Scion on a Stock Producing siRNAs of Potato Spindle Tuber Viroid (PSTVd) Attenuates Accumulation of the Viroid

Atsushi Kasai, Teruo Sano, Takeo Harada*
Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Japan

Abstract
Plants can attenuate the replication of plant viruses and viroids by RNA silencing induced by virus and viroid infection. In higher plants, silencing signals such as small interfering RNAs (siRNAs) produced by RNA silencing can be transported systemically through phloem, so it is anticipated that antiviral siRNA signals produced in a stock would have the potential to attenuate propagation of viruses or viroids in the scion. To test whether this is indeed the case, we prepared transgenic tobacco (Nicotiana benthamiana) expressing a hairpin RNA (hpRNA) of Potato spindle tuber viroid (PSTVd) in companion cells by using a strong companion cell-specific promoter. A grafting experiment of the wild type tobacco scion on the top of the transgenic tobacco stock revealed that accumulation of PSTVd challenge-inoculated into the scion was apparently attenuated compared to the control grafted plants. These results indicate that genetically modified rootstock expressing viroid-specific siRNAs can attenuate viroid accumulation in a non-genetically modified scion grafted on the stock.

Introduction
Viroids are the smallest known infectious agents of plants, and induce disease in a wide range of hosts including many crop species. They have been identified as non-coding, circular, single stranded RNAs ranging in size from 246 to 401 nt [1]. Viroid replication is entirely dependent on transcriptional and processing machinery supplied by the host, and transport of the resulting progeny utilizes preexisting cellular pathways [2,3]. On the other hand, exogenous RNA invader such as viroids induce RNA silencing system in the infected plants in which dicer-like (DCL) protein, argonaute (AGO) protein, and RNA-dependent RNA polymerases (RDR) function sequentially. As a result, high levels of viroid-specific small RNAs can accumulate in host plant cells [4–6]. However, viroids appear relatively resistant to RNA silencing, probably due to the highly conserved secondary structure of the genome RNA itself [7–9]. Meanwhile, transgenic tobacco plants that accumulate high levels of viroid hpRNA-derived siRNAs (hp-siRNAs) exhibited effective resistance to PSTVd infection [10,11]. Furthermore, Di Serio et al. [12] have reported that when the gene for RNA-dependent RNA polymerase 6 (RDR6), catalyzing an amplification circuit producing the double-strand precursors of secondary siRNAs, has been silenced, Nicotiana benthamiana plants accumulated increased amount of PSTVd genomic RNA comparing to the wild-type control, supporting the contention that viroid hairpin-derived siRNAs can reduce the accumulation of viroid by RNA silencing system.

RNA silencing by siRNAs in plants has the ability to spread systemically. This does not involve gradual cell-to-cell spread. The silencing signal travels along the vascular system and induces silencing in sink tissues [13,14], was first demonstrated convincingly by a grafting experiment using Nicotiana plants. Furthermore, a specific siRNAs produced in squash stock has been detected in phloem sap from a grafted cucumber scion [15]. Additionally, much of the evidence that translocation of siRNAs induces systemic silencing has been based on grafting experiments [16–21]. These silencing signals travel to metabolic sink tissues in the direction of phloem flow [22]. It has also been found that the presence of a transgene is dispensable for the RNA degradation step in plants when the endogenous gene transcript over-accumulates above the level present in wild-type plants [23]. We have also shown that root stock in which siRNAs is produced exclusively in companion cells can induce silencing of an endogenous gene in the scion, even though the silenced area is limited to that along the phloem [24].

Therefore, virus and/or viroid-derived siRNAs transported from stock harboring the siRNAs producing system would be expected to counteract the accumulation of invader RNA in the scion. If this is indeed the case, resistance of non-transgenic scion to virus and/or viroid infection might be improved by grafting on genetically-modified rootstock. Here we present for the first time the evidence that viroid-specific small RNA signals supplied from genetically-modified rootstock expressing non-infectious form of hairpin viroid RNA can attenuate the accumulation of viroid RNA in the scion.

Citation: Kasai A, Sano T, Harada T (2013) Scion on a Stock Producing siRNAs of Potato Spindle Tuber Viroid (PSTVd) Attenuates Accumulation of the Viroid. PLoS ONE 8(2): e57736. doi:10.1371/journal.pone.0057736
Editor: Frederik Börnke, Friedrich-Alexander-University Erlangen-Nurenberg, Germany
Received October 11, 2012; Accepted January 24, 2013; Published February 28, 2013

Copyright: © 2013 Kasai et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Adaptable and Seamless Technology Transfer Program through target-derived R&D of the Japan Science and Technology Agency, and by a Grant for Institutional Research at Hirosaki University. The funders of this works had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: thorada@cc.hirosaki-u.ac.jp
Results and Discussion

siRNAs in CoYMV::PSTVd N. benthamiana

The production of truncated, near-full-length, hpPSTVd-∆TLE was controlled by a companion cell-specific promoter, pCoYMV [25] (Figure 1A), to increase the potential siRNAs level in the phloem. The usefulness of pCoYMV for enhancing siRNAs signal in phloem transport had been confirmed in our previous studies [24,26]. Since the promoter sequence was linked to near full-length PSTVd cDNA lacking 12 nucleotides at the left terminal end of the genome, the resulting construct (Figure 1A) facilitated the production of non-infectious form of hpPSTVd-∆TLE sequence in the transgenic N. benthamiana companion cells.

Four CoYMV:hpPSTVd-∆TLE integrated lines of N. benthamiana were obtained. Although a certain (ca. 5.3−12.2%) proportion of the four T1 seedlings exhibited abnormal phenotypes such as dwarfism, delayed rooting, an aberrant leaf shape and so on, none of the T2 seedlings obtained exhibited any obvious phenotypical abnormalities. Lines 24 and 32 accumulated detectable levels of PSTVd-specific siRNAs by RNA-gel blot analysis, while the remaining two lines (11 and 141) did not show detectable levels of PSTVd-siRNAs (Figure 1B). As the integration of hpPSTVd-∆TLE fragment into the N. benthamiana genome was confirmed by genomic PCR (Figure S1), the difference in the viroid siRNAs accumulation levels seemed to have been caused by position effect of the integration sites. As we have already reported that the production of siRNAs exclusively in companion cells ensured silencing of the target gene expression in the remote sink tissue [24], all the four transgenic lines including lines 11 and 141 with lower levels of PSTVd-specific siRNAs accumulation were also characterized in the following experiments.

Effects on Expression of Specific Host Genes in CoYMV:hpPSTVd-∆TLE N. benthamiana

To investigate whether the production of hpPSTVd-∆TLE-derived siRNAs in companion cells affected the expression of endogenous genes, quantitative RT-PCR analyses of NbBRP (bromodomain-containing RNA-binding proteins 1 and 2, accession nos. AJ504729 and AJ504730), which are genes homologous to the tomato RNA-binding protein SlVirp1 gene [27], were performed. Although it has been reported that SlVirp1 mRNA is decreased in PSTVd-infected tomato leaves [28], the expression of both NbBRP1 and 2 was inversely up-regulated in CoYMV:hpPSTVd-∆TLE lines (Table 1). Furthermore, it was likely that a higher amount of PSTVd siRNAs led to higher NbBRP gene expression, notably in NbBRP1. Additionally, another gene, NbAGO1 (N. benthamiana ARGONAUTE1), whose mRNA level was reported to have been increased by virus infection [29,30], was analyzed. The results showed that the level of NbAGO1 mRNA in hpPSTVd-∆TLE lines was almost equivalent to that in control plants (Table 1).

Next, the transcription levels of possible target genes of small RNA of PSTVd, reported by Wang et al. [6], were investigated, since Wang et al. [6] and Navarro et al. [31] reported about the relationship between small RNAs of viroid and pathogenicity. We had already identified N. benthamiana genes that were homologous to those of possible target genes of small RNA of PSTVd in tomato. In fact, although the whole sequences of the tomato and tobacco genes showed high homology, they showed a few nucleotide differences within the possible target site (Figure S2A and B). Furthermore, in the case of SGN-U503132 (ARF GAP-like zinc finger-containing protein ZIGA3), the possible corresponding target sequence for tomato was not present in N. benthamiana (Figure S2B). The transcript of SGN-U503021 (NADPH:protochlorophyllide oxido-reductase POR) was slightly but significantly decreased in line 24, whereas the level of SGN-U567353 (phosphatase 2A) was unchanged.

Overall, it was suggested that PSTVd-specific siRNAs from the CoYMV:hpPSTVd-∆TLE construct more or less affected the expression of the specific host genes, but the changing direction was not identical to that for natural PSTVd infection in tomato, or rather somewhat opposite in some cases [32]. The level of PSTVd-specific siRNAs accumulation in N. benthamiana infected with PSTVd is overwhelmingly higher than in plants transformed with CoYMV:hpPSTVd-∆TLE. Furthermore, only phloem expression of siRNAs in CoYMV:hpPSTVd-∆TLE plants showed relatively lower amount of siRNAs accumulation than in transgenic plants driven by 35S promoter. Therefore, the different responses observed in PSTVd-infected plants were not surprising. Also, to some extent, the different responses may have been attributable to the difference in target site sequences between tomato and tobacco and also the difference in the response to PSTVd infection; i.e., “Rutgers” tomato plant showed severe stunting and epinasty, however, N. benthamiana was totally a symptomless carrier for the PSTVd isolate. Furthermore, the difference of siRNA population between cases of viroid infection and CoYMV:hpPSTVd-∆TLE expression might make the difference.

Figure 1. CoYMV:hpPSTVd-∆TLE N. benthamiana plants. (A) Schematic diagrams of the CoYMV:hpPSTVd-∆TLE construct. Arrows indicate PSTVd sequence used. pCoYMV: commelina yellow mottle virus promoter, TN: nopaline synthase terminator, TL: terminal left, P: pathogenicity, CCR: conserved central region, V: variable region, TR: terminal right. (B) Northern blot analysis of PSTVd siRNAs in the transgenic plants. Small RNA enriched nucleic acid (10 μg) was analyzed in 15% polyacrylamide gel and probed with PSTVd negative (top) and positive (middle) strand RNA. 5.8S rRNA hybridization was used as a loading control.

doi:10.1371/journal.pone.0057736.g001
Challenge Inoculation of Viroid into CoYMV:hpPSTVd-TLE Lines

To examine whether PSTVd accumulation can be attenuated in CoYMV:hpPSTVd-TLE lines, we performed northern blot analyses to detect the mature PSTVd positive strand molecules in line 24 and 32 which accumulated detectable levels of the PSTVd siRNA by gel blot analysis (Figure 2). At 14 dpi, plants of all lines showed PSTVd accumulation. However, the signal intensities in lines 24 and 32 were weaker than those in the lines Empty control (Figure 2A and B). That is, PSTVd accumulation was suppressed in the two CoYMV:hpPSTVd-TLE lines, in agreement with a previous report indicating that PSTVd resistance is correlated with the level of hpPSTVd siRNAs accumulation [11]. Although the period of attenuation was not prolonged and differences in the signal intensity between the lines were not observed at 22 dpi anymore, PSTVd accumulation was evidently restrained at 14 dpi. To confirm the attenuating effect of hpPSTVd-TLE small RNAs on PSTVd accumulation, we performed another experiment using eight individual plants of CoYMV:hpPSTVd-TLE lines including line 11 and 141 which did not show detectable levels of PSTVd-siRNAs (Figure S3). Dot-blot hybridization experiment was done as substitute for northern hybridization because of the concise analysis. As shown in Figure 3, although the levels of accumulation varied considerably within the same lines, all plants in line 24 and especially those in line 32, exhibited lower accumulation levels relative to the other three lines. Thus, it is appeared that PSTVd siRNAs production only in the companion cells was at least among the promising methods to attenuate PSTVd accumulation in the genetically modified plant.

Attenuation of PSTVd Accumulation in the Scion Grafted on Genetically Modified Stock Containing CoYMV:hpPSTVd-TLE Lines

Finally, to clarify whether PSTVd accumulation can be attenuated by a RNA silencing signals transferred from hpPSTVd-TLE stock to the scion, we performed grafting experiments using wild-type N. benthamiana as the scion and CoYMV:hpPSTVd-TLE lines as the stock. As shown in Figure 4A, to enhance the movement of the RNA silencing signals from the stock to the scion, the leaves of scion plants were removed except for one leaf, which was inoculated with PSTVd. At 12, 16, 19 and 25 dpi, the most newly expanded leaf at each time point was sampled for RNA extraction. All hybridization data were normalized by the 5.8s rRNA concentrations as the internal control (Figure S4), and then the levels of PSTVd accumulation in each plant were converted to a heat map (Figure 4B). At 12 dpi it was evident that 12 out of 21 wild type (WT) scions on the three lines of CoYMV:hpPSTVd-TLE stocks gave no accumulation signs of PSTVd infection. Furthermore, even at 16 dpi, five out of a total 21 plants in the three lines of wild type WT/CoYMV:hpPSTVd-TLE combinations continued to be negative for PSTVd, whereas all the seven plants in WT/Empty combination turned PSTVd-positive by 16 dpi. Furthermore, although all the plants showed PSTVd-positive at 25 dpi, the positive signals were somewhat dense in WT/Empty combination plants (Figure 4B). The changes in the levels of PSTVd accumulation from 12–25 dpi were quantified by using the sum of sores in the seven representative plants. As the result, it was apparent that all the three WT/CoYMV:hpPSTVd-TLE combination plants attenuate PSTVd accumulation in the scions (Figure 4C). Interestingly, line 141, in which PSTVd-specific siRNAs accumulation was not evident (Figure 1), also attenuated PSTVd accumulation. As shown in Figure 3 and Figure S3, some of line 141 plants exhibited the attenuating efficacy of PSTVd accumulation. Therefore, line 141 is thought to have small amount level of the siRNA which was detectable by northern blot analysis. Despite the lack of very clear efficacy of the siRNA transporting from stock to the scion, our observations have indicated that further research into the use of the siRNA producing stock as a mean of viroid disease resistant scion production is promising.

Future Perspective

The results presented here provided a potentially useful technique for preventing plant from virus and/or viroid infections, in which we have constructed a hair-pin structure of near full-length (non-infectious form) of PSTVd and expressed under the control of companion cell–specific CoYMV promoter in N. benthamiana plants. Both of the genetically modified N. benthamiana plant itself and the wild type N. benthamiana scion grafted onto the genetically modified N. benthamiana stock successfully suppressed PSTVd accumulation in the early stage of infection. The plhemo transportation system to deliver RNA silencing signals from genetically modified rootstock to scion has already been succeed in an endogenous tobacco gene, glutamate-l-semialdehyde aminotransferase (GSA), in our previous study [24], and now the system is also promising for future improvement of plant resistance to virus and/or viroid infection. The improved scion variety resulting from transportation of RNA silencing signals such as siRNAs from the genetically modified rootstock does not contain the inserted transgene itself, meaning for examples that the fruits bearing on the scion are also free from transgene [33]. Thus there is an extensive merit in the system in associated with a possible risk of genetic contamination of ecosystem by pollen escaping to surrounding native vegetation either. It is anticipated that further application of stock delivering specific RNA silencing signals for scion improvement will lead to the development of rapid, easy,

| Table 1. The expression levels of viroid responsive genes in CoYMV:hpPSTVd-TLE plants. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gene            | Empty           | CoYMV:hpPSTVd-TLE |
|                 | 11              | 141             | 24              | 32              |
| NbBRP1          | 92.90 ± 15.12   | 98.12 ± 7.21    | 121.76 ± 33.35  | 172.89 ± 20.52***| 149.82 ± 15.0***|
| NbBRP2          | 877.23 ± 110.47 | 884.68 ± 182.54 | 1029.34 ± 212.35| 1065.33 ± 88.40 | 1161.01 ± 214.91 |
| NbAGO1-1        | 98.05 ± 21.09   | –               | –               | 112.70 ± 35.81  | 92.86 ± 10.85    |
| NbAGO1-2        | 105.89 ± 14.43  | –               | –               | 114.07 ± 30.68  | 86.55 ± 7.11     |

*The level of each gene expression was normalized based on NbUbi. One resulted value of NbBRP1 and NbAGO1-1 was based as a benchmark of 100, respectively. *– means no trial. Triple asterisk indicates significant difference (p < 0.01) from the Empty.*

doi:10.1371/journal.pone.0057736.t001

PLOS ONE | www.plosone.org

February 2013 | Volume 8 | Issue 2 | e57736
and environmentally systems for the improvement of crops, or particularly those such as fruit trees, in which grafting is commonly used for renewing the varieties [34].

Materials and Methods

Plasmid Construction

A truncated, near full-length, PSTVd-Intermediate strain sequence (PSTVd-DTLE; lacking the terminal left end nucleotides from No. 353 to No. 5 in the genome [35]), was amplified by PCR using primers tPSTVd sFw and tPSTVd sRv and subcloned into the EcoRV site of the pBluescript II SK+ plasmid (Stratagene, La Jolla, CA, USA). Then the CAT1 intron [36] amplified by PCR using primers intFw and intRv was integrated into the AatII/HindIII sites of the plasmid. Finally, an additional PSTVd-DTLE sequence, amplified by PCR using primers tPSTVdaFw and tPSTVdaRv, was integrated into the HindIII/KpnI sites, to make a hairpin (hp) structure of PSTVd-DTLE, in which a pair of PSTVd-DTLE is located in both boundaries of CAT1 intron by head to head orientation. The hpPSTVd-DTLE fragment from the plasmid was inserted into the BamHI/KpnI sites of CoYMV-nos [24] based on pIG121 [37], and the resulting plasmid was termed CoYMV:hpPSTVd-DTLE. Construction of an empty vector as a control was done as described previously [24]. The DNA sequences of the plasmids were confirmed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Plant Transformation and Growth Conditions

Transformation of tobacco (Nicotiana benthamiana) was performed as described previously [24]. The single functional T-DNA insert and the presence of the hpRNA structure were identified by 3:1 segregation for kanamycin resistance in seeds obtained from the

---

**Figure 2.** PSTVd accumulation in the CoYMV:hpPSTVd-ΔTLE lines. (A) Detection of viroid quantity by northern blot analysis of RNA extracted from the leaves of transgenic lines 24 and 32 at 7, 14 and 22 days post inoculation (dpi). Total RNA (100 ng) was hybridized with the PSTVd negative strand RNA probe. The results of 18S RNA qRT-PCR show equal loading of RNA. (B) Accumulated levels of the PSTVd RNA at 14 dpi and 22 dpi are shown diagrammatically. Quantity of total RNA was normalized against 18S rRNA. Note that hybridization of each dpi membrane was done for separate experiments.
doi:10.1371/journal.pone.0057736.g002

**Figure 3.** PSTVd accumulation in the CoYMV:hpPSTVd-ΔTLE lines at 14 dpi. The data were obtained by dot-blot hybridization (Figure S3) using 8 individual plants of each line.
doi:10.1371/journal.pone.0057736.g003
selfed primary transformants. Genomic DNA of each transgenic line was extracted using DNeasy (QIAGEN, Frankfurt, Germany) in accordance with manufacturer’s instructions. Genomic PCR analysis was performed using OneTaq DNA polymerase (New England BioLabs Inc., MA, USA). The primers used for genomic PCR are described in Table S1. Homozygotes for the integrated gene in 2-nd self progeny were confirmed by absence of antibiotic sensitivity. Two weeks after sowing, transgenic tobacco seedlings were transferred to rockwool (Nitto Boseki Co., Tokyo, Japan) in a standard nutrient solution (Ohtsuka House, Ohtsuka Chemical Co., Osaka, Japan) and grown in a plant growth room at 24 °C under a 16-h light/8-h dark cycle with cool fluorescent light at about 100 μmol m⁻² s⁻¹.

Viroid Strains and Infections

*N. benthamiana* plants were mechanically inoculated with PSTVd. The inoculum contained 50 μg of PSTVd-enriched low molecular weight nucleic acid extract from tomato plants (*Solanum lycopersicum*, cultivar Rutgers) infected with PSTVd-Intermediate strain (accession no. AY937179) in 150 μl of 50 mM phosphate buffer (pH 7.0) containing 1 mg/ml bentonite. The 7-th true leaf of one-month-old plants were dusted with 600-mesh carborundum powder, and then gently rubbed with the inocula. Diethylpyrocarbonate-treated water was used for mock inoculation.

**Extraction of Small RNAs and Detection of siRNAs**

Extraction of small RNAs and detection of siRNAs were performed essentially as described by Kasai et al. [24]. The digoxigenin-labeled truncated PSTVd sense and antisense riboprobes were synthesized using DIG RNA labeling Mix and T3, T7 RNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), respectively. The riboprobes were hybridized to small RNAs at 55 °C in DIG Easy Hyb solution (Roche). The membrane was washed twice with 2×SSC/0.5% SDS at 55°C. The hybridized probe was visualized using CDP-star solution (Roche) and detected using a ChemiDoc XRS (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

---

**Figure 4. PSTVd accumulation in the WT scion on a CoYMV:hpPSTVd-ΔTLE stock.** (A) Illustration of the experimental procedure. At 13 dpi, leaf disks were harvested from the most expanded leaf. Then, sampling was done every 3 days. Seven grafted plants per line were tested. Accumulation level of the PSTVd RNA was analyzed by dot-blot hybridization (Figure S4). (B) Levels of PSTVd accumulation in the WT scion presented in the form of a heat map. The intensity of the hybridization signals was normalized relative to the 5.8S RNA signal, and sorted out from 0 no signal to 7 maximal. (C) Changes in the accumulation levels of PSTVd. Total scores for 7 grafts in each line are represented.

doi:10.1371/journal.pone.0057736.g004
Total RNA Extraction and qPCR Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and treated with TURBO DNA-free (Applied Biosystems, Ambion, USA). In accordance with the manufacturer’s instructions, cDNA was synthesized with 1 μg RNA as a template using Superscript VILO (Invitrogen). The qPCR analysis was performed essentially as described in Kasai et al. [24] using SsoFast EvaGreen Supermix (Bio-Rad) with a Chroome4 realtime PCR detector (Bio-Rad). Information on the PCR primers for the N. benthamiana BRP, AGO1, ZIGA3, and NbPDD1 genes was obtained from the EMBL database. The primers used for qPCR are described in Table S1.

Dot and Northern Blot Hybridizations

The amount of PSTVd in N. benthamiana plants was assessed by blot analyses. Two discs (1.0 cm in diameter) were sampled from the upper most expanding leaves and subjected to extraction of the RNA fraction, as referenced [30]. One-microgram-RNA samples were denatured with denaturing buffer (0.3×SSC, 25% formamide, 8.75% formaldehyde) and denatured at 65°C. Then, the RNA solutions were spotted onto a nylon membrane (three spots per sample). For northern blot analysis, 1 μg of the RNA was separated in a 1.5% agarose gel containing 2.2 M formaldehyde and 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS). The riboprobes were hybridized at 55°C in DIG Easy Hyb solution (Roche). The membranes were washed twice with 2×SSC at room temperature, with 2×SSC containing 1 μg/ml RNase A and then with 0.1×SSC containing 0.1% SDS at 65°C. Detection based on the hybridized signal was performed as described previously.

Micrografting and Sampling from the Scion

Micrografting was performed essentially as described by Kasai et al. [24]. After 7 days grafted plants were transferred to rockwool (Nitto Boseki Co., Tokyo, Japan) in a standard nutrient solution. After 10 days of growth, the plants were stripped of all lateral leaves and buds except for the 7-th leaf and the shoot apex, and then these were inoculated with PSTVd inocula into the 7-th leaf. At 13 days post-inoculation (dpi), two leaf discs from the uppermost expanded leaf were sampled for RNA analysis.

Supporting Information

Figure S1 Genomic PCR of the CoYMV:hpPSTVd-ATLE plants. (A) Locations of potential mRNAs in tomato (right), and the dot-blotted (B) Genomic PCR analysis of four independent transgenic lines using three primer pairs. Quantity of genomic DNA was confirmed by NbUbi. Only IR3′ fragment was performed by 40 cycles and others were by 35 cycles. (TIF)

Figure S2 The expression of possible target genes of PSTVd-mediated RNA silencing in CoYMV:hpPSTVd-ATLE plants. (A) Locations of potential mRNAs in tomato targeted by srPSTVd. (B) Alignment of possible srPSTVd target sites in tomato and N. benthamiana (left). Expression levels of three genes in transgenic tobacco whole plants harvested at one month after sowing. The levels of these mRNAs were analyzed by qRT-PCR. Error bars, s.d. (n = 3). **; significant difference from Empty (P<0.05). The amounts were normalized on the basis of NbUbi. (TIF)

Figure S3 Dot-blot analyses of PSTVd RNA in CoYMV:hpPSTVd-ATLE lines. Transgenic lines were inoculated with PSTVd. At 7 and 14 dpi, leaf disks were sampled and the extracted RNA was dot-blotted. The membrane was hybridized with the PSTVd negative strand RNA probe. 5.8S rRNA was used as an internal control for the amounts of total RNA. (TIF)

Figure S4 Dot-blot analyses of PSTVd RNA in scions grafted onto respective stocks. (A) Scion WT plants were inoculated with PSTVd (ca. 6.7 μg/20 μl) and the dot-blotted membrane was hybridized with the PSTVd negative strand RNA probe. (B) 5.8S rRNA was used as an internal control for the amounts of total RNA. (TIF)

Table S1 Sequences of primers used in gPCR and qRT-PCR.

Acknowledgements

We thank N. Olszewski for providing the pCOI plasmid.

Author Contributions

Conceived and designed the experiments: AK TH. Performed the experiments: AK TH. Analyzed the data: AK TH. Contributed reagents/materials/analysis tools: AK TS. Wrote the paper: AK TS TH.

References

1. Di Serio F, Randles JW, Flores R, Owens RA, Sano T, et al. (2011) Viroids. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus Taxonomy, 9th Report. Waltham, MA: Academic Press. Pp. 117–139.

2. Flores R, Hernández C, Martínez de Alba AE, Daro´s JA, Di Serio F (2005) Viroids and viroid-host interactions. Annu Rev Phytopathol 43: 117–139.

3. Ding B (2003) The biology of viroid-host interactions. Annu Rev Phytopathol 41: 103–131.

4. Iwaya A, Fokinovion M, Matsuda Y, Nelson RS, Ding B (2003) Potato spindle tuber viroid as inducer of RNA silencing in infected tomato. Mol Plant Microbe Interact 14: 1332–1334.

5. Papaefthimiou I, Hamilton AJ, Denti MA, Baulcombe DC, Tsagris M, et al. (2009) Jackalope: an integrated resource for RNA silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J 28: 4738–4748.

6. Voinnet O, Baulcom DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.

7. Palaquin JC, Elmany T, Pollen JM, Vassetter H (1997) Systemic acquired silencing: transgenic-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J 16: 4738–4745.

8. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus Taxonomy, 9th Report. Waltham, MA: Academic Press. Pp. 117–139.

9. Michel C, Yamahata N, Watanuki H, Owens RA, Sano T (2007) Successive accumulation of two size classes of viroid-specific small RNA in potato spindle tuber viroid-infected tomato plants. J Gen Virol 88: 2819–2830.

10. Corni EA, Martinez de Alba AE, Flores R, Gago S (2008) Large and small RNA interference in transgenic tobacco expressing a viroid hairpin RNA construct. Mol Plant Pathol 10: 439–469.

11. Di Serio F, Martinez de Alba AE, Nabarro B, Gisel A, Flores R (2010) RNA-dependent RNA polymerase 6 delays accumulation and precludes meristem invasion of a viroid that replicates in the nucleus. J Virol 84: 2477–2485.

12. Gomez G, Pallais V (2007) Mature monocistronic forms of Hop stunt viroid RNA silencing in transgenic plants. Plant J 51: 1041–1049.

13. Vassiliou V, Baulcombe DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.

14. Vassiliou V, Baulcombe DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.

15. Vassiliou V, Baulcombe DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.

16. Vassiliou V, Baulcombe DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.

17. Vassiliou V, Baulcombe DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.

18. Vassiliou V, Baulcombe DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.

19. Vassiliou V, Baulcombe DC (1997) Systemic signification in RNA silencing. Nature 395: 533–538.
silenced and non-silenced plants, but not between silenced plants. Plant J 21: 1–8.
17. Crété P, Leuenberger S, Iglesias VA, Suarez V, Schöb H, et al. (2001) Graft transmission of induced and spontaneous post-transcriptional silencing of chitinase genes. Plant J 28: 493–501.
18. Schwach F, Vainstj FE, Jones L, Baulcombe DC (2005) An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. Plant Physiol 138: 1842–1852.
19. Shaharuddin NA, Han Y, Li H, Grierson D (2006) The mechanism of graft transmission of sense and antisense gene silencing in tomato plants. FEBS Lett 580: 6579–6586.
20. Brosnan CA, Mitter N, Christie M, Smith NA, Waterhouse PM, et al. (2007) Nuclear gene silencing directs reception of long-distance mRNA silencing in Arabidopsis. Proc Natl Acad Sci USA 104: 14741–14746.
21. Haque AK, Yamaoka N, Nishiguchi M (2007) Cytosine methylation is associated with RNA silencing in silenced plants but not with systemic and transitive RNA silencing through grafting. Gene 396: 21–31.
22. Tournier B, Tabler M, Kalantidis K (2006) Phloem flow strongly influences the systemic spread of silencing in GFP Nicotiana benthamiana plants. Plant J 47: 383–394.
23. Palauqui JC, Vaucheret H (1998) Transgenes are dispensable for the RNA degradation step of cosuppression. Proc Natl Acad Sci USA 95: 9675–9680.
24. Kasai A, Bai S, Li T, Harada T (2011) Graft-transmitted siRNA signal from the root induces visual manifestation of endogenous post-transcriptional gene silencing in the scion. PLoS ONE 6: e16895.
25. Matsuda Y, Liang G, Zhu Y, Ma F, Nelson RS, et al. (2002) The commelina yellow mosaic virus promoter drives companion-cell-specific gene expression in multiple organs of transgenic tobacco. Protoplasma 229: 51–58.
26. Bai S, Kasai A, Yamada K, Li T, Harada T (2011) A mobile signal transported over a long distance induces systemic transcriptional gene silencing in a grafted partner. J Exp Bot 62: 4561–4570.
27. Kalantidis K, Denti MA, Tzortzakaki S, Marinou E, Tabler M, et al. (2007) Virpi is a host protein with a major role in potato spindle tuber viroid infection in Nicotiana plants. J Virol 81: 12872–12880.
28. Martinez de Alba AE, Sagesser R, Tabler M, Tsagris M (2003) A bromodomain-containing protein from potato specifically binds potato spindle tuber viroid RNA in vitro and in vivo. J Virol 77: 9685–9694.
29. Havelda S, Varzalay E, Valoci A, Burgyan J (2008) Plant virus infection-induced persistent host gene downregulation in systemically infected leaves. Plant J 55: 278–288.
30. Coorba T, Lőcsa R, Huván G, Burgván J (2010) Polivirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. Plant J 62: 463–472.
31. Navarro B, Gnie A, Redel MJ, Delgado S, Flores R (2012) Small RNA containing the pathogenic determinant of a chloroplast-replicating viroid guide the degradation of a host mRNA as predicted by RNA silencing. Plant J 70: 991–1003.
32. Diermann N, Matousek J, Junge M, Riesner D, Steger G (2010) Characterization of plant miRNAs and small RNAs derived from potato spindle tuber viroid (PSTVd) in infected tomato. Biol Chem 391: 1379–1390.
33. Lasser M, Parisi C, Plan D, Rodriguez-Grezco E (2012) Deployment of new biotechnologies in plant breeding. Nature Biotech 29: 231–239.
34. Harada T (2010) Grafting and RNA transport via phloem tissue in horticultural plants. Scientia Horticulturae 125: 545–550.
35. Wang M-B, Bian X-Y, Wu L-M, Liu L-W, Smith NA, et al. (2004) On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. Proc Natl Acad Sci USA 101: 3275–3280.
36. Tanaka A, Mita S, Ohira S, Kyozuka J, Shimamoto K, et al. (1990) Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron. Nucleic Acids Res 18: 6767–6770.
37. Akama K, Shiraishi H, Ohira S, Nakamura K, Okada K, et al. (1992) Efficient transformation of Arabidopsis thaliana: comparison of the efficiencies with various organs, plant ecotype and Agrobacterium strains. Plant Cell Rep 12: 7–11.
38. Sano T, Candresse T, Hammond W, Dienner TO, Owens RA (1992) Identification of multiple structural domains regulating viroid pathogenicity. Proc Natl Acad Sci USA 89: 10104–10108.