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Discovery of Viroids and Viroid-Like RNAs in Plants

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Viroids are the smallest infectious circular RNAs (circRNAs). They lack protein-coding capacity but can autonomously replicate in host cells by hijacking the host transcription mechanism (Hao et al. 2024). Some viroids encode hammerhead ribozymes (HHRs) in both polarities, mediating the cleavage of oligomeric transcripts in the replication (Di Serio et al. 2017; Zhang et al. 2025). Given the small circular genome (234–434 nt) and the HHRs, viroids were regarded as the relics of the RNA World (Diener 2016; Flores et al. 2022). Recently, a large number of viroid-like RNAs were identified from animals, fungi, humans and environmental samples (Edgar et al. 2022; Forgia et al. 2023; Lee et al. 2023; Zheludev et al. 2024), indicating the great genetic diversity and providing an evolutionary space to understand the origin and evolution of viroids. However, only four known viroids were retrieved from the 1000 Plant transcriptome (1KP) dataset and few viroid-like RNAs were identified from plants (Lee et al. 2023; Raza et al. 2025), leaving their full extent in plants largely unexplored. Here, we identified three new HHR-containing viroids and more than 30 viroid-like RNAs in plants, expanding the diversity of these elements in the plant kingdom.

We initially conducted a BlastN search against own RNA sequencing (RNA-seq) data and transcriptome shotgun assembly sequence databases of green plants (taxid: 33090) in GenBank using the HHR sequences of viroid and viroid-like RNAs. Following filtering for sequences encoding HHRs in both polarities and clustering at 90% nucleotide identity, 43 unique candidates were obtained. Most (37) of these RNAs were predicted to be circular due to 5′-3′ overlaps (Tables S1 and S2). These 43 novel HHR-containing RNAs, ranging from 273 to 996 nt in size, share no significant identity with any entries in GenBank and are distinct from previously documented sequences (Lee et al. 2023; Raza et al. 2025). They were found in 16 plant species across different families (Figure S1–S3, Table S1), including *Ulva lactuca*, a green alga presumed to be an ancestor of higher plants. The circular RNA identified in *U. lactuca* was 468 nt in length with a GC content of 58.8% (Figure S3B). To date, this is the first report of a viroid-like RNA in a lower plant.

Five circular RNAs identified in own data were experimentally verified, resulting in the discovery of three new viroids, rose

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Zhiyou Xuan, Fei Xing and Xinying Yang contributed equally to this work.

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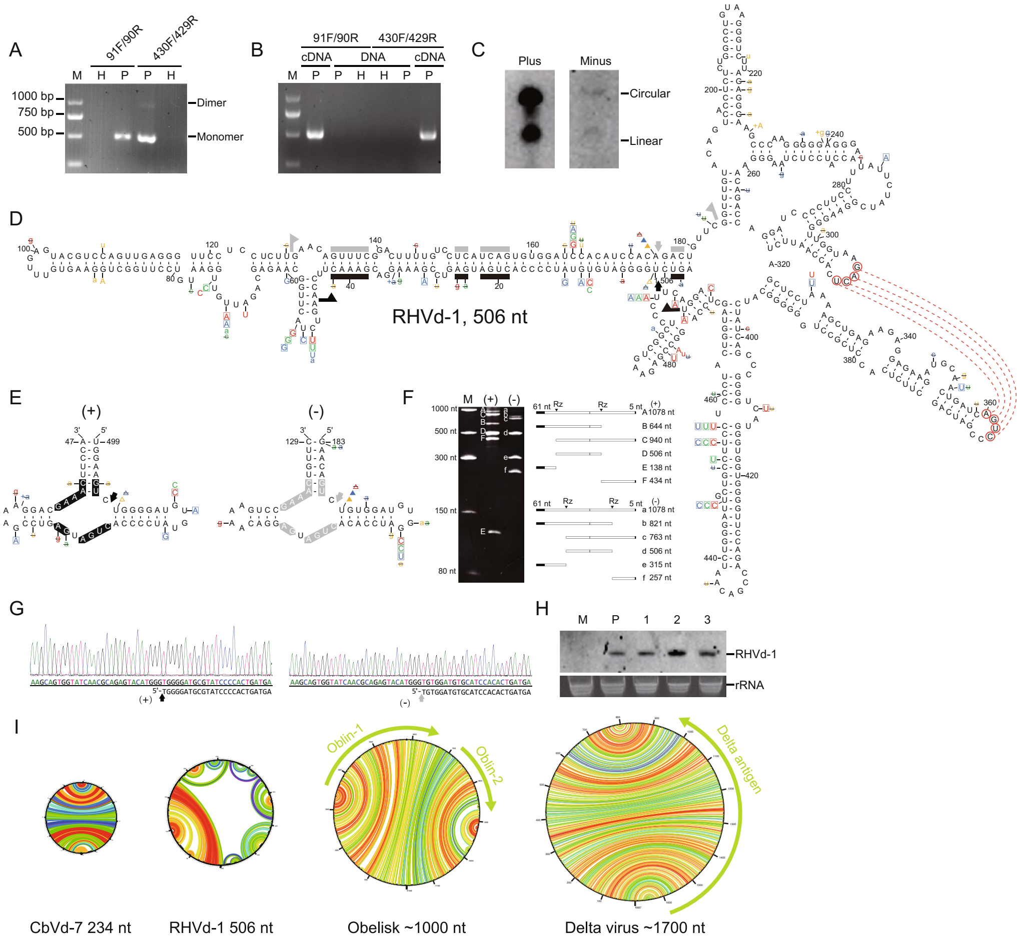

FIGURE 1 | Molecular characterisation of RHVd-1. (A) One step RT-PCR detection of RHVd-1 using RNA preparation from healthy plant (H) and positive plant (P), M: DL2000 marker. (B) PCR detection of DNA from H and P, cDNA of positive plant was used as positive control. (C) Northern blot detection of circular and linear forms of both polarity strands of RHVd-1. The total RNA of positive plant was separated by 8% denaturing polyacrylamiyde gel electrophoresis (PAGE). (D) Predicted secondary structure of RHVd-1. The HHR region and the conserved nucleotides are indicated by flags and boxes, the self-cleavage sites are marked by arrows, black and white symbols refer to plus and minus strands, respectively. The red dashed lines refer to a potential kissing-loop interaction. Site mutations of clones sequenced from different positive plants are denoted by different colour characters and symbols, the symbols (+) and (▲/△) refer to insertions and deletions, the upper characters and (▲) denote these changes occurred in more than 50% clones, the lower characters and (△) denote less than 50% clones. The changes within symbols (□) or with a strikethrough denote they were found in 100% clones or only in one clone, respectively. (E) Predicted structure of hammerhead ribozymes in (+) and (−) polarities of RHVd-1. (F) In vitro self-cleavage analysis of RHVd-1, the dimeric (+) and (−) transcripts and self-cleavage products were separated by 8% denaturing PAGE and stained by ethidium bromide, their expected size are shown in the right of the panel. The black and white bars denote vector and viroid sequences, respectively, the arrowheads denote the ribozyme (Rz) self-cleavage sites. (G) Determination of the self-cleavage sites by 5′ RACE. (H) Northern blot detection of plants inoculated by dimeric transcripts of RHVd-1, 1–3: Three RHVd-1 inoculated plants, M, the mock-inoculated plants; P, positive control. (I) Genome organisation of the smallest (coleus blumei viroid 7, CbVd-7) and largest (RHVd-1) viroid, and two kinds of viroid-like RNAs, obelisk, and delta virus.

hammerhead viroid 1, 2 (RHVd-1, RHVd-2) and lychee hammerhead viroid (LHVd), and two new viroid-like RNAs, *psidium guajava* hammerhead viroid-like RNA 1 (PgHVd-LR1) and citrus hammerhead viroid-like RNA 2 (CHVd-LR2).

These five circular RNAs were successfully amplified by RT-PCR of extracted total RNAs using two pairs of adjacent primers (Table S3), but not by PCR of DNA preparation (Figure 1A,B and Figures S4–S7). The sense strand of RHVd-1, RHVd-2, LHVd and PgHVd-LR1, and their circular forms were identified by northern blot hybridization (Figure 1C, Figures S4B, S5C, S6C,D). Their incidences in the field were 15.6% (10/64) for RHVd-1, 4.8% (12/252) for RHVd-2, 26.6% for (17/64) LHVd, 5.7% (7/122) for PgHVd-LR1, and 1.2% (1/82) for CHVd-LR2. These results confirm their genuine existence as exogenous, circular RNAs.

Sanger sequencing confirmed the complete genomes of RHVd-1 (506 nt), RHVd-2 (485 nt), LHVd (483 nt), PgHVd-LR1 (622 nt) and CHVd-LR2 (323 nt), each with approximately 50% G+C content (Table S1). RHVd-1, RHVd-2, LHVd and PgHVd-LR1 were predicted to adopt branched secondary structures with a base-paired arm that encodes HHRs in both polarities and a highly branched region with potential kissing-loop interactions (Figure 1D, Figures S4C, S5D, S6E), similar to the secondary structure of pelamoviroids (Di Serio et al. 2017). CHVd-LR2 was predicted to assume a quasi-rod conformation (Figure S7C). The HHRs embedded in these RNAs really have enzyme activities since the in vitro transcripts self-cleaved into expected bands (Figure 1F, Figures S4E, S5F, S6G and S7E). The cleavage sites were verified by 5′-RACE analysis of the cleaved fragments (Figure 1G, Figures S4F, S5G and S6H, S7F).

Cloning and sequencing, followed by sequence alignment, showed that the populations of the five circular RNAs contained different variants, reflecting distinct genetic diversity within the corresponding plants (Figure 1D, Figure S8–S11). Most of the polymorphic positions located in loops, co-mutated for a base pair and converted canonical into wobble base pairs, or vice versa, keeping the stability of the proposed secondary structures (Figure 1D, Figures S4C, S5D and S7C). Thus, these circular RNAs may replicate in plants. Bioassays verified the autonomous replication of RHVd-1, −2 and LHVd. Dimeric in vitro transcripts of the five circular RNAs were inoculated into the corresponding healthy plants by slashing young stems. Northern blot of the upper non-inoculated leaves detected RHVd-1 and RHVd-2 in China rose, with infection rates of 10% (3/29) and 41% (9/22), respectively, and LHVd in lychee plants with an infection rate of 32% (7/22) (Figure 1H, Figures S4G, S5H). No obvious symptoms were observed in the positive plants. PgHVd-LR1 and CHVd-LR2 failed to infect seedlings of *Psidium guajava* and citrus, even under different inoculation methods, including slashing inoculation, grafting and agroinfiltration.

The viroid-like circular RNAs identified from sequencing data of grapevine and *Areca catechu* were not detected in field-collected samples (Figures S1 and S2). In the sRNA-seq data from one grapevine, two of the circular RNAs and a narna-like mycovirus exhibited similar sRNA profiles that differed from those of plant viroids and viruses (Figure S12A). The differences were mainly reflected in abundance (< 2000 reads vs. > 10 000 reads), primary sizes (22 nt and 23 nt vs. 21 nt and 22 nt), and predominant nucleotides at the 5′ terminal (U vs. C and U). For sRNA profiles of the five circular RNAs detected in field plants (Figure S12B), RHVd-1, RHVd-2 and LHVd resembled plant viroids and viruses, whereas PgHVd-LR1 and CHVd-LR2 were similar to the two circular RNAs from grapevine. These results suggest that sRNA profiles can, to some extent, distinguish bona fide viroids from viroid-like circular RNAs.

Based on these observations, we conclude that RHVd-1, −2 and LHVd are three new viroids. Given their HHR activities, branched secondary structures, potential kissing-loop interactions, distinctive sRNA profiles, and the phylogenetic status (Figure S13), they should belong to the genus *Pelamovirod* in the family *Avsunviroidae* (Di Serio et al. 2017). PgHVd-LR1 and CHVd-LR2 are probably associated with microorganisms in guava and citrus.

In conclusion, we experimentally identified three new viroids and two new viroid-like RNAs and found more than 30 viroid-like RNAs in plants including a green alga, further expanding the known diversity of HHR-containing viroids and viroid-like circular RNAs in plants. The novel viroid RHVd-1 has the largest genome among known viroids, extending the upper size limit by 16.6%—from 434 nt (apple hammerhead viroid, AHVd) to 506 nt. This discovery narrows the size gap between viroids and viroid-like circular RNAs with known hosts (e.g., obelisks; Figure 1I). Although most of the viroid-like RNAs we identified have yet to be experimentally verified, their findings highlight that HHR-containing circular RNAs are more diverse in plants than previously expected. Thus, the growing number of viroid-like circular RNAs being discovered will further blur the boundaries between viroids and other RNA species.

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Data Availability Statement

The data reported in this paper have been deposited in the GenBase in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences/China National Center for Bioinformation, under accession number C_AA125966.1 to C_AA126007.1 and C_AA126280.1 that is publicly accessible at https://ngdc.cncb.ac.cn/genbase.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1. Figure S2. Figure S3. Figure S4. Figure S5. Figure S6. Figure S7. Figure S8. Figure S9. Figure S10. Figure S11. Figure S12. Figure S13. Tables S1–S3.**