Functional analysis of a viroid RNA motif mediating cell-to-cell movement in *Nicotiana benthamiana*

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**Abstract**

Cell-to-cell trafficking through different cellular layers is a key process for various RNAs including those of plant viruses and viroids, but the regulatory mechanisms involved are still not fully elucidated and good model systems are important. Here, we analyse the function of a simple RNA motif (termed ‘loop19’) in potato spindle tuber viroid (PSTVd) which is required for trafficking in *Nicotiana benthamiana* leaves. Northern blotting, reverse transcriptase PCR (RT-PCR) and *in situ* hybridization analyses demonstrated that unlike wild-type PSTVd, which was present in the nuclei in all cell types, the trafficking-defective loop19 mutants were visible only in the nuclei of upper epidermal and palisade mesophyll cells, which shows that PSTVd loop19 plays a role in mediating RNA trafficking from palisade to spongy mesophyll cells in *N. benthamiana* leaves. Our findings and approaches have broad implications for studying the RNA motifs mediating trafficking of RNAs across specific cellular boundaries in other biological systems.

RNA plays many important roles in regulating plant physiology and development, innate defence and establishment of systemic infection by viruses and viroids [1, 2]. Certain RNAs can systemically traffic by passing through different cellular layers from one cell to another after proper processing and intracellular transport [3–9]. Systemic trafficking of RNAs is achieved through the plasmodesmata (cell-to-cell trafficking) and the plant vascular system (long-distance trafficking) [10, 11]. Increasingly, experimental evidence indicates that this process is regulated by specific RNA structural motifs that provide one possible means to regulate RNA intercellular trafficking [2, 12–14]. Identification of RNA motifs regulating intercellular RNA trafficking is crucial to help us investigate the RNA structure–function relationships [15]. In this aspect, potato spindle tuber viroid (PSTVd) provides a unique model to investigate the structure and functions of RNA motifs due to its non-coding feature and proposed secondary structures with typical loops and stems [14]. In fact, most loop structures often form recurrent three-dimensional (3D) structures through base–base interactions including non-Watson–Crick base pairing and stacking [16]. These 3D structures are functional in the interactions between RNA and RNA/protein/other ligands [16, 17]. Zhong et al. [13] identified 11 loops in PSTVd genomic RNA that are involved in its systemic trafficking in *Nicotiana benthamiana*. Loop19 containing nucleotides A135 and C227 is flanked by canonical *cis*-Watson–Crick base pairs in PSTVd genomic RNA and is categorized as an A/C loop (Fig. 1a, PSTVd-Int, GenBank accession no. NC002030). Base substitutions of A to G or C to U close this loop, resulting in viroid mutants that are able to replicate but lose the ability to move from cell to cell [13]. Interestingly, a new A/C loop is spontaneously formed in inoculated plants via mutations next to the mutated pair that restore PSTVd trafficking [18]. Thus, this A/C loop presents a simple model to investigate how a 3D structural motif may mediate an important biological function such as cell-to-cell trafficking of a non-coding RNA.

In order to gain insights into functions of loop19 in PSTVd systemic trafficking, the abilities of 15 loop19 mutants to traffic systemically were compared. Results showed that six trafficking-competent mutants and five trafficking-alteration mutants were observed, but four of them (i.e. A135/C227G, A135G/C227G, A135G/C227U and A135U/C227G) could not be detected in systemic leaves [18]. Possible explanations for these four trafficking-defective mutants include the following; (1) these mutants replicate too poorly to support systemic trafficking; (2) loop19 is required for trafficking among different cell types in the inoculated leaves, and these mutants failed to exit the inoculated leaves;
Fig. 1. Replication and trafficking analysis of loop19 mutants and wild-type of PSTVd. (a) Secondary structure of PSTVd and loop19. (b) Northern blotting for RNAs of loop19 mutants of PSTVd and PSTVd-WT from inoculated (left) and systemically infected leaves (right), respectively. Lanes 1–10, A135C/C227G; lanes 11–20, A135G/C227G; lanes 21–30, A135G/C227U; lanes 31–40, A135U/C227G; lanes 41–50, WT; M, mock; PC, positive control; c-PSTVd, (+)-circular PSTVd. (c) Representative images of whole-mount in situ hybridizations comparing replication of loop19 mutants of PSTVd and PSTVd-WT. The purple dots, some indicated by arrows, indicate viroid hybridization signals in the nuclei. Bar, 50 µm. (d) Detection of loop19 mutants of PSTVd and PSTVd-WT in inoculated and systemically infected leaves by reverse transcriptase PCR (RT-PCR). Amplification of 5.8S rRNA serves as an internal control. M, Mock. (e) Replication efficiencies of PSTVd loop19 mutants as determined by whole-mount in situ hybridization. Average numbers of PSTVd signals from those pieces of leaf were used for statistical analysis. Data are expressed as percentages of PSTVd-WT (set to 100 %), and error bars indicate standard deviations from the respective means. (f) Schematic representation of a transverse section of the N. benthamiana leaf. (g) In situ hybridization analysis of 14 µm paraffin sections of N. benthamiana leaves inoculated with PSTVd-WT, PSTVd-loop19 mutants or water (mock). The purple dots, some indicated by arrows, indicate viroid hybridization signals in the nuclei. uEp, Upper epidermis; Pm, palisade mesophyll; Sm, spongy mesophyll. Bar, 50 µm.
(3) these mutants failed to traffic long distances after leaving the inoculated leaves. In this study, replication efficiencies and trafficking abilities of the four trafficking-defective mutants of loop19 were compared by whole-mount in situ hybridization, Northern blotting and reverse transcriptase PCR (RT-PCR). In situ hybridization demonstrated that the failure of loop19 mutants to establish systemic infection is not due to deficiency in replication but rather to an inability to cross the cellular boundary between palisade mesophyll and spongy mesophyll in *N. benthamiana* leaves.

A plasmid containing a full-length cDNA derived from the type strain of PSTVd (PSTVd-Int, GeneBank accession number NC002030 and the nucleotide sequences begin at the eighty-sixth position) was constructed by Hu et al. [19] and was a gift from Dr Robert Owens. All mutants described in this study were generated by site-directed mutagenesis [13] based on this template. Plasmids containing the resulting PSTVd cDNAs were linearized by digestion with *Hind*III before use as templates for in vitro transcription using T7 RNA polymerase. In our experiments, linear in vitro transcripts of plasmids containing the full-length PSTVd cDNAs were inoculated on the expanding leaves of *N. benthamiana* plants. Replication and movement of these RNAs were analysed by Northern blotting and RT-PCR. Northern blotting allows the identification of and discrimination between linear viroid RNAs, which could be residual transcript inoculum, and circular RNAs, which are evidence of viroid replication. For RT-PCR analysis, we used primers P1F (CGGAATCAAATCGTGTTTC) and P1R (AGGAACAAACTGCGGTCCAG) that are located at nt 1–20 and 338–359 of PSTVd-Int and produce a product of 359 nt only when circular, replicating viroid RNA is present as a template in the plant. Antisense PSTVd digoxigenin-labelled riboprobes for hybridization were prepared by in vitro transcription using T7 MAXIScript kit (Ambion) and SpeI-linearized PSTVd-Int (→) plasmid as the template. All RNA transcripts were purified by MEGAClear kit (Ambion) after the DNA templates were removed by digestion using RNase-free DNase I and quantified by UV spectrometry. *N. benthamiana* plants were grown in the greenhouse maintained at 16 h light/8 h dark cycles at 27°C.

For the biological assays, RNA transcripts of each mutant were inoculated onto the upper epidermis of young greenhouse-grown *N. benthamiana* leaves (200–300 ng per leaf) dusted with carbo-rundum powder. Inocula containing wild-type PSTVd (PSTVd-WT) or water served as positive and negative controls (mock), respectively. The inoculated leaves and the connected petioles were collected at 18 days post-inoculation (p.i.), and total RNA was extracted for Northern blotting and RT-PCR analysis. Meanwhile, parts of inoculated leaves were harvested and chemically fixed for whole-mount in situ hybridization and in situ hybridization using digoxigenin-labelled PSTVd-specific riboprobes. Tissue fixation and in situ hybridization processing followed previously described protocols [20] with some modifications. PSTVd-accumulating cells were counted by visual inspection under the light microscope.

For whole-mount in situ hybridization samples, the counting was conducted in a 1×1 mm area per leaf sample, and the average of numbers from those pieces of leaf was analysed statistically for replication efficiency analysis of loop19 mutants. For thin section in situ hybridization samples, the number of PSTVd-accumulating cells was counted per section, which was 0.5×0.5 mm, and the average of numbers from those sections was also used for statistical analysis. The data were analysed by t-test using Microsoft Excel (two-tailed distribution, two-sample unequal variance). When the *P* value was <0.05, it was treated as a significantly different value and is indicated in the text. The ability of each mutant to move systemically was assayed by Northern blotting and RT-PCR analysis of RNA extracted from upper, non-inoculated leaves (i.e. eleventh and twelfth true leaves, collectively termed 'systemic leaves') harvested at 30 days p.i. The assay was repeated with three biological replicates for each mutant.

Northern blotting revealed the presence of (+)-circular PSTVd in the leaves inoculated with the four loop19 mutants of PSTVd and PSTVd-WT and its absence in the leaves inoculated with water (Fig. 1b). Whole-mount in situ hybridization also revealed the presence of PSTVd in leaves inoculated with the four loop19 mutants of PSTVd and PSTVd-WT, although the intensity of hybridization signals differed from sample to sample (Fig. 1c). RT-PCR using SuperScript III Reverse Transcriptase (Invitrogen) and Taq DNA Polymerase (Sigma) was conducted on the RNA samples from the inoculated leaves, and specific bands of 359 nt were obtained for all the four loop19 mutants of PSTVd and PSTVd-WT (Fig. 1d). Sequence analysis of the resulting RT-PCR products revealed that dominant sequences among the progenies of all the four loop19 mutants maintained the same sequence as the respective inocula (Table 1), indicating that all four mutants could replicate in inoculated leaves.

Fig. 1(e) compares the replication efficiencies of all four trafficking-defective mutants based on results from three independent whole-mount in situ hybridization analyses, and WT signal intensity was set as 100% replication efficiency. Mutant A135U/C227G exhibited the highest replication efficiency (47.3%), followed by A135G/C227U (42.6%), A135C/C227G (26.8%) and A135G/C227G (19.8%). Northern blotting and RT-PCR analysis revealed (i) the presence of all four loop19 mutants in inoculated leaves and (ii) their absence from systemic leaves; thus, none of the four loop19 mutants were capable of systemic infection. Takeda [18] has shown that a replication efficiency of 20% is sufficient to support systemic trafficking, which was also verified in this study. Although the replication efficiency of A135G/C227G mutant was somewhat lower than 20%, each of the other three mutants (A135U/C227G, A135G/C227U and A135C/C227G) exhibited replication levels high enough to achieve systemic trafficking. The failure of these three mutants to establish systemic infection is thus unlikely to be due to deficiency in replication.

One possible explanation for the failure to achieve systemic infection is that these mutants failed to exit the inoculated leaves. Failure to traffic long distances after leaving the
inoculated leaves is also a possibility. To distinguish between these two possibilities, RT-PCR was conducted with the RNA samples collected 18 days p.i. from the petioles of leaves inoculated with all the four mutants. PSTVd-WT could be detected in the petioles of inoculated leaves at 6 days p.i.; thus, 18 days p.i. should be sufficient time for the loop19 mutants to enter the petioles of inoculated leaves. There was no evidence for the presence of PSTVd in the petioles of leaves inoculated with any of the four mutants, however, suggesting that these mutants were unable to exit the inoculated leaves (data not shown). The A135G/C227G mutant had lower level of replication efficiency and showed no trafficking capability. We cannot exclude the possibility that this mutant fails to develop systemic infection due to impaired intracellular trafficking such as replication or in vivo stability.

Whole-mount in situ hybridization does not allow assessment of localization of loop19 mutants in distinct cell types, and so, the cellular boundary at which loop19 functions to mediate trafficking remained unclear. In our final analyses, inoculated N. benthamiana leaves were chemically fixed at 18 days p.i. to obtain paraffin sections for in situ hybridization. Fig. 1(f) displays the leaf cell types in a transverse view and shows that PSTVd inocula were applied to the upper epidermis. As shown in Fig. 1(g), comparison of typical transverse sections of N. benthamiana leaves inoculated with the four loop19 mutants, PSTVd-WT or water (mock) demonstrated that hybridization signals were present in the nuclei in all cell types including upper epidermal, palisade mesophyll and spongy mesophyll cells of leaves inoculated with PSTVd-WT. In contrast, all four loop19 mutants were visible only in the nuclei of upper epidermal and palisade mesophyll cells. Unlike PSTVd-WT, none of the mutants were observed in spongy mesophyll, bundle sheath or phloem cells. Finally, hybridization signals were totally absent from any cells in water-inoculated leaves. These results demonstrate that loop19 plays a role in PSTVd trafficking from palisade to spongy mesophyll cells of N. benthamiana leaves.

In summary, a symmetric A/C loop provides a useful model system in which to investigate the relationship between the 3D structure of a non-coding RNA and its biological functions. Our work provides evidence that loop19 in PSTVd plays a role in mediating RNA trafficking from palisade mesophyll to spongy mesophyll in N. benthamiana leaves. Further study of the 3D structure of this simple loop may shed light on the mechanisms involved. Our experimental approaches may be useful for identifying motifs in other RNAs that mediate trafficking between specific cells, thereby illuminating the pathogenic mechanism of plant pathogens including viruses and viroids.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

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