application in the field. Still, reducing the viroid titer would be useful to improve sanitation efficiency when using *in vitro* culture of shoot tips or shoot-tip grafting *in vitro* to obtain viroid-free mother plants for propagation. Of particular interest are RNAi tools based on art-sRNAs because of their high specificity. However, the design of highly specific art-sRNAs is limited to plant species with well-annotated transcriptomes, which currently includes several model (e.g. *Arabidopsis thaliana, N. benthamiana* or *Brachypodium distachyon*) and crop (e.g. tomato, potato, rice or maize) plants. Hence, a wider biotechnological use of art-sRNAs in agricultural

species will require to expand the current catalogue of crop species with a sequenced genome. In any case, the recently described high-throughput systems for designing, producing and screening in a time and cost-effective manner large amounts of amiRNAs in transient assays in *N. benthamiana* have facilitated the identification of highly effective art-sRNAs targeting PSTVd RNAs of both polarities. Thus, it is very likely that transgenic plants simultaneously expressing several of these highly active anti-PSTVd art-sRNAs (for example, from a single syn-tasiRNA construct) would be highly resistant. Alternatively, antiviroid resistance may also be achieved with art-sRNAs targeting endogenous genes coding for proteins that interact with the viroid and facilitate its replication such as VIRP1 (Kalantidis et al., 2007; Maniataki et al., 2003), CUCUMBER PROTEIN 2 (Gomez and Pallas, 2001), the avocado chloroplastic replicating protein PARBP33 (Daros and Flores, 2002), the DNA ligase (Nohales et al., 2012a), or the chloroplastic tRNA ligase (Nohales et al., 2012b), as previously proposed (Dalakouras et al. 2015).

Finally, the development of highly efficient methodologies for the production and topical delivery to plants of art-sRNA precursors is key for a broader use of these technologies for antiviroid applications in line with current regulations applying to genetically modified organisms. Moreover, a deeper knowledge of the basic mechanisms dictating art-sRNA biogenesis, mode of action and anti-viroid targeting will definitely help refining these art-sRNAi tools not only for enhanced anti-viroid protection but also to engineer high-performant next-generation crops.

## CRediT authorship contribution statement

**Alberto Carbonell:** Conceptualization, Investigation, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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