Molecular Plant Pathology

WILEY

Molecular Plant Pathology

SHORT COMMUNICATION OPEN ACCESS

DNA Ligase I Circularises Potato Spindle Tuber Viroid RNA in a Biomolecular Condensate

Yunhan Wang[1] | Junfei Ma[2] | Jie Hao[2] | Bin Liu[3] | Ying Wang[1,2]

[1]Plant Molecular and Cell Biology Program, University of Florida, Gainesville, Florida, USA | [2]Department of Plant Pathology, University of Florida, Gainesville, Florida, USA | [3]Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida, USA

Correspondence: Ying Wang (ying.wang1@ufl.edu)

Received: 11 October 2024 | **Revised:** 21 November 2024 | **Accepted:** 10 December 2024

Funding: This work was supported by National Science Foundation (Grants IOS-2410009, MCB-1906060, and MCB-2350392).

Keywords: biomolecular condensate | DNA ligase I | RNA processing | viroid

ABSTRACT

Viroids are single-stranded circular noncoding RNAs that mainly infect crops. Upon infection, nuclear-replicating viroids engage host DNA-dependent RNA polymerase II for RNA-templated transcription, which is facilitated by a host protein TFIIIA-7ZF. The sense-strand and minus-strand RNA intermediates are differentially localised to the nucleolus and nucleoplasm regions, respectively. The factors and function underlying the differential localisation of viroid RNAs have not been fully elucidated. The sense-strand RNA intermediates are cleaved into linear monomers by a yet-to-be-identified RNase III-type enzyme and ligated to form circular RNA progeny by DNA ligase I (LIG1). The subcellular compartment for the ligation reaction has not been characterised. Here, we show that LIG1 and potato spindle tuber viroid (PSTVd) colocalise near the nucleolar region in *Nicotiana benthamiana* protoplasts. The colocalised region is also the highly condensed region of sense-strand PSTVd RNA, indicating that PSTVd RNA and LIG1 form a biomolecular condensate for RNA processing. This finding expands the function of biomolecular condensates to the infection of subviral pathogens. In addition, this knowledge of viroid biogenesis will contribute to exploring thousands of viroid-like RNAs that have been recently identified.

Viroids, the smallest nucleic acid-based pathogens known to date, are single-stranded circular noncoding RNAs that mainly infect crops (Ma et al. 2023; Wang 2021; Ortolá and Daròs 2023). There are two families of viroids, *Pospiviroidae* and *Avsunviroidae*. Members of the two families differ by their sites of replication, structural features and replication modes (Di Serio et al. 2023). Members of *Pospiviroidae* (also termed nuclear-replicating viroids) replicate in the nucleus. Upon infection, host ViRP1 protein recognises the C-loop RNA motif in the viroid genome, and the Importin alpha4 protein shuttles the viroid/ViRP1 RNA–protein complex into the nucleus (Ma et al. 2022). Within the nucleus, viroids, aided by a specific splicing variant of TFIIIA protein (TFIIIA-7ZF), redirect host DNA-dependent RNA polymerase II for de novo RNA-templated transcription (Dissanayaka Mudiyanselage et al. 2022; Wang et al. 2016; Rackwitz, Rohde, and Sanger 1981; Dissanayaka Mudiyanselage and Wang 2020). Interestingly, there is an active shuttling process to enrich sense-strand (+) viroid intermediates, but not the minus-strand intermediates, to the nucleolar region (Qi and Ding 2003). However, the exact nature and function of this condensed RNA region remain a mystery.

For members of *Pospiviroidae*, linear multimeric intermediates are processed by a yet-to-be-identified RNase III-type enzyme to become linear monomers (Gas et al. 2008), which are subsequentially circularised by host DNA ligase I (LIG1) during the natural infection process (Nohales, Flores, and Daros 2012). LIG1 recognises a conserved position in the viroid loop E motif or equivalent positions in members of *Pospiviroidae* (Nohales, Flores, and Daros 2012; Gas et al. 2007). This processing is

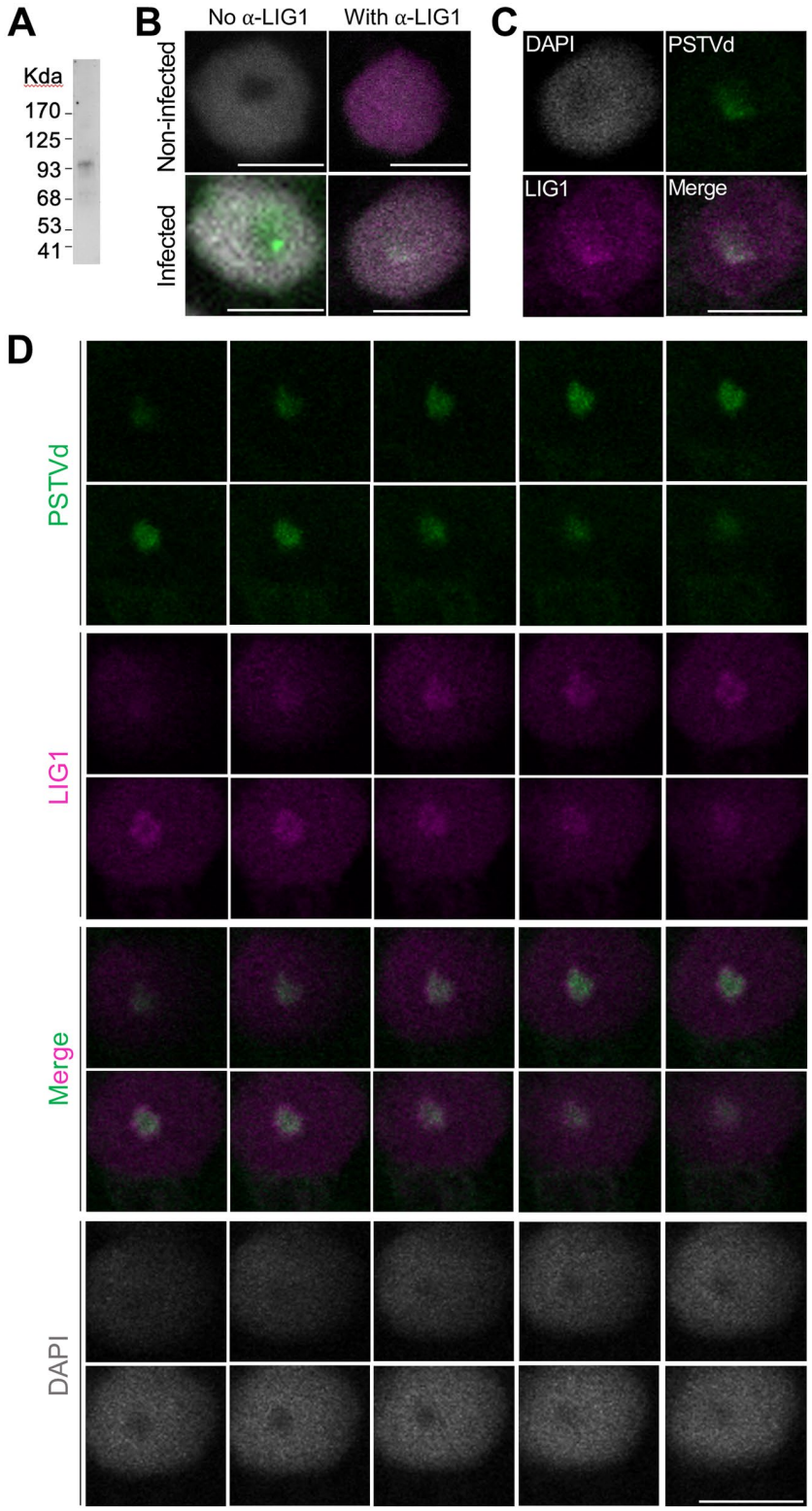

FIGURE 1 | DNA ligase 1 (LIG1) and PSTVd colocalised in the biomolecular condensate in *Nicotiana benthamiana* protoplasts. (A) An immunoblot shows the specificity of α-LIG1 primary antibody (ThermoFisherSci). (B) Test of the new method in combining immunofluorescence and RNA fluorescence in situ hybridisation. DAPI (grey) stains the nucleus. Specific PSTVd riboprobe with Alexa Fluor 488 labelling only gave signals (green) in PSTVd-infected cells. Alexa Fluor 594-conjugated secondary antibody only gave signals (magenta) in cells incubated with α-LIG1. Detailed protocol can be found in Ma et al. (2024). (C) Single channels and the merge of PSTVd and LIG1 of the bottom right image in (B). (D) A representative *z*-stack series showing the colocalisation of PSTVd (green) and LIG1 (magenta). Scale bar, 5 μm.

accompanied by a series of drastic RNA structural rearrangements (Gas et al. 2007). Despite the importance of this processing, the exact subcellular compartment for the ligation step has not been elucidated (Ma et al. 2023).

In *Arabidopsis*, LIG1 is distributed throughout most of the nucleus and is slightly enriched in the nucleolus (Li et al. 2015), as observed by using immunofluorescence (IF). Although it is convenient to observe LIG1 subcellular localisation using fluorescent protein-based live cell imaging or immunofluorescence (IF), it is technically challenging to simultaneously observe protein and RNA in plant samples, particularly for those localised in the nucleus. The MS2-MCP system has been useful to track RNA localisation in live cells; it uses bacteriophage MS2 coat protein (MCP) tagged with green fluorescent protein (GFP) to recognise a conserved RNA hairpin motif derived from the bacteriophage genome (Syed and Lim 2024). However, this system cannot be applied to viroids, because insertion of the MS2-binding hairpin eliminates viroid infectivity. The procedures of traditional IF and RNA fluorescence in situ hybridisation (FISH) are generally incompatible, because formamide and high temperature treatment in FISH denatures antibodies and hampers antibody specificity (Meyer, Garzia, and Tuschl 2017). To address this, we recently developed a new method that allows simultaneous observation of protein and RNA in the nucleus (Ma et al. 2024). In this method, we first incubate primary and Alexa Fluor-conjugated secondary antibodies with freshly prepared *Nicotiana benthamiana* protoplasts infected with potato spindle tuber viroid (PSTVd). Protoplasts are then fixed and treated with RNA FISH procedures (Qi and Ding 2002) before observation under microscope.

By using a specific antibody against endogenous LIG1 (Figure 1A), we observed the distribution of LIG1 resembling the previous report (Figure 1B). There was no signal in cells treated with secondary antibody but without primary antibody (Figure 1B), indicating that the IF result is specific. In addition, only PSTVd-infected cells showed a positive signal (Figure 1B), indicating the signal specificity of RNA FISH. Interestingly, we found that LIG1 and PSTVd mostly colocalised near the nucleolar region (Figure 1B,C), which had the strongest (+) PSTVd signal akin to the previously reported pattern of (+) PSTVd (Qi and Ding 2003).

By observing the serial *z*-stack images obtained using a laser scanning confocal microscope (Figure 1D), we further confirmed that LIG1 colocalises with (+) PSTVd only in the PSTVd-enriched region that is near the nucleolar region, inferred by the obvious hollow region in the DAPI staining. This pattern was repeatedly observed in our samples. Therefore, the PSTVd-enriched region is a highly organised of protein–RNA complexes, which is a biomolecular condensate by definition (Miao, Chodasiewicz, and Fang 2024; Banani et al. 2017). Given that LIG1 is the known enzyme catalysing the ligation reaction, this overlapping region is probably the viroid RNA ligation site. In addition, we did notice that PSTVd was not completely colocalised with LIG1 in the condensate, as indicated by the existence of a few PSTVd green dots in the merge panel in addition to the overlapping dots showing a pale white colour (Figure 1D), implying the presence of additional factor(s).

Our discovery provides answers to the long-standing question regarding the mysterious viroid enrichment near the nucleolar region. This (+) viroid-enriched region is the biomolecular condensate that contains viroid RNA (probably the sense-strand intermediates) and at least the ligation enzyme LIG1. Whether any RNase III-type enzyme also resides in the biomolecular condensate for cleavage remains to be determined after the identification of the enzyme. Interestingly, there appears to be only one PSTVd-containing biomolecular condensate in infected cells, as repeatedly observed in this study and in the previous report (Qi and Ding 2003), suggesting some level of coordination of viroid RNA processing and trafficking within the nucleus. It also explains why the minus-strand viroid RNA does not form a biomolecular condensate (Qi and Ding 2003), because minus-strand viroid RNA in the nucleus only serves as a template but is not further processed like (+) viroid RNA intermediates. It is well known that this viroid-containing biomolecular condensate is close to, but has very limited overlap with, the nucleolus as indicated by the localisation of PSTVd RNA and U3/U14 RNAs (Qi and Ding 2003). The organisation of a viroid biomolecular condensate close to the nucleolus will possibility impact the function of the nucleolus, which has been implicated by the report that viroid infection affects the 18S rRNA maturation process (Cottilli et al. 2019).

The discovery of PSTVd-LIG1 biomolecular condensate expands our understanding of pathogen-formed biomolecular condensates, which is now confirmed to occur during the infection of subviral pathogens. This supports the idea that formation of biomolecular condensates is a fundamental process to concentrate protein and RNA for specific functions. In addition, knowledge of viroid biogenesis will be useful for exploring recently discovered thousands of viroid-like RNAs (Hao, Ma, and Wang 2024; Navarro and Turina 2024).

Acknowledgements

We are grateful for the constructive comments from Svetlana Y. Folimonova at the Department of Plant Pathology, University of Florida. We apologise to colleagues whose work was not cited due to the page limits. This work is supported by US National Science Foundation (MCB-1906060, MCB-2350392 and IOS-2410009) to Ying Wang. Yunhan Wang is supported by CALS Deans' scholarship from University of Florida.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Banani, S. F., H. O. Lee, A. A. Hyman, and M. K. Rosen. 2017. "Biomolecular Condensates: Organizers of Cellular Biochemistry." *Nature Reviews. Molecular Cell Biology* 18: 285–298.

Cottilli, P., B. Belda-Palazon, C. R. Adkar-Purushothama, et al. 2019. "Citrus Exocortis Viroid Causes Ribosomal Stress in Tomato Plants." *Nucleic Acids Research* 47: 8649–8661.

Di Serio, F., R. A. Owens, B. Navarro, et al. 2023. "Role of RNA Silencing in Plant–Viroid Interactions and in Viroid Pathogenesis." *Virus Research* 323: 198964.

Dissanayaka Mudiyanselage, S. D., and Y. Wang. 2020. "Evidence Supporting That RNA Polymerase II Catalyzes de Novo Transcription Using Potato Spindle Tuber Viroid Circular RNA Templates." *Viruses* 12: 371.

Dissanayaka Mudiyanselage, S. D., J. Ma, T. Pechan, O. Pechanova, B. Liu, and Y. Wang. 2022. "A Remodeled RNA Polymerase II Complex Catalyzing Viroid RNA-Templated Transcription." *PLoS Pathogens* 18: e1010850.

Gas, M. E., C. Hernandez, R. Flores, and J. A. Daros. 2007. "Processing of Nuclear Viroids In Vivo: An Interplay Between RNA Conformations." *PLoS Pathogens* 3: e182.

Gas, M. E., D. Molina-Serrano, C. Hernandez, R. Flores, and J. A. Daros. 2008. "Monomeric Linear RNA of Citrus Exocortis Viroid Resulting From Processing In Vivo Has 5′-Phosphomonoester and 3′-Hydroxyl Termini: Implications for the RNase and RNA Ligase Involved in Replication." *Journal of Virology* 82: 10321–10325.

Hao, J., J. Ma, and Y. Wang. 2024. "Understanding Viroids, Endogenous Circular RNAs, and Viroid-Like RNAs in the Context of Biogenesis." *PLoS Pathogens* 20: e1012299.

Li, Y., C. G. Duan, X. Zhu, W. Qian, and J. K. Zhu. 2015. "A DNA Ligase Required for Active DNA Demethylation and Genomic Imprinting in *Arabidopsis*." *Cell Research* 25: 757–760.

Ma, J., S. D. Dissanayaka Mudiyanselage, J. Hao, and Y. Wang. 2023. "Cellular Roadmaps of Viroid Infection." *Trends in Microbiology* 31: 1179–1191.

Ma, J., S. D. Dissanayaka Mudiyanselage, W. J. Park, et al. 2022. "A Nuclear Import Pathway Exploited by Pathogenic Noncoding RNAs." *Plant Cell* 34: 3543–3556.

Ma, J., Y. Wang, S. Y. Folimonova, B. Liu, and Y. Wang. 2024. "Simultaneous Observation of pol II and Potato Spindle Tuber Viroid RNA in the Nucleus by Combining Immunofluorescence and RNA In Situ Hybridization." *bioRxiv*, 2024.2009.2027.615539. [Preprint].

Meyer, C., A. Garzia, and T. Tuschl. 2017. "Simultaneous Detection of the Subcellular Localization of RNAs and Proteins in Cultured Cells by Combined Multicolor RNA-FISH and IF." *Methods* 118-119: 101–110.

Miao, Y., M. Chodasiewicz, and X. Fang. 2024. "Navigating Biomolecular Condensates in Plants From Patterns to Functions." *Molecular Plant* 17: 1329–1332.

Navarro, B., and M. Turina. 2024. "Viroid and Viroid-Like Elements in Plants and Plant-Associated Microbiota: A New Layer of Biodiversity for Plant Holobionts." *New Phytologist* 244: 1216–1222.

Nohales, M. A., R. Flores, and J. A. Daros. 2012. "Viroid RNA Redirects Host DNA Ligase 1 to Act as an RNA Ligase." *Proceedings of the National Academy of Sciences of the United States of America* 109: 13805–13810.

Ortolá, B., and J. A. Daròs. 2023. "Viroids: Non-coding Circular RNAs Able to Autonomously Replicate and Infect Higher Plants." *Biology (Basel)* 12: 172.

Qi, Y., and B. Ding. 2002. "Replication of Potato Spindle Tuber Viroid in Cultured Cells of Tobacco and *Nicotiana benthamiana*: The Role of Specific Nucleotides in Determining Replication Levels for Host Adaptation." *Virology* 302: 445–456.

Qi, Y., and B. Ding. 2003. "Differential Subnuclear Localization of RNA Strands of Opposite Polarity Derived From an Autonomously Replicating Viroid." *Plant Cell* 15: 2566–2577.

Rackwitz, H. R., W. Rohde, and H. L. Sanger. 1981. "DNA-Dependent RNA Polymerase II of Plant Origin Transcribes Viroid RNA Into Full-Length Copies." *Nature* 291: 297–301.

Syed, S., and B. Lim. 2024. "Multi-Labeling Live Imaging to Quantify Gene Expression Dynamics During *Drosophila* Embryonic Development." *Methods in Molecular Biology* 2805: 137–151.

Wang, Y. 2021. "Current View and Perspectives in Viroid Replication." *Current Opinion in Virology* 47: 32–37.

Wang, Y., J. Qu, S. Ji, et al. 2016. "A Land Plant-Specific Transcription Factor Directly Enhances Transcription of a Pathogenic Noncoding RNA Template by DNA-Dependent RNA Polymerase II." *Plant Cell* 28: 1094–1107.