Viroid Affairs: Exploring the Hidden Network of RNA-Protein Interactions

V. Sánchez-Camargo[1*], K. Katsarou[2.3*], E. Bardani[2], L. Michailidou[2], S. Roussaki[2], H. van den Burg[1], K. Kalantidis[2.3]

[1] *Molecular Plant Pathology, Swammerdam Institute for Life Sciences (SILS), University of Amsterdam, 1098 XH Amsterdam, The Netherlands*

[2] *Department of Biology, University of Crete, Voutes University Campus, Heraklion, Crete, Greece*

[3] *Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Heraklion, Crete, Greece*

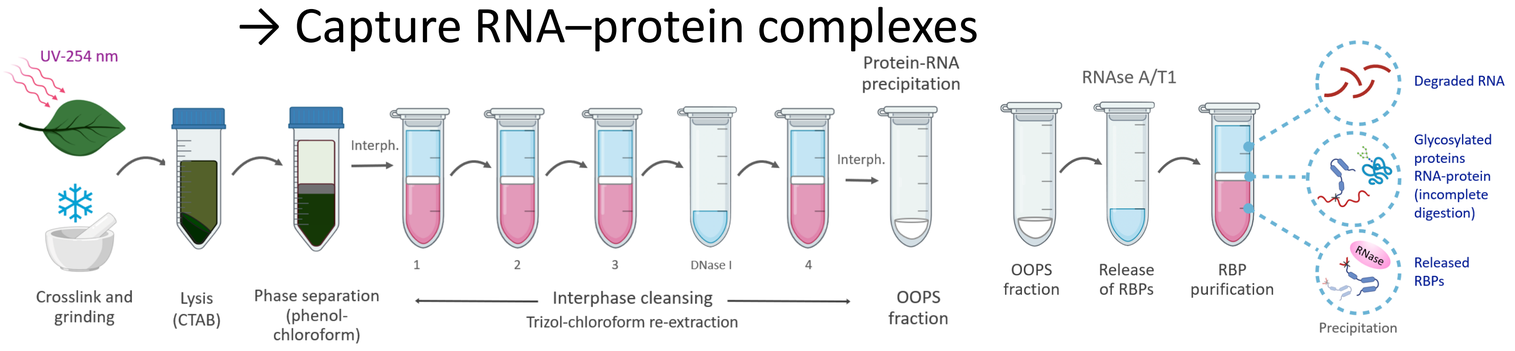
* Authors contributed equally

Viroids are commercially relevant plant pathogens characterized by an RNA genome that does not encode proteins. Despite more than five decades of viroid research, only a handful of host proteins have been identified as critical for the viroid biological cycle. Viroid-binding protein 1 (VIRP1) is one of the most studied viroid binding host factors, although a few others such as RNA Polymerase II (RNA PolII), transcription factor TFIIIA-7ZF and Phloem Protein 2 (PP2), have been described.

This study aims to identify novel viroid partners by employing a recently developed method, termed Orthogonal Organic Phase Separation (OOPS), coupled with proteomics analysis. This technique involves in vivo UV-crosslinking of leaves, stabilizing RNA-protein interactions, followed by multiple purification steps. Finally, the isolated RNA-binding proteins (RBPs) are subjected to proteomics profiling. By comparing samples from plants infected with Potato Spindle Tuber Viroid (PSTVd) and healthy *Nicotiana benthamiana* plants, we identified 125 differentially regulated RBPs. Based on their predicted biological function, a number of these were selected as possible candidates for further functional characterization using Virus-Induced Gene silencing (VIGS), followed by PSTVd challenge. Understanding how viroids exploit host factors is essential for elucidating the molecular mechanisms underlying their pathogenicity and could pave the way for developing viroid-resistant plant varieties.

A Orthogonal Organic Phase Separation: OOPS → Proteomics → Identify RNA-binding proteins

→ Capture RNA–protein complexes



Protocol for capturing the RNA-binding proteome from plants using orthogonal organic phase separation
Victor A. Sánchez-Camargo, Gertjan Kramer, Harrold van den Burg
doi: https://doi.org/10.1101/2025.07.29.667348

(A) Overview of the OOPS protocol. Plant leaves are harvested and crosslinked using UV light at 254 nm. The tissue is then frozen and ground, and lysis is performed using a custom CTAB buffer, followed by clarification of the lysate. Phase separation with phenol-chloroform produce two main phases (aqueous and organic) and an interphase containing crosslinked RNA-protein complexes. Successive rounds of organic re-extraction (Trizol-chloroform cycles) remove contaminants from the interphase and DNase treatment eliminates residual DNA. RNA-protein complexes are then subjected to RNase A/T1 digestion at 37 °C for 3 h, and the reaction is stopped with Trizol LS and chloroform, releasing RNA-binding proteins (RBPs). Released proteins are precipitated, resuspended in triethylammonium bicarbonate (TEAB), and processed for proteomics. Sample preparation includes reduction, alkylation, and trypsin digestion prior to LC-MS/MS analysis.

Selection

B

Results

→ **125** uniquely identified proteins in PSTVd-infected *Nicotiana Benthamiana* plants

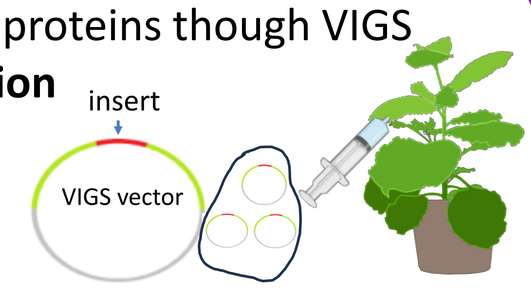
→ At least 10 key proteins chosen for further study

Proteins
Protein A
Protein B
Protein C
Protein D
Protein E
Protein F
Protein G
Protein H
Protein I
Protein J

(B) Selection of candidate proteins from PSTVd-infected *Nicotiana benthamiana*. Proteomic analysis identified 125 unique proteins, of which 10 were prioritized for initial study. These were chosen as they represent diverse functions relevant to host-pathogen interactions, and additional candidates will be tested in future work.

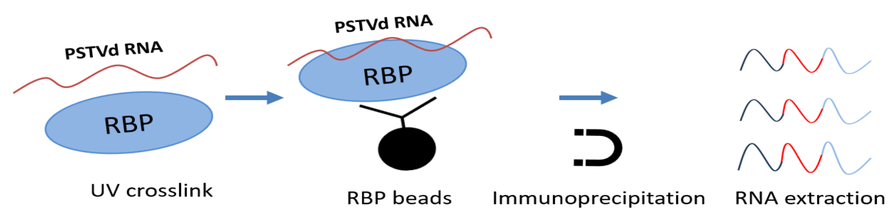
Functional Validation

C Test candidate proteins though VIGS **+ PSTVd infection**



(C) Functional validation of candidate proteins using VIGS and PSTVd infection. Candidate genes were cloned into a VIGS vector (insert indicated in red) and delivered into plants by Agrobacterium-mediated infiltration. Gene-silenced plants were then challenged with PSTVd infection to assess the contribution of candidate proteins to host-pathogen interactions.

D Test candidate proteins + PSTVd through RIP



(D) Functional validation of candidate proteins using RNA immunoprecipitation (RIP). PSTVd RNA was UV crosslinked to candidate RBPs, which were captured on RBP-specific beads. Following immunoprecipitation, bound RNAs will be extracted and analyzed to determine interactions between candidate proteins and PSTVd RNA.

Conclusions

- Adapted the **OOPS method** for studying viroid–host RNA–protein interactions.
- Using OOPS and proteomics, identified **125 candidate RBPs** associated with PSTVd.
- Selected the first **10 top candidates** for functional validation with VIGS.
- Developing an optimized RIP protocol for improved RNA-protein validation.
- **Findings reveal potential novel host factors** and inform strategies for viroid control.

ViroiDoc project is funded by the European Union within the Horizon Europe MSCA Doctoral Networks, Reference Number HORIZON-MSCA-2023-DN-01-01, Marie Curie Grant Agreement Number: 101169421. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or European Research Executive Agency. Neither the European Union nor the granting authority can be held responsible for them.