Viroid-derived small RNA induces early flowering in tomato plants by RNA silencing

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RESEARCHPAPER

Alterations of the viroid regions that interact with the host defense genes attenuate viroid infection in host plant

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ABSTRACT

Understanding in intimate details how the viroid interaction with host's defense genes is a cornerstone for developing viroid resistant plants. In this present study, small RNAs (sRNA) derived from *Potato spindle tuber viroid* (PSTVd) were studied *in silico* in order to detect any interactions with the serine threonine kinase receptor, a transmembrane protein that plays a role in disease resistance in plants. Using molecular biology techniques, it was determined that PSTVd infection negatively affects at least three serine threonine kinase receptors as well as with three other genes that are known to be involved in the overall development of the tomato plants. The transient expression of these putative PSTVd-sRNAs, using the microRNA sequence as a backbone, in tomato plants induced phenotypes similar to viroid infection. Mutants created by altering the sequence of PSTVd in these regions failed to infect the tomato plant. The data presented here illustrates the importance of these regions in viroid survival, and suggests a possible avenue of exploration for the development of viroid resistant plants.

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KEYWORDS
host defense genes; mutants; PSTVd; serine threonine kinase receptor; vd-sRNA

Introduction

Viroids are small (246 to 401 nucleotides, nt), single-stranded, non-coding RNA molecules which exclusively infect and cause disease in several economically important plants [1]. More than 32 viroid species have been characterized to date and they have been classified into two families: the *Avsunviroidae* and the *Pospiviroidae* [2]. Since viroids do not encode for any proteins, they rely entirely on their sequence and on host factors for their replication. That said, upon infection they induce a wide array of symptoms in host plants, including stunting, leaf epinasty, leaf distortion, flower distortion and a reduced number of flowers [3–5]. Due to their highly base-paired structures and RNA-RNA mode of replication, viroids are both inducers and targets of RNA silencing [6]. Indeed, 21- and 24-nt viroid derived small RNAs (vd-sRNA) have often been recovered from viroid infected plants, supporting the hypothesis that viroid infection triggers RNA silencing [7,8]. Since viroids are non-coding RNAs, one possibility is that vd-sRNAs might play a role in symptom induction. The RNA inteference (RNAi)-mediated down-regulation of the host's mRNAs by the interaction with vd-sRNA has been discussed by several groups using different host-viroid combinations [9–14]. More recently, transcriptome analyses of PSTVd infected tomato plants have revealed the genome-wide changes in the host plant. This suggests the possible involvement of either secondary small RNAs (sec-siRNA), or phased secondary small RNAs (phasiRNAs), in the effects observed during the PSTVd infection in tomato plants [15,16].

Plants, unlike their animal counterparts, lack antibodies and specific cells that defend against pathogen invasion. That said, they have developed a wide array of strategies in order to detect and adapt to environmental cues by the use of a wide range of receptors within and at their cell surface [17]. Proteins involved in plant development or defense fall mainly into two broad groups: the nucleotide-binding site leucine-rich repeat (NBS-LRR) class and the receptors such as receptor serine threonine kinases (RSTK) [18]. The RSTK is a transmembrane protein that interacts with other protein classes in order to provide both an efficient disease resistance and developmental regulation [19,20]. Nevertheless, how exactly a highly structured, non-coding RNA affects both the overall development and the defense of the plant in order to ensure its accumulation remains elusive.

Although an RNA silencing-based strategy presents a very attractive method with which to create viroid-resistant/tolerant plants, transgenic tomato and *N. benthamiana* plants expressing PSTVd-specific small RNAs (PSTVd-sRNA) failed to resist PSTVd infection [21,22]. Hence, an understanding of the viroids host defense genes are of the utmost importance in the development of alternative viroid resistant strategies. In order to address how viroids affect overall plant development and defense genes expression, and to determine the importance of the regions of the viroid that are predicted to target these genes the possible targets of vd-sRNA on the genes involved in both the tomato plant's development and in its defense were

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analyzed by employing various molecular biology and cellular biology approaches. Using three variants of PSTVd for the bioassays, the results presented here indicate that the variants of PSTVd differentially affect both the RSTK genes and other host developmental genes, and that alterations in the portion of viroid that is predicted to bind to the host's defense genes adversely affects viroid accumulation.

Results

vd-sRNAs of PSTVd variants predicted to target both the serine threonine protein kinase mRNA and other genes involved in development of tomato plants

As viroids are highly structured and non-coding pathogens, it is critical for them to maintain their structure intact in order to protect themselves from the host's defense system and defense mechanisms. It is now well established that viroid infection triggers host RNA silencing and induce disease symptoms through vd-sRNA [9–13]. In the context of investigating the possible interaction between vd-sRNA and host defense genes such as NBS-LRR and RSTK, we investigated publicly available tomato transcriptome datasets using the WMD3 Web-based tool (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) against sRNAs derived from three PSTVd variants (PSTVd-mild [PSTVd-M], PSTVd-Intermediate [PSTVd-I] and PSTVd-RG1) that are known to induce different symptoms in tomato cv. Rutgers upon infection [23–25]. The free energy for each PSTVd-sRNA:target duplex was calculated using PairFold [26]. Interestingly, at least two vd-sRNAs derived from the plus (+) and one derived from the minus (−) strands of the PSTVd variants were predicted to target the RSTK gene, among other targets (Fig. 1A). Specifically, for the (+) strand, a vd-sRNA derived from the pathogenicity (P) domain of all 3 of the PSTVd variants (i.e. positions 45 to 65; vd-s:+45) was predicted to target the putative LRR receptor-like serine/threonine-protein kinase At2g24230-like (LRR-RSTPK; GenBank Acc. No. XM_004242540), while the vd-sRNA derived from the variable (V) domains of PSTVd-I and PSTVd-RG1 (i.e. positions 121 to 141; vd-s:+121) was predicted to target the serine/threonine-protein kinase gene (STPK; GenBank Acc. No. XM_004229847). Strikingly, the vd-sRNA derived from the Terminal Left (TL) domain of (−) strands of all 3 PSTVd variants (i.e. positions 353 to −333; vd-s:-353) was predicted to target the putative receptor-like serine/threonine-protein kinase At5g57670 (RSTPK; GenBank Acc. No. XM_004230991). This kinase is a transmembrane protein, which is involved in various pathways implicated in plant development as well as in disease resistance [18].

Interestingly, both vd-s:+45 and vd-s:-353 were found to target other genes which are important in the plant's growth and development. Specifically, vd-s:+45 was predicted to target the *Phosphatidylinositol 4-kinase alpha* mRNA (PI4KA; GenBank Acc. No. XM_004250677). PI4KA plays an important role during the production of inositol-1,4,5,-trisphosphate [27,28]. Similarly vd-s:-353 was predicted to target the *Vacuole membrane protein 1* (VMP1; GenBank Acc. No. XM_004247320) as well as the pentatricopeptide repeat-containing protein At2g17033 (PPR; GenBank Acc. No. XR_743486). VMP1 is a putative transmembrane protein that has been associated with different functions including autophagy, cell adhesion and membrane trafficking [29]. The PPRs are prevalent in plants, where they have specific roles in the post-transcriptional regulation of organelle gene expression [30].

To understand the effect of predicted target mRNAs on host morphology, the target genes were knocked-down by virus induced gene silencing (VIGS). To achieve this, the predicted target genes were amplified from the tomato cv. Rutgers using gene specific primers and were then expressed in the pTRV2 vector under the control of the 35S promoter [31]. The resulting binary vectors were named pTRV2:LRR-RSTPK (targets LRR-RSTPK-mRNA), pTRV2:PI4KA (targets PI4KA-mRNA), pTRV2:STPK (targets STPK-mRNA), pTRV2:RSTPK (targets RSTPK-mRNA), pTRV2:VMP1 (targets VMP1-mRNA) and pTRV2:PPR (targets PPR-mRNA). The binary vector constructs were then transformed into the *Agrobacterium tumefaciens* strain GV3101, and were then used for agroinfiltration into tomato seedlings as previously described [31]. pTRV2:PDS, which is capable of silencing the *Phytoene desaturase gene* (PDS), was expressed in tomato plants as a positive control, while a negative control was obtained by agro-infiltrating the plants with empty pTRV2 vectors. At approximately 3-weeks post infiltration (wpi), all silenced plants exhibited morphological abnormalities as compared to control plants (Fig. 1B). The pTRV2:STPK silenced plants were pale and developed a fewer leaves, while the plants agro-infiltrated with either pTRV2:LRR-RSTPK and pTRV2:RSTPK had yellowish leaves. The knockdown of PI4KA resulted in the puckering of leaves and branches. VMP1 silenced plants showed necrotic spots on their leaves and stems along with leaf curling. The PPR knockdown plants exhibited a severe phenotype which included severe leaf curling, necrosis and drooped branches. These plants died at approximately 6-wpi illustrating the importance of the PPR in the plant's life cycle as the products derived from the PPR are involved in the post-transcriptional regulation of organelle gene expression [30]. Taken together, the data presented here demonstrates the importance of these genes in the overall development of tomato plants.

Accumulation of PSTVd variants in tomato plants and analysis of PSTVd-sRNA

To investigate both the symptom induction and the accumulation of the viroid variants, time course studies were performed by inoculating the tomato plants cv. Rutgers with the PSTVd-M, PSTVd-I and PSTVd-RG1 variants. The plants inoculated with PSTVd-I and PSTVd-RG1 exhibited severe leaf curling and stunting at 2-wpi, while the PSTVd-M inoculated plants showed mild stunting and leaf curling (Fig. 2A). To monitor the accumulation levels of the viroid variants after infection, leaf samples were collected at 1 to 6-wpi and total RNA was extracted and reverse transcription–quantitative polymerase chain reactions (RT-qPCR) were performed in order to determine the PSTVd titers, as described previously (Fig. 2B) [32]. All the viroid inoculated plants exhibited viroid accumulation, while the mock-inoculated plants did not. The PSTVd-RG1 inoculated plants exhibited the highest level of viroid accumulation up to 4-wpi, while the PSTVd-M inoculated plants

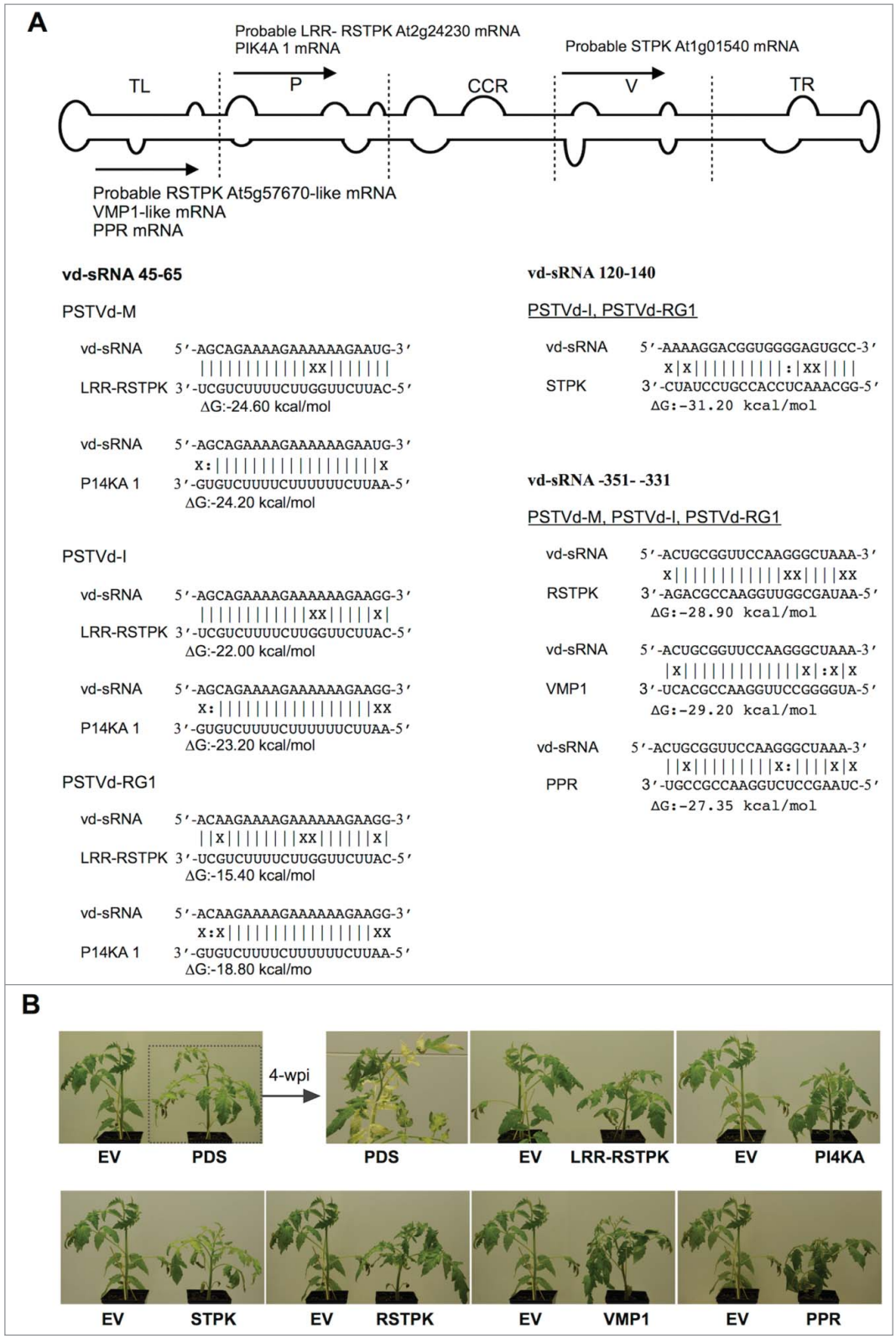

Figure 1. Schematic representation of the regions of PSTVd predicted to target the STPK genes of tomato plants. (A) Schematic view of the PSTVd structure showing the five structural/functional domains. The vd-sRNA derived from the P domain of genomic (+) strand of PSTVd were predicted to target the putative LRR- RSTPK At2g24230 and the PIK4A 1 mRNAs. The vd-sRNA derived from the V domain of the (+) strand were predicted to target the putative STPK At1g01540 mRNA. The vd-sRNA derived from the (−) strand of PSTVd was predicted to target three host genes: the putative RSTPK At5g57670-like, the VMP1-like and, the PPR mRNAs. The predicted interactions between the sRNAs derived from the PSTVd-M, PSTVd-I and PSTVd-RG1 variants with the host genes are shown. The sequences are shown in the complementary polarity. The minimum free energy (ΔG) obtained, based on secondary structures of the pairs of RNA sequences using the PairFold online tool, is shown below each vd-sRNA/target duplex. (B) The tomato plants were subjected to a knock-down assay using a VIGS technique to verify the role of the target host genes on the plant's morphology. At 3-wpi the plants exhibited phenotypic alterations similar to viroid infection. EV, TRV2 empty vector infiltrated plants; PDS, plants infiltrated with pTRV2:PDS (the knock-down of PDS is more clear at 4-wpi); LRR-RSTPK, the plant infiltrated with pTRV2:LRR-RSTPK; PI4KA, the plant infiltrated with pTRV2: PI4KA; STPK, the plant infiltrated with pTRV2: STPK; RSTPK, the plant infiltrated with pTRV2:RSTPK; VMP1, the plant infiltrated with pTRV2:VMP1; and, PPR, the plant infiltrated with pTRV2:PPR.

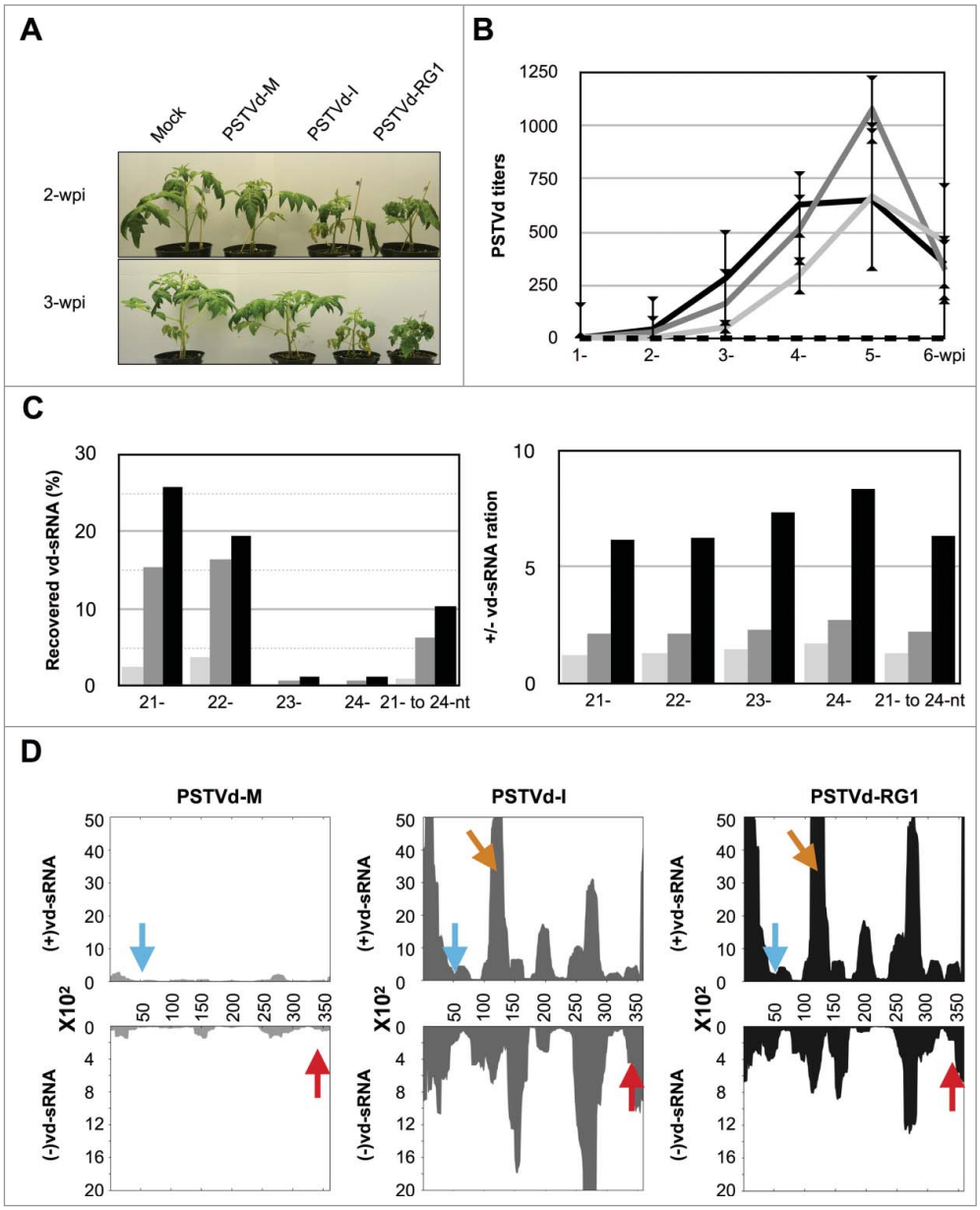

Figure 2. PSTVd variants accumulate differentially in tomato cv. Rutgers. (A) The PSTVd variants, PSTVd-M, PSTVd-I and PSTVd-RG1 were inoculated onto tomato plants cv. Rutgers. At 2-wpi, the plants inoculated with PSTVd-I and PSTVd-RG1 showed disease symptoms, while the plants inoculated with the PSTVd-M variant showed mild stunting; as compared to mock inoculated plants. (B) Total RNA extracted from tomato plants at 1 to 6-wpi were used to monitor the PSTVd titer. The dotted black line (on the X-axis) represents the mock inoculated plants, while the light grey, grey and black solid lines indicate the titers of the PSTVd-M, PSTVd-I and PSTVd-RG1 inoculated plants, respectively. The expression change is presented on a log2 scale. Each experiment was performed at least three times with true biological replicates. The error bars indicate the standard deviation (SD). (C) The vd-sRNA recovered from plants inoculated with PSTVd-M, PSTVd-I and PSTVd-RG1 were used to check the size distribution of the PSTVd-sRNAs. The horizontal axis indicates the lengths of the sRNA, and the vertical axis indicates the total number of sRNA reads expressed as a percentage (left panel). Both the (+) and (−) derived PSTVd-sRNAs were filtered and were used to verify the (+)/(−) expression ratio. The light grey bars indicate PSTVd-M, the gray bars PSTVd-I and the black bars indicate PSTVd-RG1 (right panel). (D) vd-sRNA recovered from the leaf tissues of tomato plants infected with PSTVd-M, PSTVd-I and PSTVd-RG1 were profiled on their respective (+) and (−) strands. The vertical arrows denote the three vd-sRNA populations of particular interest, namely, vd-sRNA: +45 (blue color), vd-sRNA: +120 (gold color) and vd-sRNA: -351 (red color).

showed the least amount of viroid accumulation in this time period. Interestingly, in all the cases, after the initial increase in viroid accumulation, a sudden decrease was observed. For instance, the titer of PSTVd-RG1 increased rapidly up to 4-wpi, but was then followed by a stationary phase (Fig. 2B, from 4- to 5-wpi) and a sudden decrease in viroid titer.

To validate the production of small RNA (sRNA) by the PSTVd variants upon infection, total RNA samples from the leaves collected at 3-wpi were subjected to sRNA purification followed by deep-sequencing. The obtained sRNA population of 21- to 24-nt are summarized in Table 1. The recovered vd-sRNAs identified as being from the PSTVd-M, PSTVd-I and PSTVd-RG1 inoculated plants were analyzed for their size distribution on both the (+) and (−) strands of the respective PSTVd variants. As shown in figure 2C, comparison of the vd-sRNA populations between the PSTVd variants showed a higher accumulation of vd-sRNA in PSTVd-RG1 inoculated plants, followed by the PSTVd-I inoculated plants. The least

Table 1. Summary of sRNAs identified by high-throughput sequencing.

sRNA length	Mock				PSTVd-M				PSTVd-I				PSTVd-RG1			
	sRNA	PSTVd-M sRNA	PSTVd-I sRNA	PSTVd-RG1 sRNA	sRNA	vd-sRNA	(+) sRNA	(−) sRNA	sRNA	vd-sRNA	(+) sRNA	(−) sRNA	sRNA	vd-sRNA	(+) sRNA	(−) sRNA
21-nt	93911	6/0	9/2	9/2	65734	1608	873	735	211338	32391	22008	10383	160262	41321	35533	5788
22-nt	105058	8/0	14/3	14/3	71187	2639	1501	1138	218483	35988	24650	11338	145487	28441	24528	3913
23-nt	133481	0/0	1/0	1/0	129392	167	99	68	259935	2176	1511	665	124625	1645	1448	197
24-nt	235353	0/0	2/0	2/0	209639	227	144	83	511330	4193	3057	1136	300125	3509	3135	374
Total	567803	14/2	26/5	26/5	475952	4641	2617	2024	1201086	74748	51226	23522	730499	74916	64644	10272

[a](+) sRNA/(−) sRNA.

vd-sRNA was recovered from the PSTVd-M-inoculated plants. The differences in the amounts of recovered vd-sRNA in the PSTVd inoculated plants are presumably related to the total accumulation of the PSTVd variant. Interestingly, PSTVd-RG1 produced more 21-nt long sRNA than 22-nt long sRNAs, while both PSTVd-I and PSTVd-M showed higher amounts of 22-nt long sRNAs than 21-nt long sRNAs. Detailed analysis of the (+) and (−) strand vd-sRNAs revealed that PSTVd-M produced a slightly higher amount of (+) vd-sRNA than (−) vd-sRNA. The greatest difference in the accumulation of the (+) and (−) vd-sRNA was observed for the PSTVd-RG1 variant. Specifically, it produced more than 86% of (+) strand derived sRNA, followed by PSTVd-I, which had 68% to 73% of (+) strand derived sRNA. However, all of the PSTVd inoculated plants, more (+) strand-derived sRNAs were recovered than sRNAs corresponding to the (−) strand. These results are in agree with previous results that showed both a lower accumulation of PSTVd and a lower recovery of vd-sRNA in PSTVd-M inoculated plants as compared to PSTVd-I inoculated plants [11]. This is presumably due to the higher accumulation of the (+) strand of viroid in the infected plants [11,33]. Furthermore, it was observed that the accumulation ratios of the (+) over the (−) strand sRNAs varied greatly with the PSTVd-variants (Fig. 2D). PSTVd-M inoculated plants had a (+)/(−) ratio of 1.3 for 21- to 24-nt long vd-sRNA. Similarly, both PSTVd-I and PSTVd-RG1 inoculated plants showed (+)/(−) ratios of 2.2 and 6.3, respectively. Irrespective of the PSTVd variant, the lowest (+)/(−) ratio was detected for 21-nt long vd-sRNA and the highest was for 24-nt long sRNA species. All the 21- to 24-nt long vd-sRNA populations were profiled on both the (+) and the (−) strands of the respective PSTVd variants (Fig. 2D). The data presented here demonstrate that infection with the PSTVd variants does indeed result in the production of vd-sRNAs from the strands of both polarities of the viroid variant, and that the level of vd-sRNA depends on the accumulation level of the viroid.

PSTVd infection down-regulates target genes

To determine the effect of the different PSTVd variants on the expression of genes involved in disease resistance and plant development at different time intervals (up to 3-wpi), RT-qPCR analyses were performed on the same RNA samples used to quantify viroid accumulation using gene specific primers. The expression levels were normalized to those of the mock-inoculated plants. For almost all of the putative target genes of vd-sRNA, the PSTVd inoculated plants showed the greatest degree of repression at 3-wpi (Fig. 3). It is very interesting to note that except for PI4KA, all the plants inoculated with PSTVd-RG1 revealed the highest level of down-regulation, followed by PSTVd-I, while the plants inoculated with PSTVd-M showed the least amount of down-regulation.

To verify the correlation between the level of gene repression and the vd-sRNA counts, the vd-sRNA for each PSTVd variant directed against the genes of interest mRNA were analyzed in PSTVd-sRNA libraries (Table 2). The data revealed that PSTVd-RG1 had the highest number of sRNAs, followed by PSTVd-I and the PSTVd-M. This may be attributed to that fact that higher amounts of vd-sRNAs are recovered from PSTVd-RG1 inoculated plants than in the other PSTVd variant inoculated plants. The data presented here clearly demonstrates that all the variants of PSTVd are capable of negatively affecting the putative target transcripts, and all have the ability of producing vd-sRNAs against such target mRNAs.

PSTVd-sRNA modulates plant phenotype

To demonstrate the effect of the predicted PSTVd-sRNAs on the host's phenotype, a vd-sRNA delivery system for tomato plants was developed by modifying the MIR VIGS strategy [34]. As all three of the PSTVd variants produced essentially the same vd-sRNA that are predicted to target the RSTK, the vd-sRNA sequence of the PSTVd-I variant was used to design the artificial microRNA (amiRNA); namely, amiR:I(+)45 (5′-AGCAGAAAAGAAAAAAGAAUG-3′), amiR:I(+)120 (5′-AAAAGGACGGUGGGGAGUGCC-3′) and amiR:I(−)351 (5′-ACUGCGGUUCCAAGGGCUAAA-3′). Oligonucleotides corresponding to the sequences of the vd-sRNAs were used to create, by PCR amplification, amiRNA constructs using the osa-MIR528 sequence as a backbone [15]. The resulting amiRNAs were expressed in the pCV-A vector under the control of the 35S promoter [34]. The resulting binary vectors were named pCVA-amiR:I(+)45 (targets the *LRR-RSTPK* and *PI4KA* mRNAs), pCVA-amiR:I(+)120 (targets the *STPK* mRNA) and pCVA-amiR:I(−)351 (targets the *RSTPK*, *VMP1* and *PPR* mRNAs). These vectors, upon agro-infiltration, produced vd-sRNA similar to that seen viroid during infection. This modified technique was named vd-sRNA:VIGS. If the vd-sRNA produced by the amiRNA binds a target which is involved in the host's phenotype, the plant will show phenotypic variation as binding blocks the translational processing and/or the stability of the target mRNA (Figure 4A). To demonstrate the feasibility of this technique, plants were agroinfiltrated with either empty vector, or with an amiRNA targeting PDS (pCVA-amiR:PDS) [34]. The tomato plants were then co-infiltrated with *A. tumefaciens* strain GV3101 mixtures containing pCVA-amiR and pCVB. At approximately 5-wpi, the plants agro-inoculated with pCVA:amiR-PDS and were observed to develop a typical photobleached phenotype. The

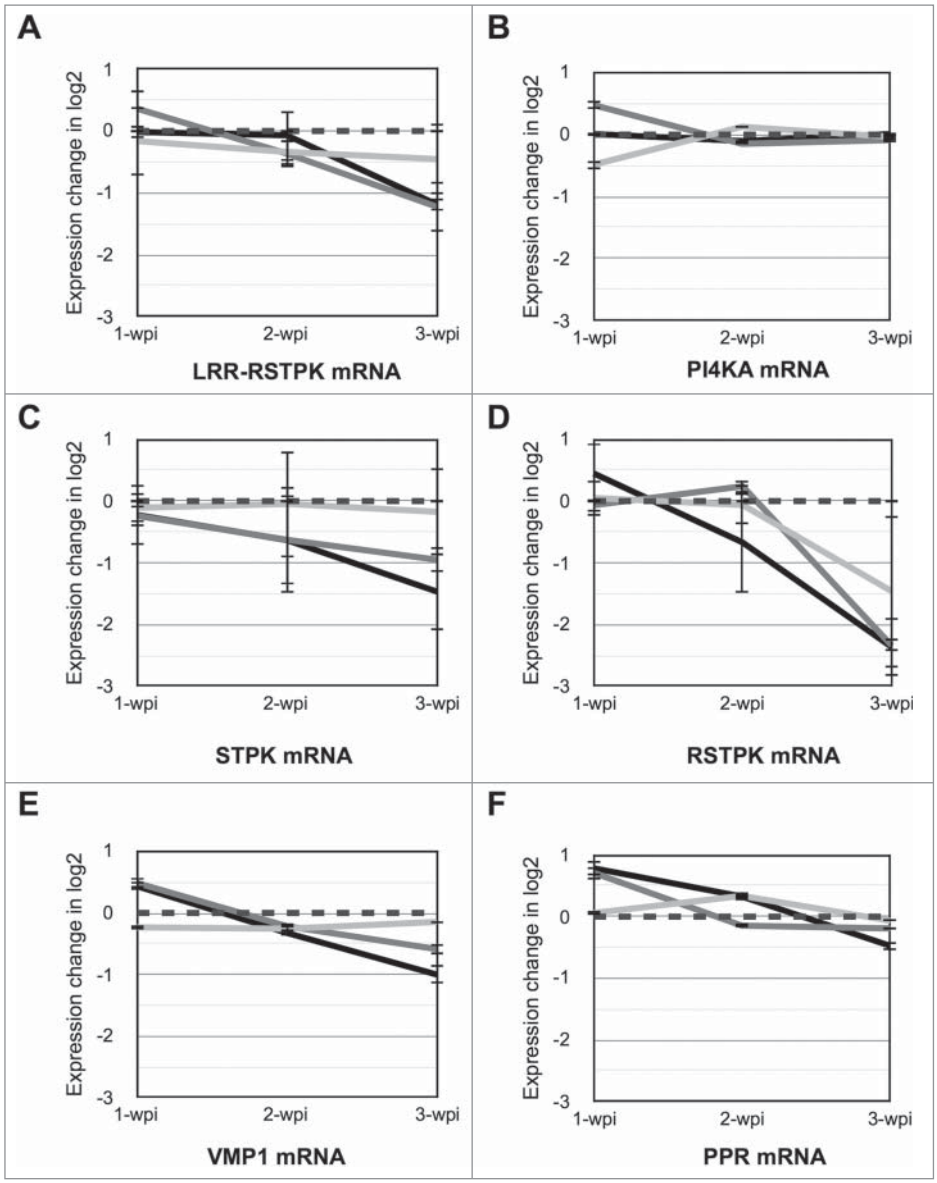

Figure 3. PSTVd infection negatively affects the genes involved in host defense and development Total RNAs extracted from tomato plants at 1-, 2- and 3-wpi were used to monitor the effect of PSTVd variants on the expression of the (A) LRR-RSTPK, (B) PI4KA, (C) STPK, (D) RSTPK, (E) VMP1 and (F) PPR. The expression change is presented on a log2 scale. Each experiment was performed at least three times with true biological replicates. The error bars indicate SD. An enlarged view of the expression of the PI4KA mRNA is presented in Fig. S1.

plants inoculated with vd-sRNA:VIGS showed phenotypes typical of viroid infection. All of the vd-sRNA:VIGS plants exhibited a decrease in development as compared to the control plants (Fig. 4B, plants inoculated with empty vector). The total RNA extracted from vd-sRNA:VIGS plants at 4-wpi were subjected to RNA gel blot hybridizations using gene-specific riboprobes in order to demonstrate the effect of the vd-sRNA on the target mRNAs (Fig. 4C–H; Fig. S2). As compared to the control plants, all of the vd-sRNA:VIGS treated plants showed the repression of their respective target mRNAs. Taken together, these data support the idea of a direct role of the vd-sRNA in viroid pathogenicity. The most plausible explanation for this phenomenon is that the down-regulation of the target mRNA induces the morphological changes seen in the host plants.

Table 2. Population of vd-sRNA detected in three variants of PSTVd that are predicted to target putative mRNAs.

Vd-sRNA	PSTVd-M	PSTVd-I	PSTVd-RG1	Target gene
vd-sRNA: +45	4	16	32	LRR-RSTPK PI4KA
vd-sRNA: +120	0	1191	895	STPK
vd-sRNA: -331	8	96	43	RSTPK VMP1 PPR

Disruption of the putative vd-sRNA proved lethal to PSTVd

To investigate the importance of the regions of PSTVd whose vd-sRNAs are predicted to form duplexes with the host defense and developmental genes, mutants were created by altering the sequence of the PSTVd. To achieve this task, the specific objectives were the disruption of the predicted vd-

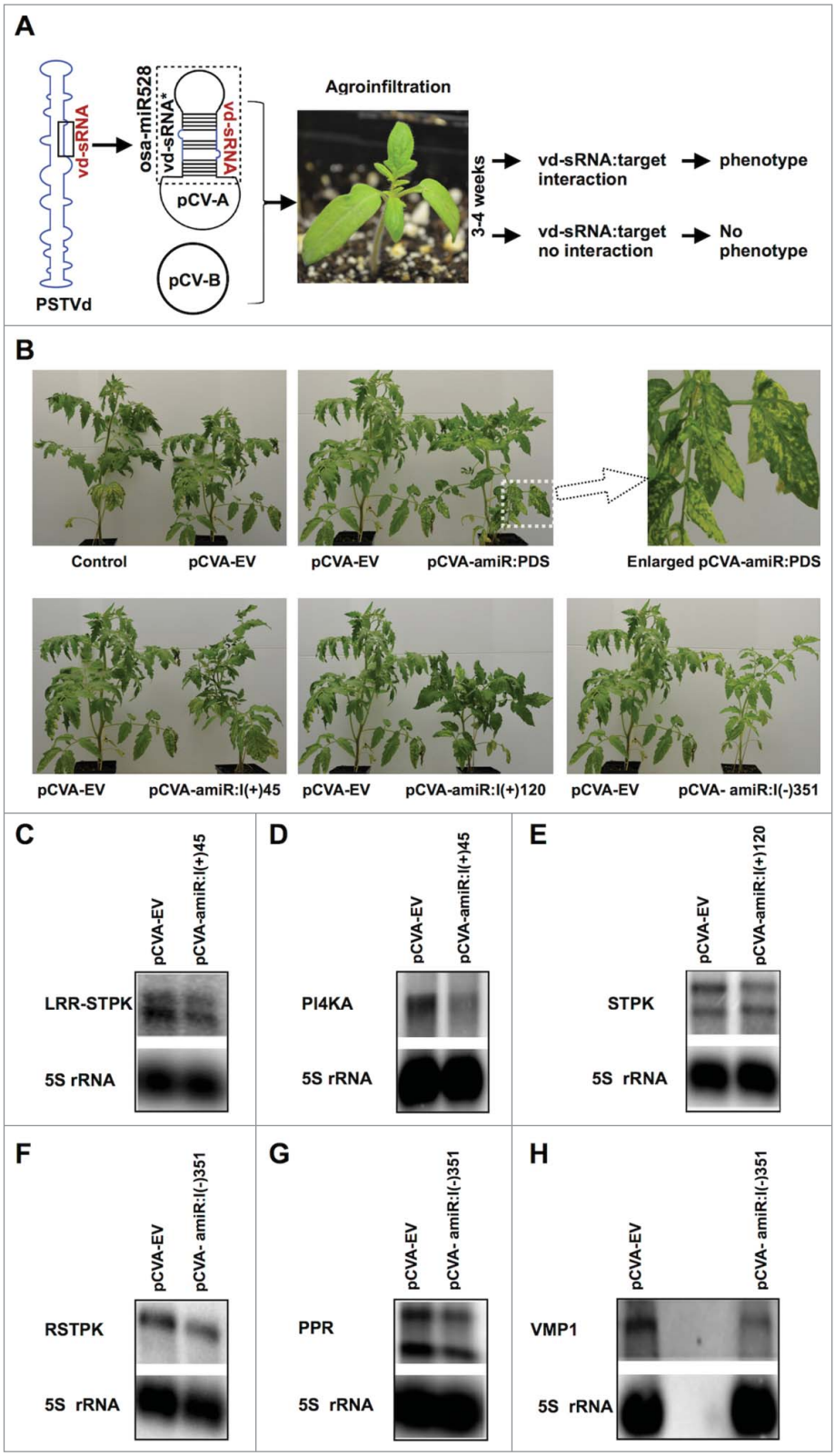

Figure 4. Transient expression of vd-sRNA induces phenotypes in tomato cv. Rutgers. (A) Flow chart illustrating the details of the vd-sRNA:VIGS experiment. The vd-sRNA expressed as an amiRNA in the vector pCVA was agroinfiltrated along with the vector pCVB, onto tomato plants at the cotyledon stage. The specific interaction of miRNA (vd-sRNA) with the target sequence leads to either RISC-mediated cleavage or translational repression, which in turn results in the phenotype of the plant. (B) The tomato plants were subjected to vd-sRNA:VIGS assay in order to evaluate the effect of the vd-sRNA on the plant's morphology. At 5-wpi the plants exhibited phenotypic alterations similar to those seen in viroid infection. pCVA-EV, pCVA empty vector infiltrated plants; pCVA-amiR:PDS, plants infiltrated with pCVA-amiR:PDS; pCVA-amiR: I(+)45, the plant infiltrated with pCVA-amiR: I(+)45; pCVA-amiR:I(+)120, the plant infiltrated with pCVA-amiR:I(+)120; and, pCVA-amiR:I(−)351, the plant infiltrated with pCVA-amiR:I(−)351. Total RNA extracted from systemic leaf samples from the agro-infitrated plants were analyzed by gel blot assay for the knock-down/suppression of (C) LRR-RSTPK, (D) PI4KA, (E) STPK, (F) RSTPK, (G) VMP1 and (H) PPR mRNAs using gene specific radiolabeled probes. The 5S-rRNA was used as a loading control. Full size RNA gel blots are presented in Fig. S2.

sRNA:target mRNA cleavage site and the modification of the structure of the PSTVd molecule. This required the mutations of several consecutive positions in each of the three targeted regions (i.e. in 4 consecutive positions). A total of seven mutants were created using PSTVd-I as the starting sequence: (i) three single mutants; (ii) 3 double mutants obtained by the combination of two single mutants; and, (iii) one triple mutant which included all the three of the single mutants (see Fig. 5A). The predicted duplexes formed from all of the mutant vd-sRNA:target duplexes significantly affected the predicted hybridization energies (Table 3). Additionally, *in silico* structure prediction using the M-fold software exhibited increased loop size in the region of the mutation, as compared to wild type PSTVd-I (Fig. 5A). This in turn affected the overall structural stability by increasing the minimal free energy (ΔG) of mutants as compared to wild type PSTVd-I.

It was envisaged that, upon infection, the mutants would accumulate to a lesser extent than the wild type as the mutants: (i) produce altered vd-sRNA directed against the host defense genes which are less effective than the wild type vd-sRNAs; and, (ii) have bigger loops in the altered sequences, thus making them more prone to host RNA silencing mechanism. It was also predicted that the viroid accumulation level would decrease in the order single mutants followed by double mutants, and least accumulation would be observed in triple mutant inoculated plants. Surprisingly, none of the mutant inoculated plants showed any symptoms, even at 3-wpi although the PSTVd-I inoculated plants exhibited disease symptoms at 12-days post inoculation (data not shown). Total RNA was extracted at 3-wpi from control, PSTVd-I and mutant inoculated plants and subjected to RT-qPCR to verify the accumulation of the different mutants. Only PSTVd-I$^{mut-331}$ inoculated plants showed a very small almost negligible amount of PSTVd (Fig. 5B). However, the RNA gel blot assay for the same samples did not reveal viroid accumulation in any of the mutant inoculated plants (Fig. S3). Taken together, the mutation of the nucleotides of the vd-sRNA:target cleavage sites increased the size of certain loops, and this proves to be lethal to the viroid, clearly indicating the importance of these vd-sRNAs and loops in determining viroid survival in host plants.

Discussion

Despite more than four decades of extensive studies on viroid RNA structure and function, developing viroid resistant plants remains an unachieved goal. Although RNA silencing strategies have been extensively used against RNA virus pathogens, transgenic plants containing hairpin constructs derived from the

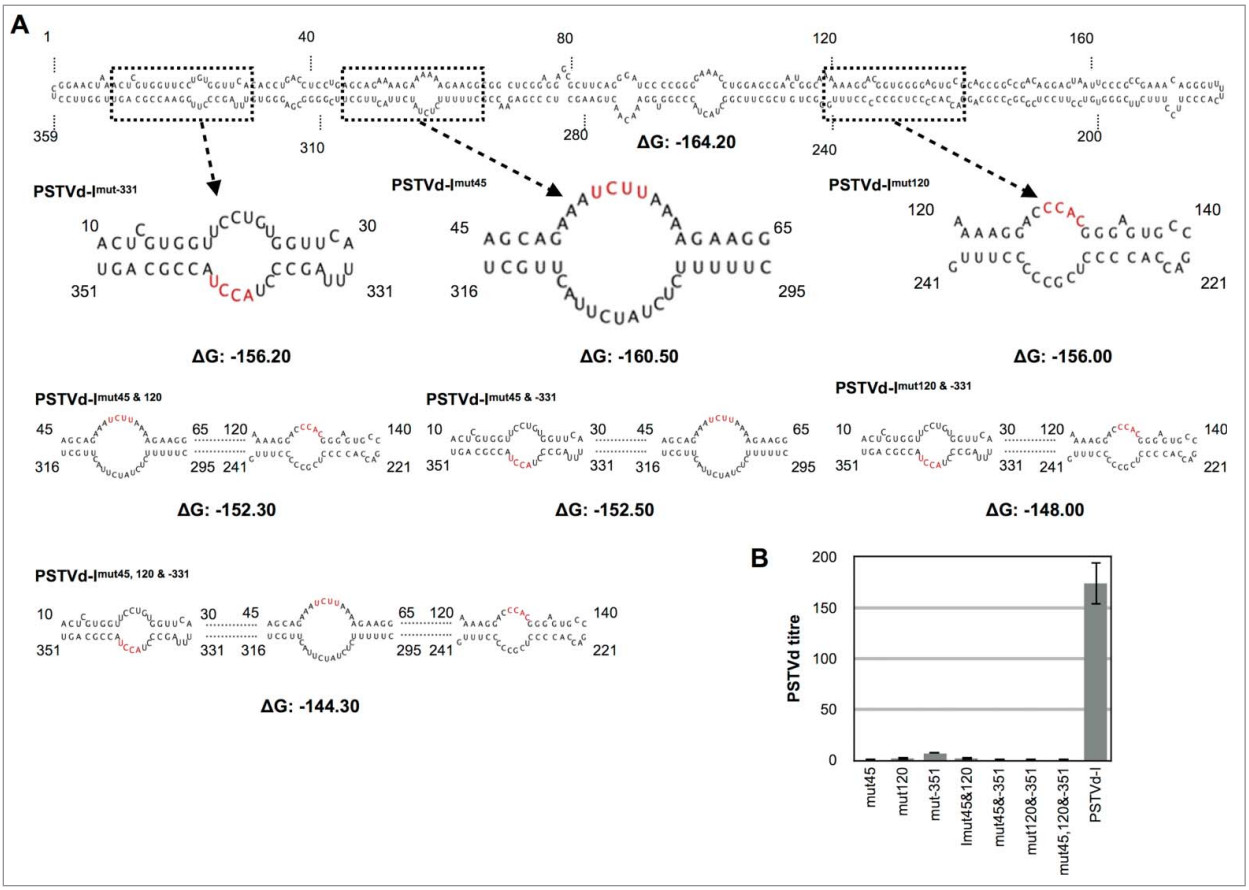

Figure 5. Comparison of the computer-predicted secondary structures of PSTVd-I and its mutants. (A) The secondary structure of PSTVd-I was predicted using the M-fold online tool. It was compared to the secondary structures obtained for the single mutants (PSTVd-I^{mut45}, PSTVd-I^{mut120} and, PSTVd-I$^{mut-351}$), the double mutants (PSTVd-I$^{mut45\&120}$, PSTVd-I$^{mut45\&-351}$ and, PSTVd-I$^{mut120\&-351}$) and the triple mutant (PSTVd-I$^{mut45,\ 120\ \&\ -351}$). The mutated sequences are shown in red. At 3-wpi, total RNA was extracted from systemic leaf and subjected to (D) RT-qPCR assay to monitor the PSTVd accumulation. The expression change is presented on a log2 scale. Each experiment was performed at least three times with true biological replicates. The error bars indicate SD.

Table 3. Mutation of the PSTVd-I in a Predicted targeting vd-sRNA.

vd-sRNA	Mutants	Target genes	vd-sRNA:mRNA duplex	ΔG[a,b]	% pairing
vd-s: +45	PSTVd-I^{mut45}	LRR-RSTPK	**UCUU**AA G 5′-AGCAGAAA AAGAA G-3′ 3′-UCGUCUUU UUCUU C-5′ UCUUGG A	-22.0	66.7
		P14KA 1	AG **UCUU** GG 5′- CAGAAA AAAAGAA -3′ 3′- GUCUUU UUUUCUU -5′ GU UCUU AA	-23.2	61.9
vd-sRNA: +120	PSTVd-I^{mut120}	STPK	A A **CCAC** G AG 5′- A AGGAC G G UGCC-3′ 3′- U UCCUG C C ACGG-5′ C A CCAC U AA	-31.2	57.1
vd-sRNA: -331	PSTVd-I$^{mut-331}$	RSTPK	A **AGGU** GG AA 5′- CUGCGGU A GCUA -3′ 3′- GACGCCA U CGAU -5′ A AGGU GG AA	-28.9	57.1
		VMP1	C **AGGU** G UA A 5′-A UGCGGU AGG C A -3′ 3′-U ACGCCA UCC G U -5′ C AGGU G GG A	-29.2	57.1
		PPR	U **AGGU**AG A A 5′-AC GCGGU GGCU A -3′ 3′-UG CGCCA CCGA U -5′ C AGGUCU A C	-27.35	57.1

[a]The PairFold online tool was used to predict the minimum secondary structure free energy.
[b]Hybridization energy in kcal/mol.
Altered nucleotides are denoted in bold letters.

different regions of PSTVd failed to confer resistance against viroid infection [22]. In this scenario, understanding the relationship between the viroid genome and that of host defense genes are of the utmost important as this can provide a clue towards designing novel strategies against viroid infection. One approach is to obtain the genomic map of the vd-sRNA producing regions that interact with the host's defense genes. To investigate the vd-sRNA:RSTK gene interaction, the vd-sRNA derived from both the (+) and (−) strands of the three PSTVd variants known to induce different symptoms in tomato plants following infection were used to investigate the tomato genome. This resulted in at least 3 vd-sRNA targeting different RSTK genes, along with other genes such as PI4KA, VMP1 and PPR (Fig. 1A). As these genes play crucial roles in host defense, plant physiology and RNA editing, understanding the interactions between the regions of the viroid that are predicted to target these genes are extremely interesting for the developing novel viroid resistant strategies. Since, tomato cv. Heinz 1706 has been both sequenced and used to investigate all of the putative vd-sRNA target gene sequences, the tomato cv. Rutgers was used in this study. Hence, the sequences of the potential vd-sRNA target sites of the tomato cv. Rutgers were confirmed by sequence analysis. To verify the role of the putative target genes on the host phenotype, the VIGS technique was used to knock-down the gene of interest. All of the RNA silenced plants exhibited phenotypes (Fig. 1B), suggesting the importance of these genes in the overall development of tomato plants.

To verify how three PSTVd variants that induces array of symptoms upon infection accumulate over time, total RNA was extracted from inoculated plants at intervals of one week for up to 6 weeks and the extract was subjected to RT-qPCR analysis. PSTVd-RG1 accumulated to much higher levels and induced severe disease symptom while PSTVd-M accumulated at a lower rate and resulted in mild symptoms in tomato plants (Fig. 2). Irrespective of the PSTVd variant, the viroids showed the log, stationary (absent PSTVd-M and -I) and lag phases of accumulation. In the case of PSTVd-RG1, the stationary phase was reached at 4-wpi. In the case of the PSTVd-I inoculated plants, the viroid titer declined steeply from the 5th to 6th week. In both cases, the plants showed severe disease symptoms and eventually died. Although PSTVd-M inoculated plants showed an accumulation curve similar to that of PSTVd-I inoculated plants, viroid accumulation level was much lower than that of PSTVd-I inoculated plants. As the PSTVd-M titer fell in the 6th week, the plants started to recover from viroid infection. Such recovery of the host from viroid infection has been observed previously [35,36].

Since all of the PSTVd variants were in log phase, the RNA samples extracted at 3-wpi were used for the analysis of vd-sRNA as well as for the effect of the viroids on the putative target genes. A direct correlation was found between the amount of PSTVd accumulation and the number of vd-sRNAs which accumulated (Fig. 2B, C). In other words, PSTVd-RG1 accumulated at a higher rate and produced more vd-sRNA, while PSTVd-M inoculated plants exhibited a lower amount of both viroid accumulation as well as vd-sRNA. Also, the profiling of vd-sRNAs obtained on its respective PSTVd variant revealed the production of vd-sRNA from the strands of both polarities, in agreement with previous data [8,37]. It is interesting to note that PSTVd-M, which produced milder symptoms and accumulated less in the plants, produced almost equal amounts vd-sRNAs from both the (+) and (−) strands while PSTVd-RG1, which accumulated at a higher rate and induced severe symptoms in tomato plants, showed the highest amount of (+)

strand derived sRNAs. In other words, a correlation was observed between the amount of (+) vd-sRNA, viroid accumulation and disease symptoms. Although PSTVd variants produced different amount of sRNAs, all had a similar pattern, in agreement with previous studies [7,35,36].

To evaluate the effect of the PSTVd variants on the putative target genes, time course analyses of the RNA extracted up to 3-wpi were performed. All three PSTVd variants used in this study had negative effects on LRR-RSTPK, RSTPK, VMP1 and PPR-mRNA as compared to control plants (Fig. 3). Only the PSTVd-I and PSTVd-RG1 inoculated plants exhibited the down-regulation of the STPK transcript. It should be noted that PSTVd-M lacks vd-sRNA that can target the STPK transcript. Although the *in silico* prediction showed the possible binding of vd-sRNA 45–65 with PI4KA, none of the viroid-infected plants exhibited noticeable changes to transcript levels. Possibly there is no effect of PSTVd on the PI4KA transcript. However, the binding of vd-sRNA can lead to translational suppression. This possibility cannot be verified because of the unavailability of the specific antibodies required.

The involvement of vd-sRNA in the RNA silencing of host transcripts has been well established by several research groups [9,11,15,14]. However, to show the direct effect of vd-sRNA on the host's phenotype, Eamens et al. [10], used amiRNA capable of expressing vd-sRNA in transgenic *N. benthamiana*. This technique is laborious and time consuming. Therefore, much less is known about the direct involvement of vd-sRNA in inducing a phenotype in viroid-infected plants. Previously, MIR VIGS was developed to study the functional genomics of microRNAs in *N. benthamiana* plants [34]. In this technique, amiRNA was constructed using the miR319 sequence as the starting point, and was then transiently expressed by co-agroinfiltrating pCVA and pCVB vectors. However, the production of the miR319-based amiRNA required multiple PCR reactions on the miR319 backbone. Previously, we and others evaluated the effectiveness of a truncated, 120-nt long, osa-MIR528 sequence for the production of an amiRNA [11,38]. Hence, in the present study, the miR319 backbone was replaced with the smaller, osa-MIR528 backbone, which requires only a single PCR for the construction of the amiRNA. Expression of amiRNA targeting PDS on an osa-MIR528 backbone was used to evaluate the functionality of the vd-sRNA:VIGS technique in tomato plants (Fig 4). As all of the RSTPK genes are involved in host development and defense systems, the transient expression of vd-sRNAs in tomato plants, resulted in phenotypes similar to viroid infection. The vd-sRNA:VIGS technique will be very useful for researchers in evaluating the role of vd-sRNA in different host plants.

Previous studies on loop 4 (where vd-sRNA -351 is located); loop 9 (where vd-sRNA 45 is located), and loop 18 (where vd-sRNA 120 is located) of the PSTVd structure revealed the importance of these loops in both the viroid's replication and its systemic accumulation (Fig. S4) [39]. As the PSTVd-sRNAs produced in these regions are capable of binding RSTK and the host defense genes, any modification of these regions of PSTVd should be detrimental for PSTVd's survival. To evaluate this hypothesis, mutagenesis experiments were performed by modifying 4 bases of the vd-sRNA producing regions of PSTVd-I (Fig. 5). This resulted in (i) the disruption of the cleavage site of the vd-sRNA/RSTK duplexes; and, (ii) an increased loop size in the vd-sRNA producing regions of PSTVd-I. As expected, these mutants either didn't survive, or accumulated at much lower levels in the inoculated tomato plants, indicating these regions are critical for the viroid's survival.

The vd-sRNA:VIGS technique developed here to evaluate the role of the predicted vd-sRNA on the host phenotype's will help viroid researchers evaluate the effects of individual vd-sRNA on host morphology, and also to understand functional genomics in terms of viroid biology. The data presented here shows that PSTVd variants are capable of down-regulating host genes that are involved in both host defense and plant development. Any alteration in the sequence of the viroid that is shown to interact with RSTK is detrimental to its survival. In summary, the data presented in this study, reveals the regions of a viroid that can be explored to develop novel viroid resistant strategies for sustainable agriculture.

Materials and methods

Viroid bioassay, RNA extraction and RT-qPCR

The dimeric constructs of PSTVd-M (GenBank Acc. No. AB623143), -I (GenBank Acc. No. AY937179) and –RG1 (GenBank Acc. No. U23058) were used to synthesize infectious dimeric transcripts as described previously [11,15]. 1 μg of the RNA transcripts of the respective viroid variants were mechanically inoculated into tomato plants and incubated as before [11]. Leaf samples were collected at 1-, 2-, 3-, 4-, 5- and 6-wpi for total RNA extraction as described previously [11].

Complementary DNA (cDNA) was prepared by reverse transcribing 1 μg of the total RNA using SuperScript III Reverse Transcriptase in the presence of random hexamers according to the manufacturer's instructions (Invitrogen; Cat. No. 18080093). For the evaluation of both the PSTVd titer and of the gene expression levels, 10 ng of cDNA were used for qPCR with the appropriate primer combinations (Table S1). RT-qPCR data were obtained for the three house-keeping genes were used for normalization as described previously [11]. At least three true biological replicates were used for each experiment. Every qPCR run included a negative control. qBASE framework was used to calculate gene expression levels [40]. All RT-qPCR analyses were performed commercially at the Laboratoire de Génomique Fonctionnelle de l'Université de Sherbrooke (http://palace.lgfus.ca).

Small RNA extraction, high-throughput sequencing and data analysis

For the high-throughput sequencing of the small RNAs, sRNAs of 15–50 nt in length were purified from the total RNA extracted from 3-wpi plants, and were subjected to sRNA library preparation commercially at the Laboratoire de Génomique Fonctionnelle de l'Université de Sherbrooke (http://palace.lgfus.ca) using an Illumina Mi-Seq platform and a multiplex strategy. sRNAs (21- to 24-nt long) were filtered after trimming of the adapters as described previously [41]. vd-sRNAs of 21- to 24-nt were filtered and were plotted against

the strands of both polarities of the respective variants after normalizing to the Reads Per Million (RPM) scale [42,43].

Construction of the vectors for VIGS and vd-sRNA:VIGS

To produce the pTRV2 derivatives targeting either LRR-RSTPK. PI4KA, STPK, RSTPK, VMP1 or PPR, portions of the respective genes were amplified from tomato cv. Rutgers using gene specific primers (Table S2). The resulting amplicons were ligated into the binary vector pTRV2. The resulting recombinant binary vectors were then transformed into the *A. tumefaciens* strain GV3101, and were then used for agro-infiltration as previously described [15].

To evaluate the role of vd-sRNA on the tomato plant's phenotype, amiRNAs for the different vd-sRNA sequences were initially synthesized on an osa-MIR528 backbone using the appropriate oligonucleotide pairs in the PCR reaction (Table S3). The PCR products were ligated into the binary vector pCV-A after digestion with the restriction endonucleases *Xba*I and *Kpn*I. The resulting binary vectors were then transformed into the *A. tumefaciens* strain GV3101 and were used for agroinfiltration in tomato plants. Agroinfiltrated plants were grown at 23°C with 16 h of light and 8 h of darkness.

Probe preparation and RNA gel blot assay

To detect LRR-RSTPK, PI4KA, STPK, RSTPK, VMP1 and PPR, the riboprobes were prepared by using the previously amplified genes from tomato cv. Rutgers which had been cloned in the pBlueScript KS(+) vector. The details of the primers used to amplify the genes are presented in Table S4. A dimeric PSTVd-I construct was used to produce the (−) PSTVd riboprobe. To prepare the riboprobes, either the T3 (Ambion; Cat. No.AM1316) or the T7 MAXIscript kit (Ambion; Cat. No.AM1312) was used after linearization of the plasmid with the appropriate restriction endonuclease. A DNA probe (60 nt in size) was used to verify the expression level of the 5S rRNA, which was used as the loading control for the RNA gel blot hybridizations. In order to detect both the host genes and the vd-sRNA, 5.0 μg of the total RNA samples extracted at 4-wpi of vd-sRNA:VIGS was used. The gel blots were performed as described previously [11].

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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