LETTER

Symptomatic plant viroid infections in phytopathogenic fungi: A request for a critical reassessment

Pedro Serra[a], Alberto Carbonell[a], Beatriz Navarro[b], Selma Gago-Zachert[c], Shifang Li[d,e], Francesco Di Serio[b,1], and Ricardo Flores[a,1]

Since their discovery (1), viroids—small (~250 to 430 nt), non–protein-coding, circular RNAs—are thought to infect and cause disease only in plants (2); thus, the report that they infect and incite symptoms in filamentous phytopathogenic fungi (3) is surprising. Viroids are classified into two families (4). Members of the *Pospiviroidae*, including potato spindle tuber viroid (PSTVd) (1, 5), replicate in the nucleus through an RNA–RNA rolling-circle mechanism catalyzed by host enzymes (RNA polymerase, RNase, and RNA ligase). Members of the *Avsunviroidae*, like peach latent mosaic viroid (PLMVd) (6), form hammerhead ribozymes (HHRz) that functionally substitute the RNase during replication in chloroplasts (4). The host range of the second family is restricted to plant species (or relatives) in which the viroids were described. Unexpectedly, Wei et al. (3) report that seven viroids, including PLMVd and avocado sunblotch viroid (ASBVd) (both of the *Avsunviroidae*), infect *Nicotiana benthamiana*, a known host for only some members of the *Pospiviroidae*.

Here, we inoculated blocks of *N. benthamiana* with the following: 1) buffer, 2) an ASBVd preparation from infected avocado leaves containing circular forms, 3) dimeric head-to-tail transcripts of the same PSTVd variant (3), and 4) dimeric head-to-tail transcripts of PLMVd variant gds6 infectious to peach (7). The dimeric PLMVd RNAs produced during transcription the expected fragments from self-cleavage by the two HHRz (including the full-length monomeric forms with the specific termini needed for the circularization by a chloroplastic tRNA ligase) (8), while those of PSTVd remained unprocessed (Fig. 1*A*). Conversely, the inocula used before (3) were monomeric RNAs with arbitrary termini flanked by vector sequences that may reduce/abolish their infectivity (8, 9) and that were not bioassayed in diagnostic host plants (3). In a second experiment, blocks of *N. benthamiana* were inoculated, instead of mechanically, by agroinfiltration (far more efficient) with *Agrobacterium tumefaciens* harboring plasmids for expressing dimeric head-to-tail transcripts of PSTVd, PLMVd, and ASBVd. In both experiments, only PSTVd infected *N. benthamiana*, as revealed by RNA gel-blot hybridization and RT-PCR (Fig. 1 *B* and *C*).

A limitation of the results in question (3) is that they were obtained by RT-PCR, prone to generate false positives (potentially explaining some of the observations in *N. benthamiana* and fungi). Only one RNA gel-blot hybridization is shown (ref. 3, figure S4B), with the doublets attributed to circular and linear forms of ASBVd and hop stunt viroid (HSVd) (*Pospiviroidae*), but without controls from infected plants (3). Debilitation of *Valsa mali* growth and virulence by HSVd (3) should be substantiated with sequence analysis of the viroid progeny, expected to accumulate changes in its adaptation to the fungus. Additionally, the seven tested viroids are reported to infect *Saccharomyces cerevisiae* (3), extending a proposal for ASBVd (10). However, that detecting ASBVd after 25 yeast generations (10) reveals autonomous viroid replication (launched from a plasmid) is difficult to reconcile with observations in avocado, wherein RNA polymerization, self-cleavage, and circularization of both ASBVd strands occur in chloroplasts, absent in yeasts and filamentous fungi (3).

Therefore, a reassessment (with a second detection technique, appropriate controls, and further experiments) is needed to support infectivity and symptoms of viroids in fungi. Extraordinary claims demand extraordinary evidence.

[a]Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas–Universidad Politécnica de Valencia, 46022 Valencia, Spain; [b]Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche, 70126 Bari, Italy; [c]Department of Molecular Signal Processing, Leibniz Institute for Plant Biochemistry, 06120 Halle (Saale), Germany; [d]Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; and [e]Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China

Author contributions: F.D.S. and R.F. designed research; P.S. performed research; A.C. and S.G.-Z. contributed new reagents/analytic tools; P.S., A.C., B.N., S.G.-Z., S.L., F.D.S., and R.F. analyzed data; and P.S., A.C., B.N., S.G.-Z., S.L., F.D.S., and R.F. wrote the paper.

The authors declare no competing interest.

[1]To whom correspondence may be addressed. Email: rflores@ibmcp.upv.es or francesco.diserio@ipsp.cnr.it.

First published April 21, 2020.

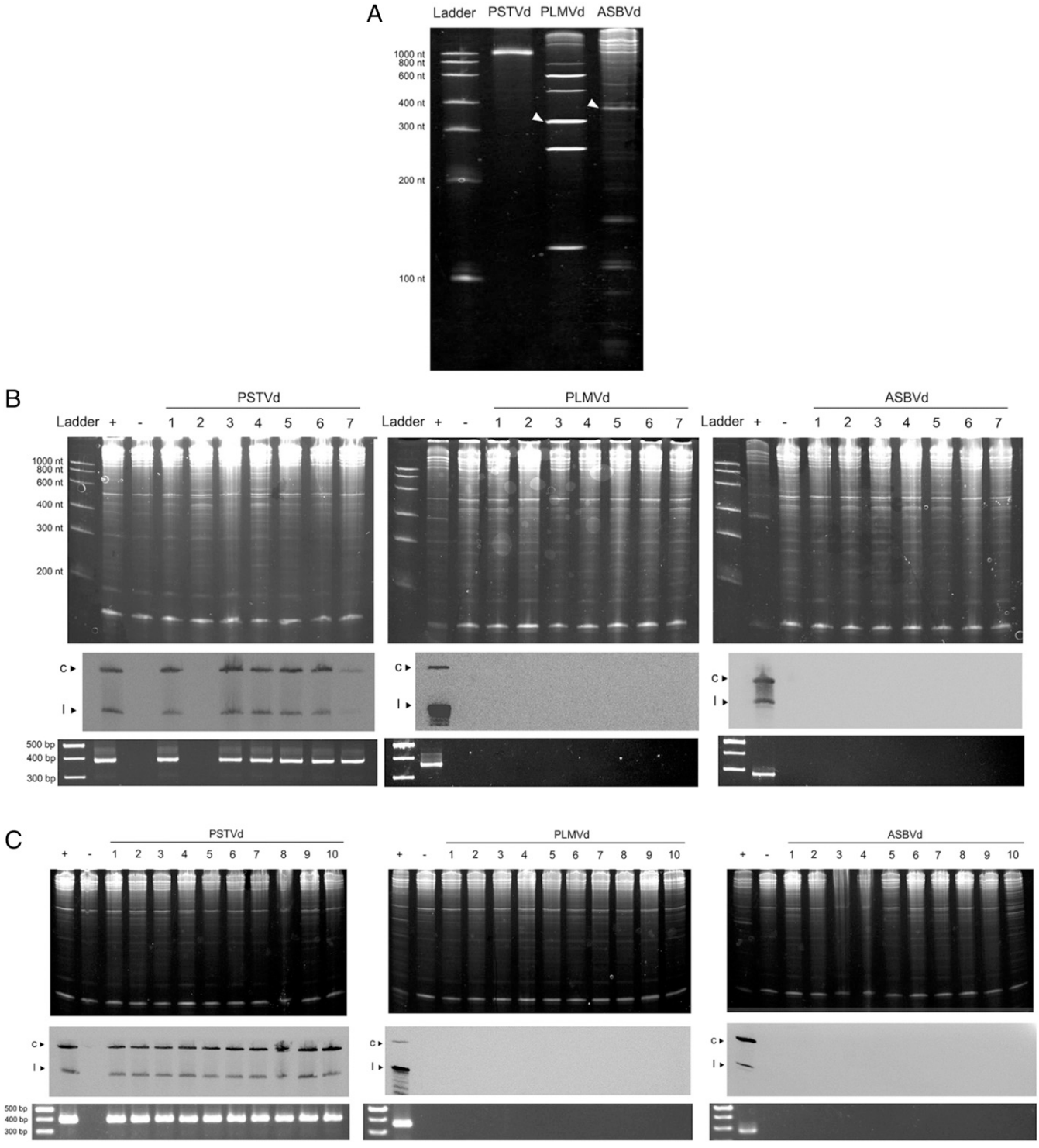

Fig. 1. (*A*) Denaturing 5% PAGE stained with ethidium bromide showing preparations used for mechanical inoculations. PSTVd and PLMVd, dimeric head-to-tail transcripts of PSTVd (variant X00009, 359 nt) and PLMVd (variant AJ005303, 337 nt), respectively, with the PLMVd self-cleaved monomeric linear forms marked by one arrowhead; ASBVd, preparation from ASBVd-infected avocado, with the monomeric circular forms (variant J02020 with a C213→U substitution, 247 nt) marked with one arrowhead; ladder, RNA size markers. (*B*) Blocks of *N. benthamiana* plants mechanically inoculated with PSTVd, PLMVd, and ASBVd examined by denaturing 5% PAGE stained with ethidium bromide (*Upper*) followed by RNA gel-blot hybridization with riboprobes for detecting viroid plus strands (*Middle*, with viroid circular and linear forms indicated with c and l), and by RT-PCR (*Lower*) with pairs of viroid-specific abutted primers containing nonviroid 5′-tails. Symbols (+) indicate PSTVd-infected *N. benthamiana*, PLMVd-infected peach, and ASBVd-infected avocado, and (−) mock-inoculated *N. benthamiana*. The ladders in *Upper* and *Lower* panels are RNA and DNA size markers, respectively. (*C*) Assays, performed and shown as in *B*, of blocks of *N. benthamiana*, agroinoculated with *Agrobacterium tumefaciens* harboring plasmids for expressing head-to-tail dimeric transcripts of PSTVd, PLMVd, and ASBVd (the same variants as in *B*).

1 T. O. Diener, Potato spindle tuber "virus." IV. A replicating, low molecular weight RNA. *Virology* **45**, 411–428 (1971).
2 A. Hadidi, R. Flores, J. W. Randles, P. Palukaitis, Eds., *Viroids and Satellites* (Academic Press, London, 2017).
3 S. Wei *et al.*, Symptomatic plant viroid infections in phytopathogenic fungi. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 13042–13050 (2019).
4 R. Flores, S. Gago-Zachert, P. Serra, R. Sanjuán, S. F. Elena, Viroids: Survivors from the RNA world? *Annu. Rev. Microbiol.* **68**, 395–414 (2014).
5 H. J. Gross *et al.*, Nucleotide sequence and secondary structure of potato spindle tuber viroid. *Nature* **273**, 203–208 (1978).
6 C. Hernández, R. Flores, Plus and minus RNAs of peach latent mosaic viroid self-cleave in vitro via hammerhead structures. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3711–3715 (1992).
7 S. Ambrós, C. Hernández, R. Flores, Rapid generation of genetic heterogeneity in progenies from individual cDNA clones of peach latent mosaic viroid in its natural host. *J. Gen. Virol.* **80**, 2239–2252 (1999).
8 M. A. Nohales, D. Molina-Serrano, R. Flores, J. A. Daròs, Involvement of the chloroplastic isoform of tRNA ligase in the replication of viroids belonging to the family *Avsunviroidae*. *J. Virol.* **86**, 8269–8276 (2012).
9 M. A. Nohales, R. Flores, J. A. Daròs, Viroid RNA redirects host DNA ligase 1 to act as an RNA ligase. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 13805–13810 (2012).
10 C. Delan-Forino, M. C. Maurel, C. Torchet, Replication of avocado sunblotch viroid in the yeast *Saccharomyces cerevisiae*. *J. Virol.* **85**, 3229–3238 (2011).