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# Sequence Similarities between Viroids and Human MicroRNAs

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## Keywords

Viroid · microRNA · RNA interference · Human · Plants · Messenger RNA

## Abstract

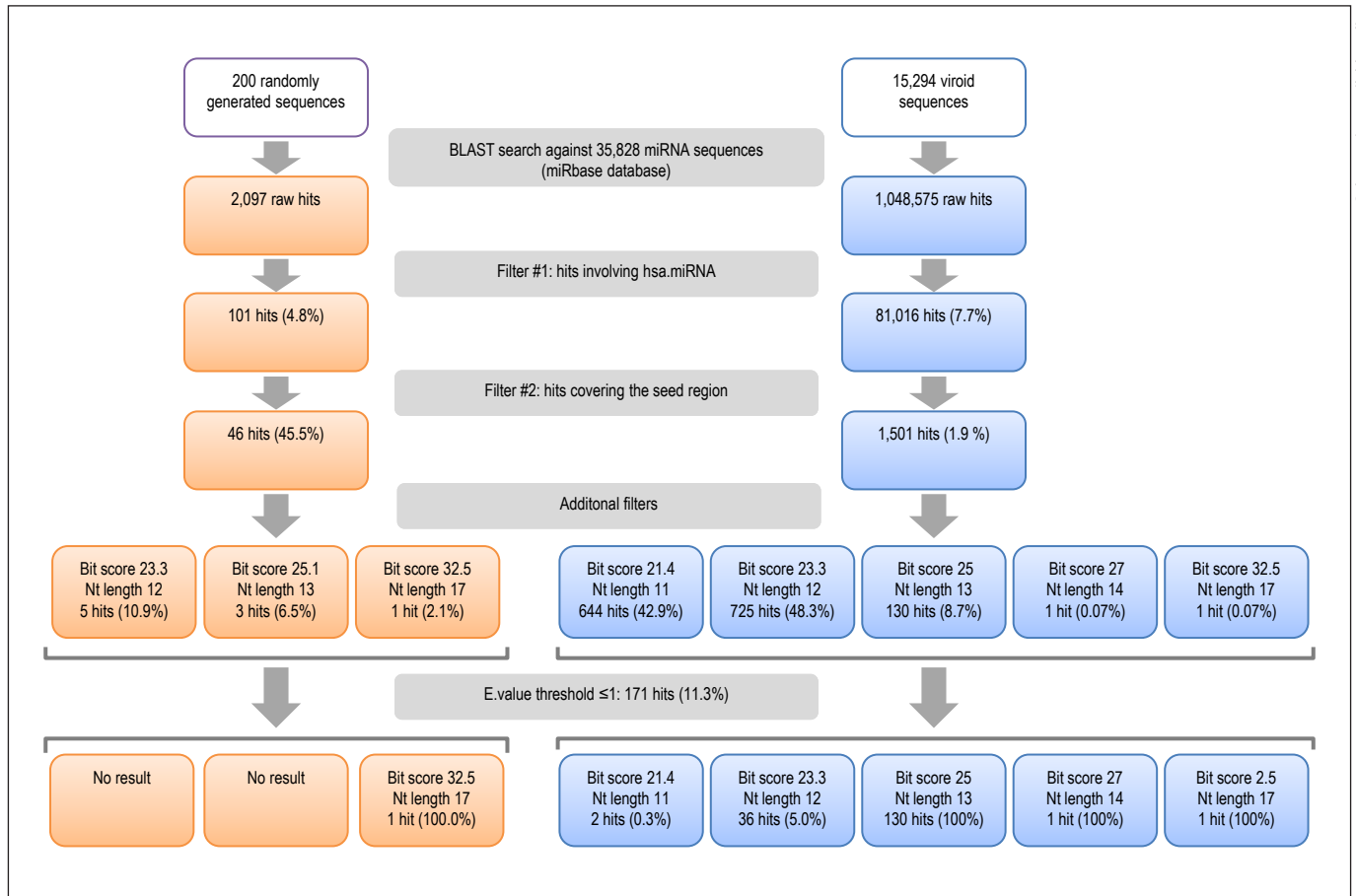
Viroids are minute unencapsidated non-coding circular RNAs known to be present and to cause diseases only in plants. Infections were associated with the occurrence of specific single-stranded RNAs similar in size to miRNAs and endogenous small interfering RNAs, and viroid pathogenicity is suspected to occur through RNA interference. We looked for sequence similarities between viroids and the seed region of human microRNAs (hsa-miRNAs). Viroid genomes were retrieved from GenBank and mature hsa-miRNAs were retrieved from miRBase. Two hundred 300-nucleotide-long sequences were randomly generated as controls. BLAST searches were performed using viroids as queries and hsa-miRNAs as subjects with relaxed parameters, and matches involving hsa-miRNA seed regions were considered. A total of 81,021 matches were found, and 1,501 that showed 100% identity with whole hsa-miRNA seed regions were selected. The most frequent matches involved *Chrysanthemum stunt viroid* or *Hop stunt viroid* species with hsa-miR-4286, in 365 and 207 cases, respectively. Three hsa-mi-

RNAs (miR-4286, miR-6808-5p, and miR-3622a-3p) were involved in 47% of all matches between viroids and hsa-miRNAs. Taken together, these findings warrant further investigation on the potential of viroids and their derived small RNAs to cross kingdoms and interact with nucleic acids in humans.

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## Introduction

Very few connections have been made between vegetal and animal pathogens, which are studied by distinct researchers [1]. Thus, viroids are very particular entities that are pathogenic for crop plants and have only been studied in plants [2, 3]. They are minute (246–401 nucleotides [nt] long) unencapsidated non-coding circular RNAs endowed with autonomous replication [3, 4]. They are highly stable due to high levels of intrasequence complementarities [5]. The mechanisms by which viroids cause diseases in plants are suspected to involve RNA interference, and RNA interference machineries from host cells [6, 7]. Several structural and functional similarities have been highlighted between viroids and pre-microRNAs (pre-miRNAs), which are hairpin-shaped stem-



**Fig. 1.** Flowchart of the BLAST searches and matches for miRNA against all generated random sequences and all the viroid sequences.

loop double-stranded RNA precursors of miRNA [8]. This is for instance the case between *Potato spindle tuber viroid* and human pre-miRNA-146a. In their mature form, miRNAs are non-coding regulatory sequences that are small, 18–25 nt in size, and interfere with host target genes in cells from plants, insects, nematodes, and vertebrates, including humans [9, 10]. A nucleotide motif located at miRNA positions 2–7 or 8, called the “seed region,” plays an active role in this RNA interference [11]. It is complementary to the 3′- or 5′-untranslated regions (UTR) that are conserved regions of host messenger RNAs (mRNAs) [10, 12]. It was shown that a perfect complementarity between this seed region and its target can induce RNA silencing in mammals [13]. Moreover, miRNA seed-matching regions have been described as being conserved in multiple mRNAs from mammals, including humans [10, 14]. Here, we sought similarities between viroids and the seed region of mature human (hsa, for *Homo sapiens*) miRNAs.

## Materials and Methods

We first retrieved all viroid genomes available from the NCBI GenBank nucleotide sequence database (<https://www.ncbi.nlm.nih.gov>) and all available mature miRNA sequences from the miRBase database ([www.mirbase.org](http://www.mirbase.org)) [15]. In addition, through the RAND-NA tool [16], we generated 200 random sequences with a size of 300 nt, similar to that of viroids, to use as controls. They consisted of 5 groups of 40 sequences with a G+C content of 40, 45, 50, 55, and 60%, chosen to cover the range of G+C-contents found in viroids. We performed BLAST searches using viroid sequences as queries and all mature miRNA sequences as subjects, with relaxed parameters including 4 as word size, 1,000 as e-value threshold, and no filter. The ten best BLAST results were thereafter filtered by keeping only viroids matching with mature hsa-miRNAs. The same protocol was used by handling the random sequences. BLAST results were sorted based primarily on a match involving the seed region of hsa-miRNAs (2–7 nt), as well as thresholds for the BLAST scores, the length of the sequence alignments, the percentage of identity between viroids and mature hsa-miRNAs, and the e-value (Fig. 1). Finally, information on hsa-miRNAs whose seed region matched with viroid RNAs according to our criteria were searched for through the miRBase database and the NCBI PubMed database (<https://www.ncbi.nlm.nih.gov/pubmed>).

**Table 1.** Top 31 most frequent matches (with a frequency  $\geq 5$ ) between viroids and seed regions of hsa-miRNAs

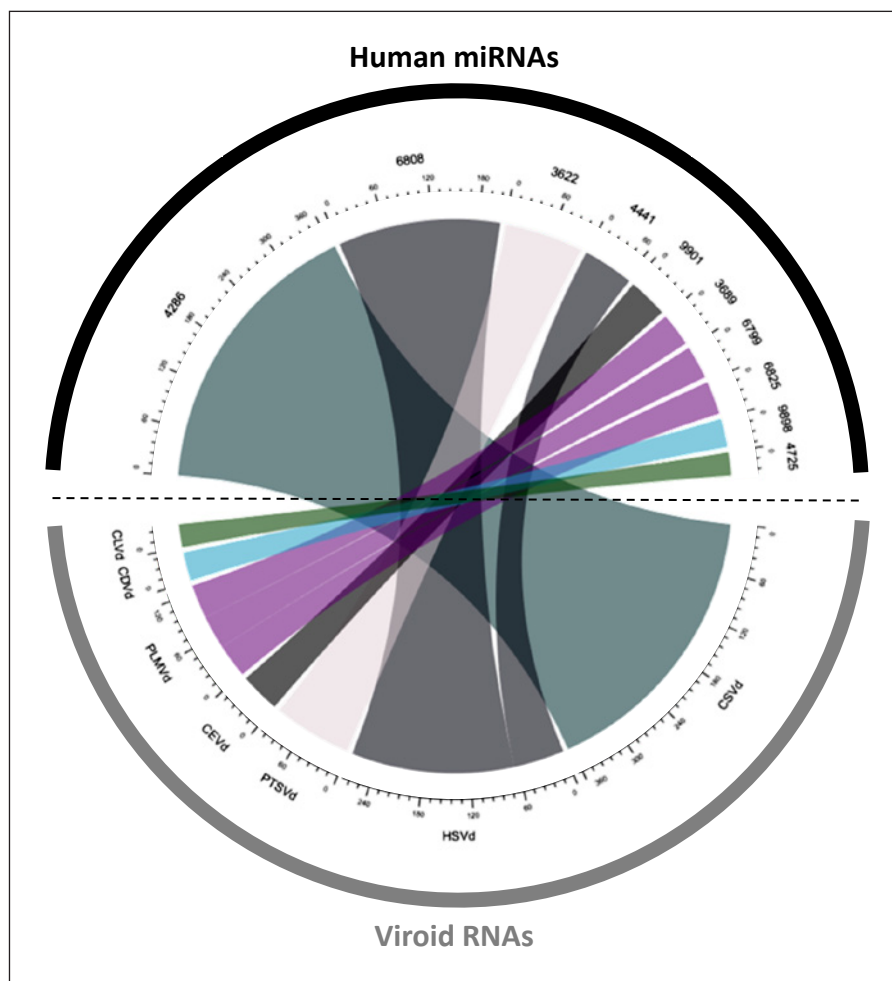
No.	Acronyms	Viroid species	hsa-miRNA	Frequency
1.	CSVd	<i>Chrysanthemum stunt viroid</i>	hsa-miR-4286	365
2.	HSVd	<i>Hop stunt viroid</i>	hsa-miR-6808-5p	207
3.	PTSVd	<i>Potato spindle tuber viroid</i>	hsa-miR-3622a-3p	102
4.	HSVd	<i>Hop stunt viroid</i>	hsa-miR-4441	65
5.	CEVd	<i>Citrus exocortis viroid</i>	hsa-miR-9901	51
6.	PLMVd	<i>Peach latent mosaic viroid</i>	hsa-miR-3689d	43
7.	PLMVd	<i>Peach latent mosaic viroid</i>	hsa-miR-6799-5p	43
8.	PLMVd	<i>Peach latent mosaic viroid</i>	hsa-miR-6825-5p	43
9.	CDVd	<i>Citrus dwarfing viroid</i>	hsa-miR-9898	37
10.	CLVd	<i>Columnea latent viroid</i>	hsa-miR-4725-5p	30
11.	HSVd	<i>Hop stunt viroid</i>	hsa-miR-1207-5p	26
12.	HSVd	<i>Hop stunt viroid</i>	hsa-miR-4756-5p	26
13.	PCFVd	<i>Pepper chat fruit viroid</i>	hsa-miR-4740-3p	26
14.	PCFVd	<i>Pepper chat fruit viroid</i>	hsa-miR-6836-3p	25
15.	ADFVd	<i>Apple dimple fruit viroid</i>	hsa-miR-9898	22
16.	CEVd	<i>Citrus exocortis viroid</i>	hsa-miR-8072	17
17.	CEVd	<i>Citrus exocortis viroid</i>	hsa-miR-4649-3p	16
18.	ASSVd	<i>Apple scar skin viroid</i>	hsa-miR-6775-3p	10
19.	GYSVd	<i>Grapevine yellow speckle viroid</i>	hsa-miR-1253	9
20.	GYSVd	<i>Grapevine yellow speckle viroid</i>	hsa-miR-6878-3p	9
21.	ELVd	<i>Eggplant latent viroid</i>	hsa-miR-514b-3p	8
22.	PSTVd	<i>Potato spindle tuber viroid</i>	hsa-miR-3074-5p	8
23.	CDVd	<i>Citrus dwarfing viroid</i>	hsa-miR-6758-5p	7
24.	CEVd	<i>Citrus exocortis viroid</i>	hsa-miR-6787-3p	7
25.	CBVd	<i>Coleus blumei viroid</i>	hsa-miR-191-5p	7
26.	MPVd	<i>Mexican papita viroid</i>	hsa-miR-3622a-3p	7
27.	PSTVd	<i>Potato spindle tuber viroid</i>	hsa-miR-4291	7
28.	PSTVd	<i>Potato spindle tuber viroid</i>	hsa-miR-4649-5p	7
29.	HSVd	<i>Hop stunt viroid</i>	hsa-miR-6876-5p	6
30.	TMCVd	<i>Tomato chlorotic dwarf viroid</i>	hsa-miR-3622a-3p	6
31.	PCFVd	<i>Pepper chat fruit viroid</i>	hsa-miR-3682-5p	5

ncbi.nlm.nih.gov/pubmed). Nucleotide diversity at viroid regions matching with mature hsa-miRNA seed regions was represented using the WebLogo online tool version 2.8.2 [17]. Statistical analyses were performed with Openepi software (v.3.03a; <http://www.openepi.com>). Proportions were compared using  $\chi^2$  or Fisher tests.  $p < 0.05$  was considered significant.

## Results

A total of 15,294 viroid sequences were used as queries for BLAST searches against a database of 35,828 mature miRNAs. This generated 1,048,575 hits (Fig. 1). Of them, 81,021 (7.7%) corresponded to matches between viroids and hsa-miRNA seed regions. After considering cases where viroid fragments were 100% similar to the whole hsa-miRNA seed region from its start (position 1 or 2), 1,501 hits (1.9%; 0.14% of the total number of hits) remained (online suppl. Table S1; for all online suppl.

material, see [www.karger.com/doi/10.1159/000509212](http://www.karger.com/doi/10.1159/000509212)). BLAST results were found to have an e value  $< 1$  in 170 (11.3%) cases. Among these 1,501 hits, 644, 725, 130, 1, and 1 had a nucleotide alignment length of 11, 12, 13, 14, and 17 nt, respectively. The longest match (17 nt) involved *Potato spindle tuber viroid* strain DI285387\_1\_KR\_1020130054489-A/33666 and hsa-miRNA-6774-5p, with a BLAST bit score of 32.5. The second longest match (14 nt) was between *Potato spindle tuber viroid* strain A/2147 and hsa-miRNA-3145-5p (BLAST bit score of 27). After removing duplicates, 25 different viroid species were involved in matches with the seed region of 212 different hsa-miRNAs (Table 1). A same procedure was applied to the 200 random sequences of 300 nt used as controls for specificity assessment. BLAST searches using these sequences against the miRNA database generated 2,097 hits. Out of them, 101 (4.8%) involved the whole seed region of several hsa-miRNAs with an identity of 100%. Among



**Fig. 2.** Chord diagram representing the frequency of matches between the 10 viroid species and hsa mature-miRNAs the most frequently involved in matches. Numbers on the inner circle indicate numbers of interactions between viroids and hsa-miRNAs.

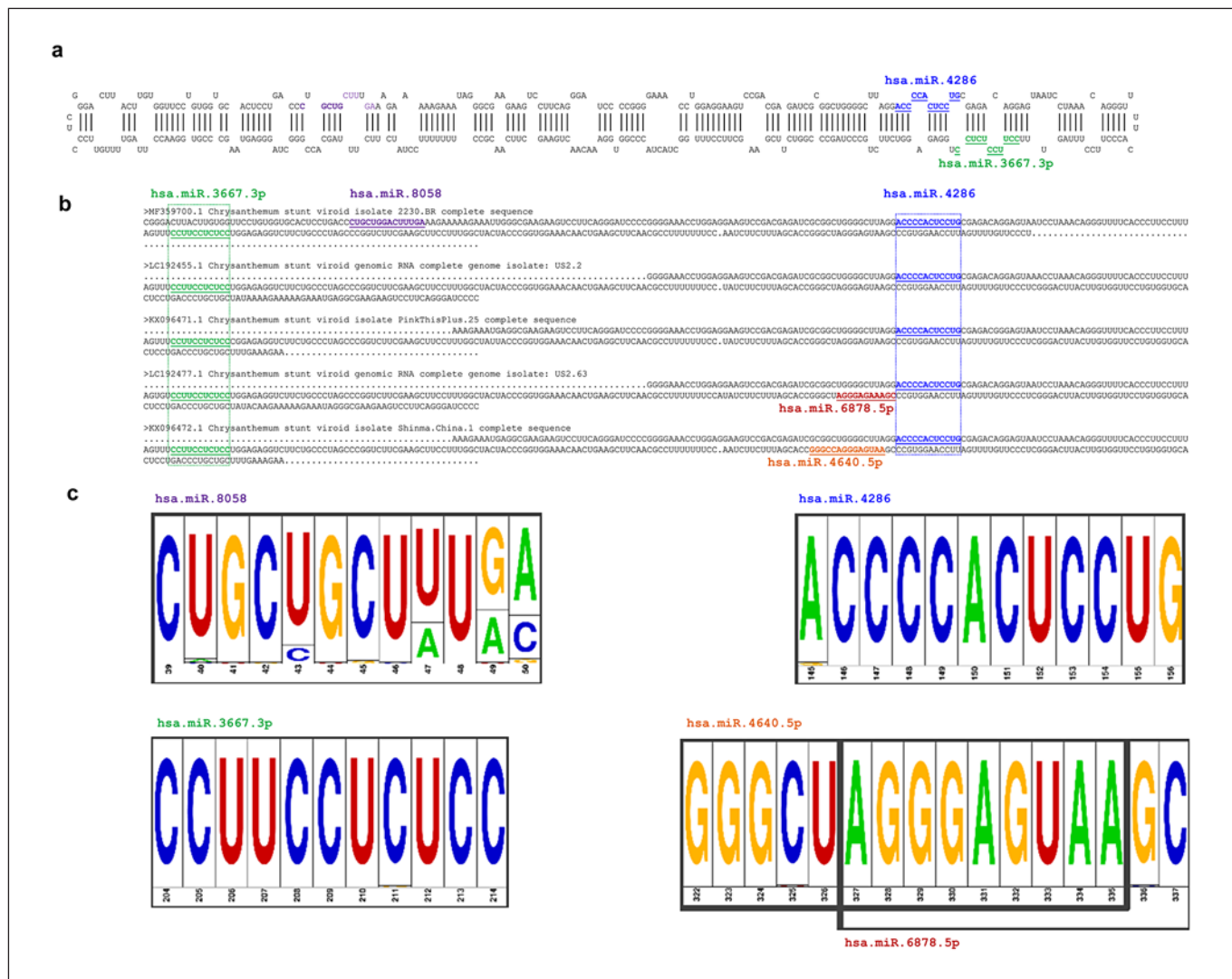
these 101 hits, 5, 3, and 1 had a nucleotide alignment length of 12, 13, and 17 nt, respectively. Hence, the proportion of these hits with an alignment length  $\geq 12$  nt was higher for viroids than for controls (57 vs. 20%;  $p < 1e-3$ ).

Overall, the most frequent matches involved *Chrysanthemum stunt viroid* or *Hop stunt viroid* species (online suppl. Fig. S1) with hsa-miR-4286, in 365 and 207 cases, respectively (Table 1). *Potato spindle tuber viroid* species matched 102 times with hsa-miR 3622. Nineteen of the 25 species of viroids for which fragments were found to match significantly with the seed region of hsa-miRNAs are known to infect fruits or vegetables that are common in human food, which suggests that humans can be exposed to them (online suppl. Table S2). The other viroids have been described to infect flowers, which may also allow contacts with humans. The 10 viroid species most frequently involved in the 1,501 significant matches with seed regions of hsa-miRNAs were implicated in almost all matches, and 5 of them – *Chrysanthemum stunt viroid*,

*Hop stunt viroid*, *Potato spindle tuber viroid*, *Peach latent mosaic viroid*, and *Citrus exocortis viroid* – represented 83% of all matches (online Suppl. Fig. S1). Moreover, matches between 7 different viroid species and 10 hsa-miRNAs represented 66% of all matches (Fig. 2).

Thirteen hsa-miRNAs were found to match with  $>1$  viroid species (online Suppl. Table S3). The 10 hsa-miRNAs whose seed regions most frequently matched with viroids were involved in 69% of all matches (online suppl. Fig. S2). Three hsa-miRNAs – miR-4286, miR-6808-5p, and miR-3622a-3p – were involved in almost half (47%) of all matches. MiR-4286 upregulation was reported in various types of malignancies, including pancreatic cancer, glioma, esophageal adenocarcinoma, and melanoma [18, 19]. MiR-6808 has been described as part of human splicing-derived miRNAs (“mirtrons”) and was notably found to target the DVL1 gene, the dysregulation of which is associated with the progression of numerous cancers [20, 21]. MiR-3622a-3p has been identified as significant-





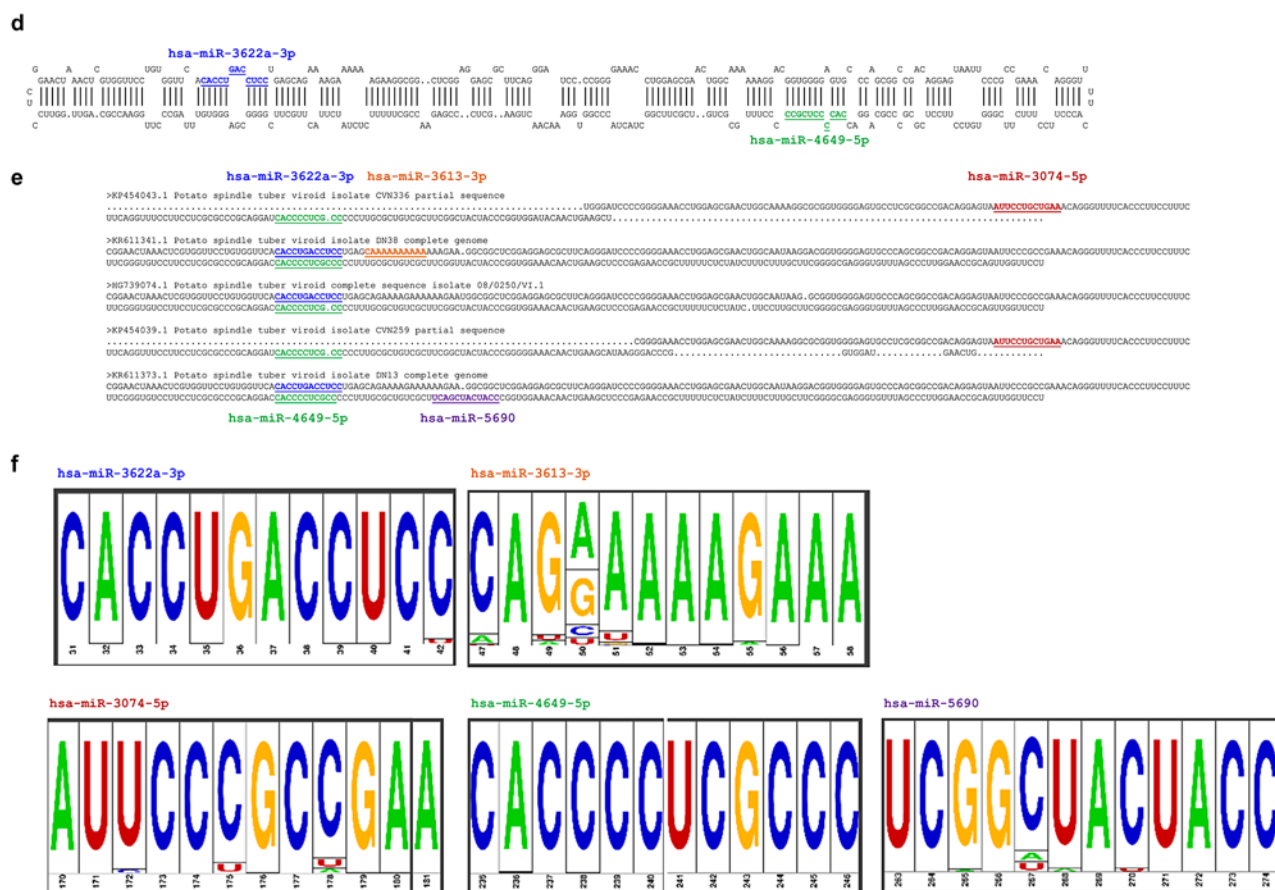
**Fig. 3.** Conservation in the genomes from different isolates of *Chrysanthemum stunt viroid* and *Potato spindle tuber viroid* species of the regions that matched with the seed regions of hsa-miRNAs. Conservation in the genomes from different isolates of *Chrysanthemum stunt viroid* and *Potato spindle tuber viroid* species of the regions that matched with the seed regions of hsa-miRNAs were represented on a viroid circularized genome (**a**, **d**), on alignments of 5 viroid sequences (**b**, **e**), and as sequence logos showing the consensus sequences corresponding only to viroid re-

gions matching hsa-miRNA seed regions (**c**, **f**). These sequence logos were built using the WebLogo online tool version 2.8.2 [17], with 91 *Chrysanthemum stunt viroid* sequences and 341 *Potato spindle tuber viroid* sequences that were downloaded from the NCBI GenBank nucleotide sequence database using viroid names and “complete” and “genome” as search terms, and that were directly alignable on their full length (see also online suppl. Fig. S3, S4 showing sequence logos for full-length viroids, and online suppl. Materials with viroid full-length sequence alignments).

(Figure continued on next page.)

ly expressed in patients suffering from bladder urothelial carcinoma and associated with longer survival times [22]. In addition, miR-4441, the fourth miRNA whose seed region most frequently matched with viroids, targets the human histone deacetylase 4 (HDAC4) gene that affects transcriptional regulation, cell cycle progression, and developmental events by altering or repressing access to tar-

get DNA promoters [23]. Finally, we analyzed the conservation of the regions that perfectly matched with the seed regions of hsa-miRNAs in the genomes of various viroid isolates from 2 species. We observed that these regions were either conserved or varied between viroid isolates in the genomes from *Chrysanthemum stunt viroid* and *Potato spindle tuber viroid* (Fig. 3).



## Discussion

Here, we describe for the first time 100% identity between several viroid fragments and the seed region that acts in the RNA interference process of various human miRNAs. Previously, cross-kingdom similarities were also noted between human miRNAs and plant virus genomes [12]. Virus-encoded microRNAs have been described or hypothesized to interact with host mRNAs [24, 25]. For instance, human cytomegalovirus-encoded-miR-UL112 was found to downregulate the major histocompatibility complex class I-related chain B gene expression during viral infection [24]. More recently, SARS-CoV-encoded small RNAs were suspected to contribute to the lung pathology associated with viral infection [26]. Viroids have stem-loop-like conformations as pri- and pre-miRNAs, the miRNA precursors [3, 5]. Viroids and pri- and pre-miRNAs are both RNA unencapsidated enti-

ties with non-coding properties that involve ribonuclease and dicer complexes to produce small RNA units [27, 28]. As a matter of fact, viroid infection was recently associated with the appearance of specific single-stranded RNA, the size of which is similar to that of miRNAs and endogenous small interfering RNAs [7, 29]. Thus, viroid-specific small RNAs comprised by 21–24 nt were detected by Northern blots in infected plants for *Potato spindle tuber viroid* [28, 30], *Citrus exocortis viroid* [31], and *Peach latent mosaic viroid* [32], while *Potato spindle tuber viroid* RNA was deemed to be opportunistic and suspected as capable of using different processing pathways in different hosts [33].

Viroids were not detected previously in humans but they could have been missed. Interestingly, although viroids were deemed to only replicate in higher plants, one of them (*Avocado sunblotch viroid*) was shown to be able to replicate in a cyanobacterium and in the yeast *Saccha-*

*romyces cerevisiae* [33–35]. In addition, exogenous small RNAs such as miRNAs were shown to be capable of spreading between cells [36]. Moreover, it was demonstrated that the miRNA-168a from rice was present in the serum samples of Chinese individuals whose main diet was based on this plant, could bind to the low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, and was able to downregulate its expression in hepatocytes [37].

Taken together, these findings question the potential of viroids and their derived small RNAs to cross kingdoms to interact with nucleic acids in humans. Viroids are prevalent in plants worldwide and could be ingested through consumption of fruits and vegetables. It can also be hypothesized that miRNA-like RNAs derived from these viroids can be either transferred from plants to humans or processed from viroids in human cells, and they could theoretically interact with human mRNAs. Further investigation is warranted to search for the presence of viroids in humans and test for their effect on mammalian cells in vitro.

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## Statement of Ethics

Not applicable: only bioinformatic analyses on sequences already available in databases were performed.

## Disclosure Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

Conceptualization: P.C. Methodology: P.C., J.G.B.-A., N.C. Analysis: J.G.B.-A., P.C., N.C., E.V. Writing – original draft preparation: J.G.B.-A., P.C. Writing – review and editing: P.C., J.G.B.-A., E.V.



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