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SYNTHESIS AND CHARACTERIZATION OF NON-INFECTIOUS HOP LATENT VIROID CIRCULAR RNA USING GROUP I SELF-SPLICING RIBOZYME SYSTEM

Aim. *Hop latent viroid (HLVd) poses a major threat to Cannabis sativa cultivation, with up to 90% infection rates and 70% THC loss. Reliable non-infectious positive controls are needed for diagnostics.* ***Methods.*** *We synthesized non-infectious HLVd circular RNA using a group I self-splicing ribozyme system. The product was characterized by RNase R digestion, RT-PCR, and sequencing to confirm circularization and the ribozyme-derived joining sequence.* ***Results.*** *Structural analysis showed the synthetic RNA retained high stability (ΔG = –90.4 kcal/mol) compared to native HLVd (ΔG = –93.8 kcal/mol), with introduced mutations preventing replication. The molecule remained stable from –20 to 37 °C for 10 days and performed consistently as a PCR positive control. The infection assays in cannabis confirmed its non-infectious nature.* ***Conclusion.*** *The synthetic HLVd circRNA provides a safe, stable positive control for diagnostics and viroid research, addressing a key gap in current detection methods.*

Keywords: *Hop latent viroid, cannabis, Group I Permutated Intron, in vitro transcription, circRNA.*

Introduction

Hop latent viroid (HLVd) was for the first time found in 1987 in two commercially grown hop cultivars (*Humulus lupulus* L.) in the northern region of Leon in Spain [1]. HLVd can affect a wide range of plants such as cucumber, grapevine, citrus, plum, peach, pear, apricot, almond [2] and

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since 2019 HLVd has been detected in Cannabis (*Cannabis sativa* L.) cultivation in California [3, 4].

HLVd is a single-stranded RNA molecule, 256 nt long that belongs to the *Cocadviroid* family of *Pospiviridae* [5, 6]. The molecule is covalently closed in a circular RNA secondary structure. HLVd is missing of the protein coat, thus for the replication it relies on the host RNA polymerase II [7, 8]. Based on the recent study by Adkar-Purushothama analyzing the sequences available on the NCBI database, only two HLVd sequence variants can be distinguished into two variants affecting cannabis. There is only one base pair mismatch between the two isolated sequences, that does not influence its RNA secondary structure [9].

HLVd remains symptomless in hop plants, but α-bitter acid and essential oil content in cones are significantly reduced, affecting hop market value [10]. In Cannabis, only in the susceptible cultivars HLVd shows symptoms of so called "dudding" disease. The described symptoms are: shorter internodal spacing, smaller leaves, stunting, malformation, chlorosis, brittle stems, reduced vigor, lower water intake, reduced flower mass and trichomes [3, 4]. At the flowering stage the susceptible plants typically show smaller and loosed buds, weaker flower smell and less trichome production. These symptoms reflect a loss of fiber production in industrial hemp varieties, and the reduction of cannabinoid contents in medical varieties, up to 70% in THC content. HLVd cannot be transmitted by seeds, but can be easily transmitted across plants by a horizontal-mechanical process such as wounds or infected cutting tools. This results in the spread of HLVd through agamic propagation from affected mother plants [11, 12].

Based on a survey carried out in California on 200.000 samples, approximately 90% of cannabis-growing facilities were positive for HLVd, of which 30% showed symptoms of the viroid's infection. A recent study analyzed the incidence of HLVd in cannabis plants from 9 different provinces of Canada between 2020 and 2022. In this research both symptomatic and asymptomatic plants were analyzed and the pathogen has been detected in 8 of the 9 provinces with an overall country wide frequency of 25.6%. The highest number of positive samples were in two regions with the greatest cannabis production (British Columbia and Ontario), followed by Alberta and Quebec, which showed infection frequencies ranging from 11.4% to 34.7% [13, 14]. These data indicate that the viroid is widespread in Canada, where the world's largest cannabis production for cannabinoids is located , resulting in a serious disease that impacts cannabis economic value.

Because HLVd is asymptomatic, it is a challenge to sustain it from the cultivation facilities. Routine scouting for the presence of disease symptoms and testing stock plants for the presence of HLVd is highly recommended. Early detection of the disease symptoms in the stock plants is important to prevent pathogens from spreading within the growing environment. There are several diagnostic approaches that have been described to detect cannabis pathogens [13, 14]. As shown by Buirs and Punjia in recent research, if plants are regularly tested (weekly) and stock plants are replaced every 3—4 months of production, and pathogen-free plants and infected plants are removed, a gradual decline in the occurrence of the diseased stock plants can be achieved [15]. Molecular diagnostic methods should be used to ensure that vegetative plants are not infected by the viroid [13, 14, 16].

The aim of this research is to synthesize *in vitro* HLVd circRNA molecule. Construction of small synthetic circRNA could be done chemically by using condensing agents such as cyanogen bromide, or through enzymatic methods using RNA or DNA ligases, or alternatively through ribozymatic methods using self-splicing introns such as Group I introns [17—19].

Circularly permutated group I introns are self-splicing elements that have been found first in *Anabena*, a filamentous nitrogen-fixing cyanobacterium [20]. In particular, group I introns are small RNAs (250—500 nt) characterized by a

linear array of conserved structural features, that through two successive transesterifications in a well defined splicing pathway can self-excise from the precursor RNA [21—23]. Group I introns are capable of self-splicing from the precursor RNA without the assistance of any proteins, thus are not influencing the expression of the host gene, but in some cases these genetic elements could be mobile, capable of transpose in homologous copies through the host genome [24]. Synthetic viroid-like ribozymes have been designed that act in *trans* to cleave RNA sequences of interest. These synthetic ribozymes consist of a target-binding domain, a catalytic domain, and a structural domain [25].

This research illustrates for the first-time how the group I introns approach has been successfully used to produce HLVd circRNA molecule. The stability of the molecule has been confirmed in a range of temperatures between –20 and 37 °C for a period of 10 days. Finally, its possible infectivity has been tested on Cannabis cultivar.

Materials and Methods

Materials. All consumables, DNA/RNA purification kits from Shanghai Gene Era Bio-Science Co., Shanghai, China. Primers synthesis service provided by Bioligo Biotechnology Co., Shanghai, China. Gene synthesis service provided by Sangon Biotech Co., Shanghai, China.

Methods. DNA template linearized and in vitro transcription. Sequence coding for HLVd genome RNA (GeneBank accession number NC_003611), was custom synthesized, and cloned into pUC-57 vector between T4 5' and 3' thymidylate synthase (td) group I intron [23] under control of T7 promotor sequence. The primers T7P and T7T (Table 1) were used for PCR amplification with *TaKaRa Taq* HS Perfect Mix, amplification program: 95 °C for 2 min (95 °C for 5 s, 55 °C for 5 s and 72 °C for 30 s) × 30 cycles. After polymerase chain reaction the PCR product was run in 2% agarose gel at 150 V for 20 minutes. The target band of the linearized DNA template was cut (613 bp) and cleaned up using DNA Gel extraction kit (Shanghai Gene Era Bio-Science Co.). Purified DNA (100ng) was added to 100 μl *in vitro* transcription (IVT) reaction mixes together with T7 RNA Polymerase (Jiangsu Shenji Biotechnology Co.). The reaction mixture was incubated for 1 hour at 37 °C, the template was digested adding 1μl of DNAse I (2.5 mg/ml) (Sangon Biotech Co.) for 10 minutes at 37 °C.

In vitro cyclization and RNAse R digestion. IVT RNA was column purified by the RNA Clean up Kit. The cyclization reagent was prepared as follows: 50mM Tris-HCl (pH 8.0), 10 mM MgCl, 2 mM GTP, 1mM DTT. Cyclization reagent was mixed with circRNA precursors and incubated at 55 °C for 20 minutes. Then, the RNAse R (Shanghai Gene Era Bio-Science Co.) was added to digest linear RNA, the sample was incubated for 30 minutes at 37 °C. At each step, the products were analyzed on agarose as described above. Then, the circRNA was precipitated from the aqueous phase using 1/10 volumes of 3 M NaOAc (pH 5.2) and 2 volumes of isopropyl alcohol, thus the pellet was dissolved in water and RNA concentration was determined by measuring the absorbance at 260 nm (Nano-300 Allsheng Instrument Co.).

Reverse transcription and cDNA synthesis. One step reverse transcription-polymerase chain reaction (RT-PCR) was carried out with One step RT-PCR Kit (Shanghai Gene Era Bio-Science Co.) using a pair of primers specific for the whole region of putative circRNA (Table 1) and according to the following program: 50 °C 10min, 95 °C 5 min, (95 °C 5 s 55 °C 5 s 72 °C 30 s) × 30 cycles. A part of the amplification products was checked on agarose as described above and another part was sent for Sanger sequencing (Sangon Biotech Co.).

CircRNA stability. The circRNA of HLVd was placed on the different temperature conditions (–20 °C, 4 °C, 25 °C, 37 °C) for 10 days. One step RT-qPCR was carried out every day using a pair of primers and probe specific for circRNA (Table 1). One step RT-qPCR Kit, was used to perform PCR program as above (Shanghai Gene Era Bio-Science Co.).

Serial dilutions and sensibility test of RT-PCR kit. The obtained circRNA of HLVd has been serially diluted from 10^5 to 10 genome copies number per µl to evaluate the sensitivity of the RT-PCR and spiked into the cannabis leaves lysate, to mimic infected plant material. RT-PCR was performed with RT-HLVd primers and probes, and TC Universal primers and probe were used as internal control [7] (Table 1). Each dilution was amplified in three technical replicates, then, Ct values were extrapolated for drawing the calibration curve.

Cannabis HLVd inoclum and RT-PCR confirmation. Ten healthy, well-developed and susceptible cannabis plants (variety Yunma) were used to test virulence of the *in vitro* prepared HLVd. One plant was used as Mock and inoculated with distilled sterilized water, the other plants were used for HLVd inoculum. The inoculum was performed by slightly cutting a leaf on its lower side with a sterile scalpel and then spread with 100 µl of HLVd RNA of a final concentration of 10^6 copies/µl).

After 10 days from inoculum, the leaves were sampled from both plants. One sample was taken directly from the inoculum site, the second from the inoculated leaf, but in a different position from the inoculum site, and the last sample was taken from another leaf located opposite the inoculum site.

Total nucleic acids were extracted from 100 mg leaves samples using 500 µl of the SDS buffer [26] with our modifications. Briefly, the samples were individually grinded with a mechanical tool and ceramic beads; then the sample was heated at 95 °C to lysate it, next centrifuged, the supernatant was transferred to a new tube and an equal volume (~400 µl) of isopropanol was added to precipitate total nucleic acids. The solution was transferred into a silica spin membrane column tube and centrifuged, removing flow through, then, membrane was washed with 75% ethanol. After centrifugation the extracted total nucleic acids was eluted with 100 µl of TE buffer and collected into a new tube through centrifugation. RT-PCR was performed with RT-HLVd and TC Universal primers and probes (Table 1) with the same protocol as described above. Each sample was amplified in three technical replicates, finally, Ct values were extrapolated as a function of Log concentration of circRNA HLVd (copies numbers).

Results And Discussion

Genetic construct design for synthesis Hop Latent Viroid Circular RNA

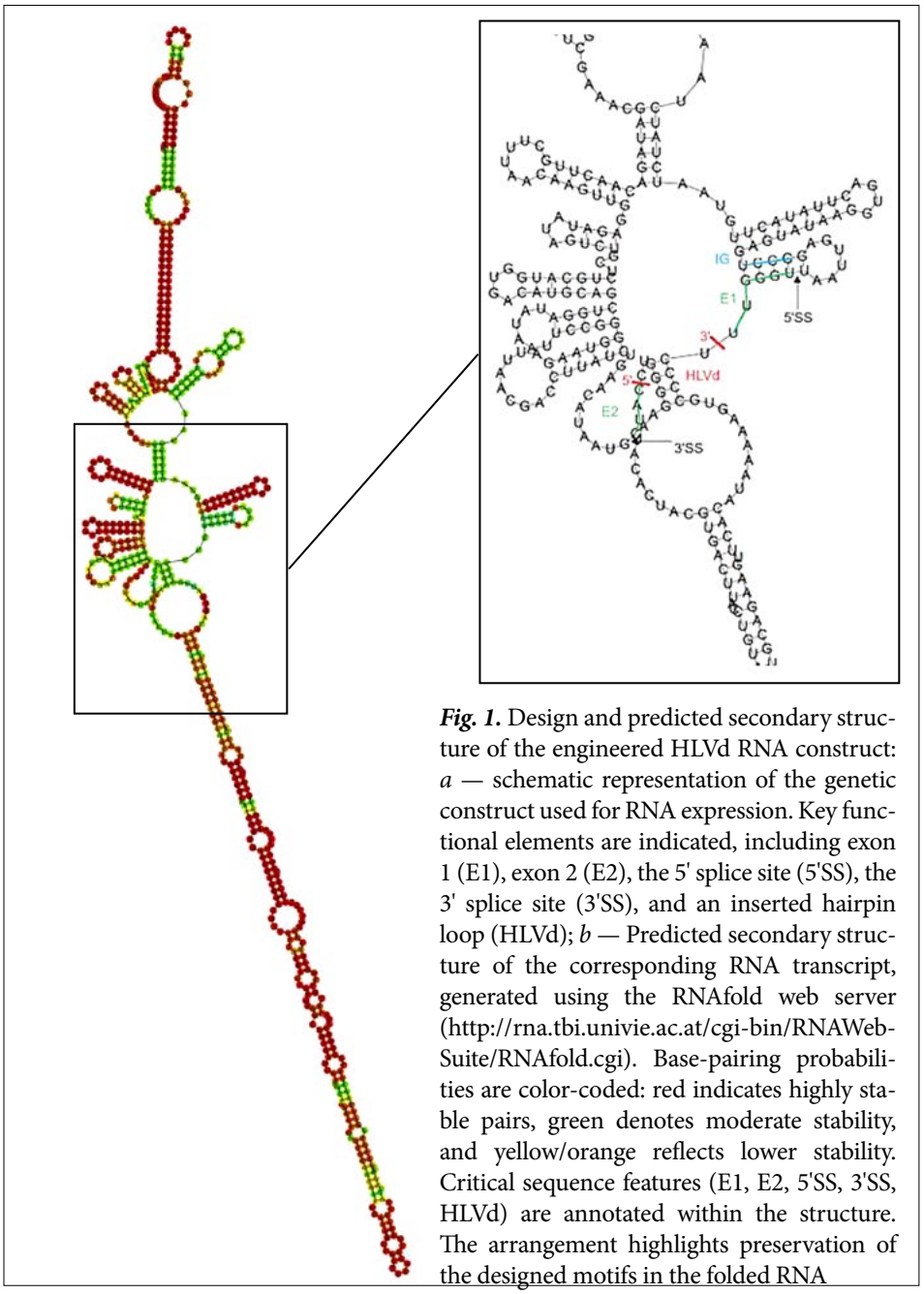
Figure 1 illustrates the design and structural analysis of the engineered RNA construct. Panel A presents a schematic representation of the genetic construct, showing the arrangement of key functional elements. It includes labeled regions

Table 1. **List of primers and sequences**

Primer name	Sequence 5' > 3'	Dye	Reference
T-7P	TAATACGACTCACTATAGG	—	This study
T-7T	GCTAGTTATTGCTCAGCG	—	
RT-HLVd-F	TTCGGCTTCTTCTTGTTC	—	
RT-HLVd-R	GCTCAAGAGTTGTATCCA	—	
RT-HLVd-P	ACGGCTCCTTCTTCACACCA	CY5	
TC-Universal CS For	TGTCCTACATATCTCAAGTCCCATTT	—	[7]
TC-Universal CS Rev	AAGGGTAGCTCCGGCTTCAA	—	
TC-Universal CS P	TA+GTAGA+CTTGA+GAAA+CATGC*	ROX	

* — + denoted LNA bases (O2'–C4' bridge in ribose backbone)

a



b

Fig. 1. Design and predicted secondary structure of the engineered HLVd RNA construct: *a* — schematic representation of the genetic construct used for RNA expression. Key functional elements are indicated, including exon 1 (E1), exon 2 (E2), the 5' splice site (5'SS), the 3' splice site (3'SS), and an inserted hairpin loop (HLVd); *b* — Predicted secondary structure of the corresponding RNA transcript, generated using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Base-pairing probabilities are color-coded: red indicates highly stable pairs, green denotes moderate stability, and yellow/orange reflects lower stability. Critical sequence features (E1, E2, 5'SS, 3'SS, HLVd) are annotated within the structure. The arrangement highlights preservation of the designed motifs in the folded RNA

corresponding to T7 promotor, exons (E1 and E2), the 5' splice site (5'SS), the 3' splice site (3'SS), and the inserted structural module (HLVd). Arrows and connecting lines indicate the orientation and relative positions of the elements within the genetic design, providing an overview of the synthetic organization intended for transcription and splicing analysis. Panel B displays the predicted secondary structure of the transcribed RNA, generated from the genetic construct shown in panel A. The structure is color-coded according to base-pairing probability: red regions correspond to highly stable base pairs, green to moderately stable pairs, and yellow/orange to less stable regions. A magnified view highlights the positioning of key features (E1, E2, 5'SS, 3'SS, HLVd) within the folded RNA, indicating that the construct maintains its designed secondary structure architecture essential for its intended functional studies.

Synthesis and Confirmation of Hop Latent Viroid Circular RNA

We successfully synthesized *in vitro* the circular RNAs of HLVd using a group I intron scaffold. The synthesized circRNA was confirmed through agarose gel electrophoresis after RNAse R digestion (Fig. 2*a*, line 4). Since RNase R specifically digests linear RNA molecules and does not cleave circular RNAs, we can prove that the products, resulting from RNase R digestion, belong to the circular type of synthesized RNA. The circRNA size is near 250 nt, which corresponds to the size of native HLVd.

To further confirm the circular nature of *in vitro* synthesized HLVd RNAs, we conducted RT-PCR with bidirectional primers that elongate the strands in opposite directions (see Fig. 2). In Fig. 2*b*, three distinct bands are visible, corresponding to triple, double, and single circle amplifications with sizes of 750 bp, 500 bp, and 250 bp, respectively. This electropherogram resembles the same amplicon bands pattern as shown in [13, 14].

Final confirmation of circular RNA HLVd was obtained through Sanger sequencing of the PCR-

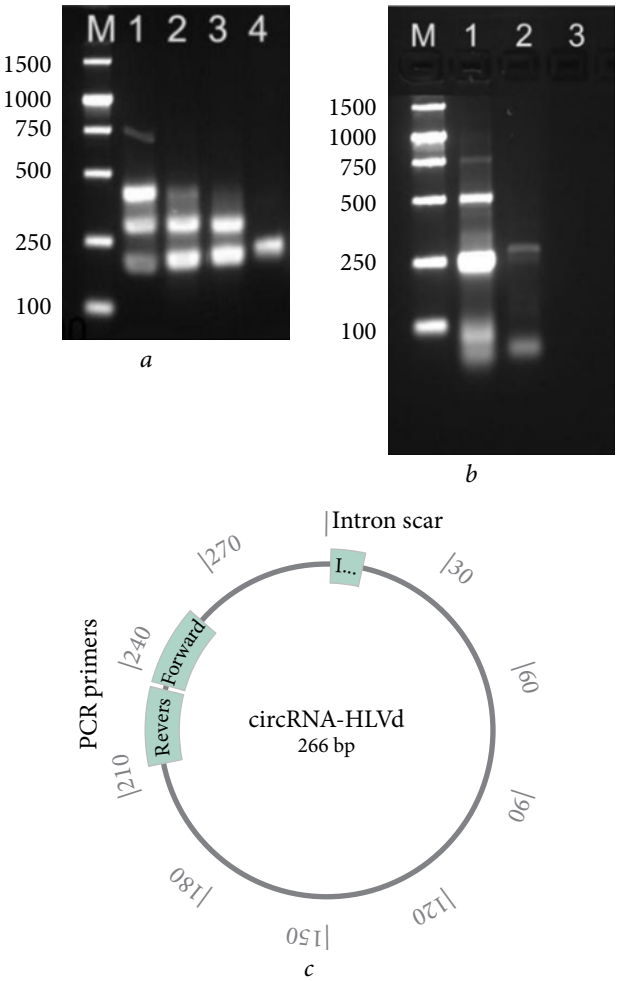
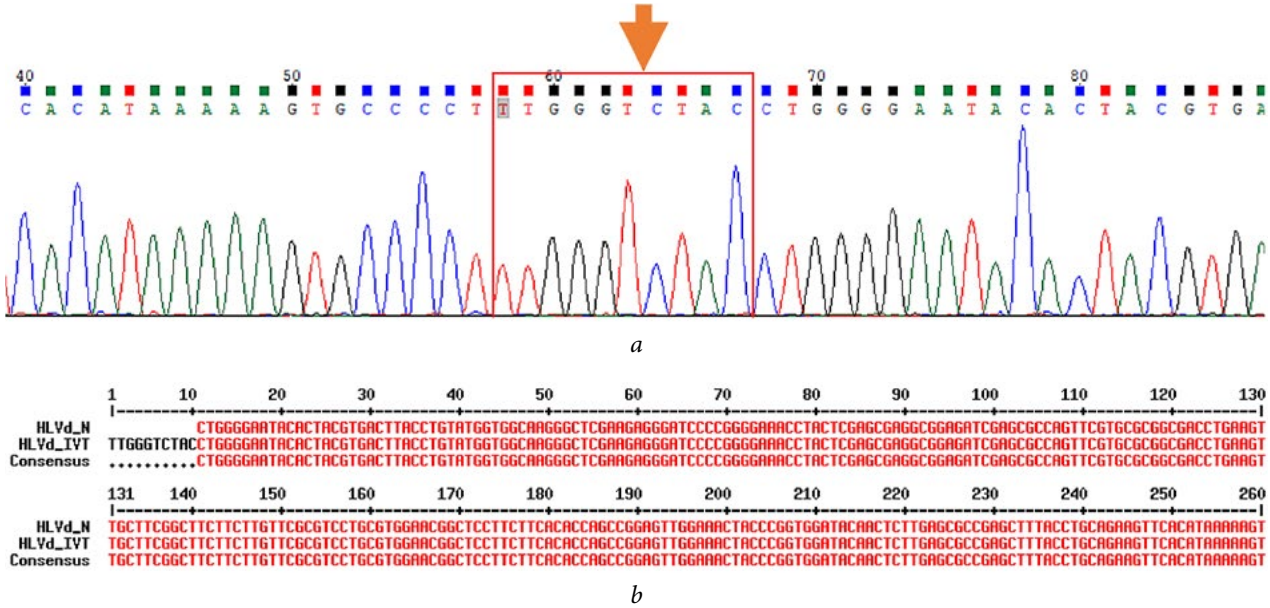


Fig. 2. Experimental validation of the engineered HLVd circRNA: *a* — agarose gel electrophoresis of different stages of HLVd circle RNA synthesis, the products: 1 — of *in vitro* transcription; 2 — after DNAse I digestion; 3 — after clean up and self-circling; 4 — after RNAse R digestions; *b* — agarose gel electrophoresis of synthetic HLVd circRNAs amplification by RT-PCR: 1 — RT-PCR products with MMLV RT; 2 — RT-PCR products without MMLV RT (minus RT control); 3 — Non template control (NTC); *c* — schematic representation of circRNA HLVd, highlighting the intron scar and the primers' annealing points

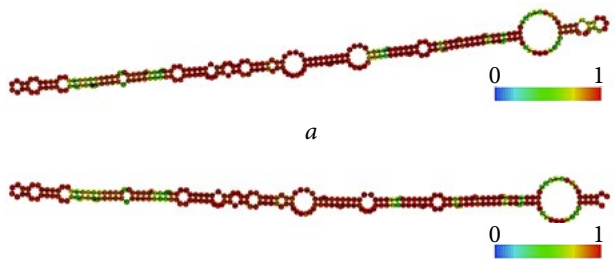
amplified product, which revealed the complete and accurate HLVd genome sequence, including the expected circular junction site and the presence of an intron-derived scar (Fig. 3*a*). The



a

b

Fig. 3. HLVd circRNA sequence analysis confirmation. *a* — Sequencing results of RT-PCR product for HLVd circRNA confirmation, arrow is indicated the joining point, intron related sequence (scar) is shown in red square. GenBank: PP829257.1. *b* — Nucleic acids sequences alignments of the HLVd-N, natural viroid RNA (NC_003611) and HLVd_IVT, synthetic viroid RNA (PP829257.1)



a

b

Fig. 4. Secondary structure comparison of synthetic (*a*) and natural HLVd (*b*) RNAs. *a* — Minimal free energy (MFE) predicted secondary RNA structure of synthetic HLV viroid (PP829257.1), ΔG –90.40 kcal/mol. *b* — Minimal free energy (MFE) predicted secondary RNA structure of natural HLV viroid (NC_003611), ΔG –93.80 kcal/mol

resulting sequences were aligned against the reference HLVd sequence (NCBI GenBank accession no. NC_003611) to verify their identity and confirm successful circularization.

The nucleotide sequence alignment shows that synthetic HLVd differs from native HLVd only by the presence of an additional sequence tag, called the 'intron scar' which originates from the E1 and E2 sequences of group I intron (Fig. 3*b*) and is involved in the cleavage and splicing of nascent circRNA [27].

We also analyzed the secondary structures of circRNA using minimal free energy prediction algorithm (RNAfold) [28, 29] between synthetic and natural viroid RNAs to compare their stability (Fig. 4). In Fig. 4 the predicted secondary structures of the native and synthetic forms of HLVd RNA are compared. Panel A displays the secondary structure model of the native HLVd RNA, highlighting the characteristic rod-like conformation with extensive internal base-pairing and a highly ordered arrangement of stems and loops. The circular nature of the molecule is reflected in the continuous folding without free termini, a hallmark of mature viroid genome. Panel B shows the secondary structure of the synthetic HLVd RNA construct generated in this study. While preserving the overall rod-shaped architecture of the na-

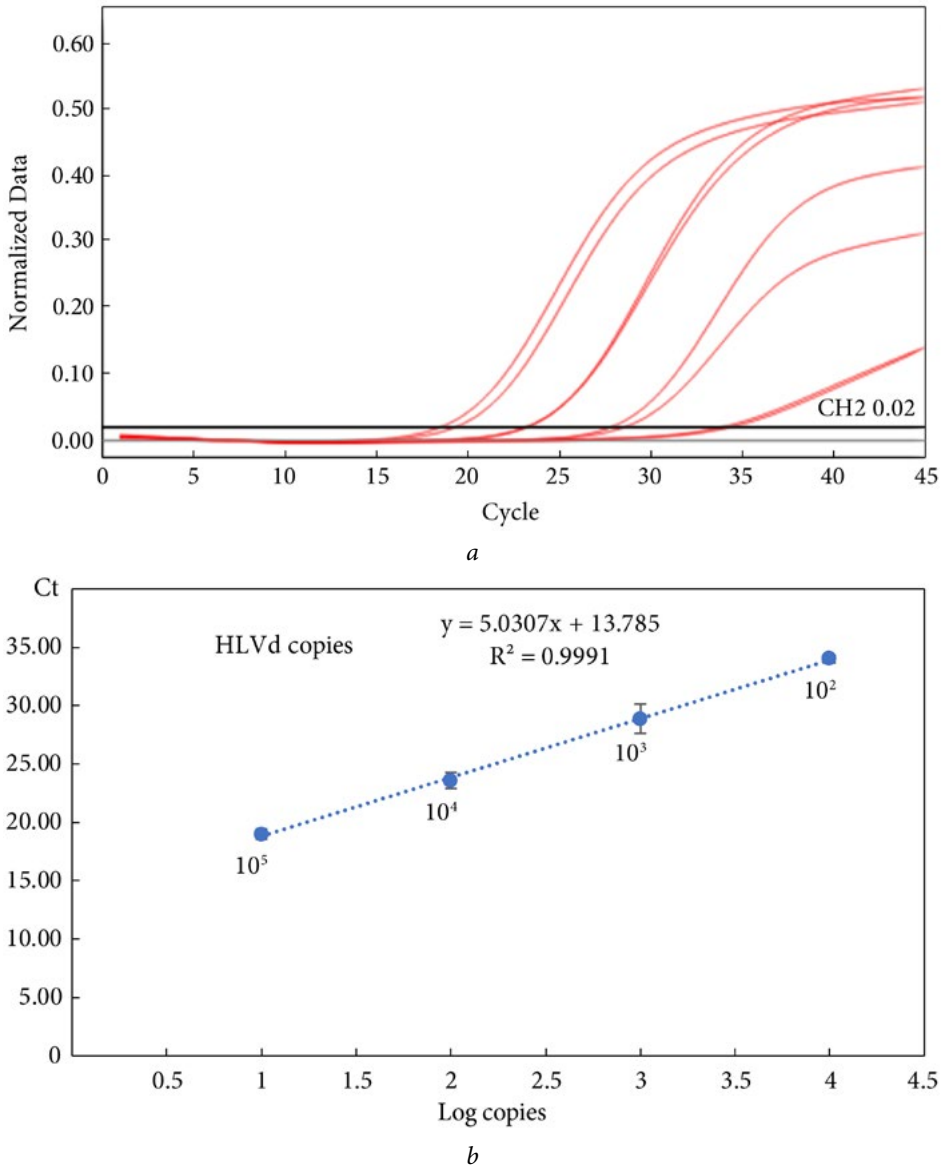


Fig. 5. Real-time qPCR for HLVd RNA: *a* — Dynamic range amplification curves from Log5 to Log2 RNAs copies; *b* — Calibration curve

tive form, the synthetic RNA exhibits minor structural variations at the engineered junction site and in the vicinity of the intron scar. These modifications are consistent with the expected design and do not disrupt the global structural organization essential for the viroid functionality.

The synthetic RNA exhibited a ΔG of –90.4 kcal/mol, while the native RNA showed a ΔG of –93.8 kcal/mol. These values indicate that both RNA structures are thermodynamically stable, with the native RNA being slightly more stable than the synthetic counterpart. The difference in ΔG values suggests minor variations in the base-pairing interactions and overall secondary structure configuration between the synthetic and native RNA, potentially affecting

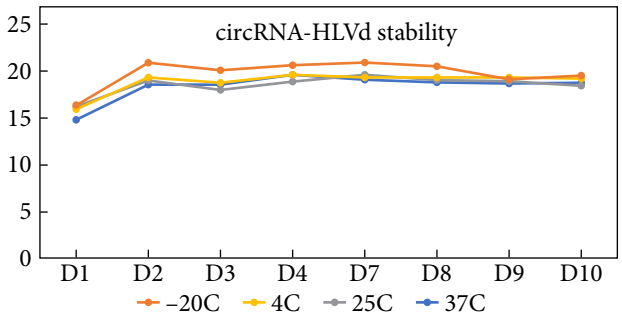


Fig. 6. Biological stability and activity testing of the synthetic HLVd circRNA in Cannabis Plants: circRNA stability test (PCR Ct as a function of time, days testing points at different temperatures)

their biological function and interaction dynamics.

The secondary HLVd RNA structure clearly shows that viroid circRNA adopt rod-shaped structures containing conserved sequences and structural motifs: the central conserved region (CCR) and the terminal conserved regions (TCR) or the terminal conserved hairpins directly involved in self-replication by asymmetric rolling circle mechanism [30].

Application as a Positive Control in PCR Kits

Our initial application of synthetic circRNA HVLd involved its use as a positive control for routine HLVd testing. Last years, several US plant farms experienced economic impacts due to the viroid infections, which were rapidly spreading through cannabis and hop cultivations. By routinely utilizing this circRNA as a positive control, users can effectively manage diagnostic procedures for HLVd detection and prevention, while also standardizing laboratory techniques through validation. Our experiments demonstrated that this circHLVd can be successfully extracted from leaf samples, even when spiked at low copy numbers, mimicking natural infections (Fig. 5). Furthermore, we evaluated the synthesized HLVd circRNA as a potential positive control for PCR kits designed to detect HLVd. Its suitability was confirmed, as it consistently produced reliable amplification signals during PCR testing, highlighting its potential utility in diagnostic applications.

Stability Assessment of the Synthesized HLVd circRNA

In order to evaluate the stability of the synthesized HLVd circRNAs, we conducted storage tests at various temperatures ranging between –20 °C and 37 °C, including 4 °C, and room temperature (25 °C).

Our results demonstrated that the circRNAs remained highly stable over extended periods under all tested conditions, maintaining both its integrity and amplification efficiency. This robustness underscores its suitability as a positive control in diagnostic assays. To further assess its utility as a spiking reagent, we investigated the circRNAs stability during storage (Fig. 6).

Our experiments revealed that the circRNA remained intact for up to 10 days, even when stored at elevated temperatures such as 37 °C. Notably, there was no significant variation in Ct values even after storge at 60 °C (data not shown).

Biological Activity Testing in Cannabis Plants

We evaluated the biological activity of the synthesized HLVd circRNA by inoculating healthy cannabis plants (n = 10). Other researches demonstrated that HLVd can be infectious in several forms such as crude sap, total RNA extract or cDNA transcript [11]. Surprisingly, in our test the inoculated plants exhibited no signs of infection or replication, suggesting that the synthesized circRNA lacked replication capability. This conclusion was further supported by the absence of symptomatic or molecular evidence of HLVd replication in the tested plants.

Conclusion

The successful synthesis and confirmation of HLVd circular RNA using the group I intron scaffold

represent a significant advancement in the development of molecular tools for the viroid research and diagnostics. The accurate sequencing and RT-PCR validation confirmed the fidelity of the synthesized circRNA, making it a reliable candidate for further applications.

One of the primary applications explored in this study was the use of the synthesized HLVd circRNA as a positive control in PCR kits for the HLVd detection. The high stability observed across various temperatures emphasizes its potential utility in diagnostic settings, where consistent performance is crucial. The robustness of this positive control can improve the reliability and accuracy of the HLVd detection assays, providing a valuable tool for both research and agricultural applications.

The inoculation experiments conducted on healthy cannabis plants revealed that the synthesized HLVd circRNA lacked functional replication activity. This finding makes us hypothesize that the intron scar superposition within the circRNA structure impairs its replication capability. The replication-deficient nature of the synthesized RNA underscores its safety for use in laboratory settings, minimizing the risk of unintended viroid spread or infection. We hypothesize that the presence of the intron scar in the terminal conserved hairpins of the synthesized HLVd circRNA negatively affects its self-replication by rolling circle mechanism that provides insights into the structural elements necessary for the viroid replication. Understanding the role of intron scars in impairing replication can inform future studies aimed at dissecting viroid replication mechanisms and potentially guide the design of replication-competent synthetic viroid RNAs for research purposes.

Conflict of Interest. The authors declare that they have no conflict of interest.

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СИНТЕЗ І ХАРАКТЕРИСТИКА НЕІНФЕКЦІЙНОЇ КІЛЬЦЕВОЇ РНК ЛАТЕНТНОГО ВІРОЇДУ ХМЕЛЮ З ВИКОРИСТАННЯМ САМОСПЛАЙСИНГОВОЇ СИСТЕМИ РИБОЗИМУ ГРУПИ І

Мета. Латентний віроїд хмелю (HLVd) становить серйозну загрозу для культивування Cannabis sativa, з рівнем зараження до 90% і втратою 70% ТГК. Для діагностики необхідні надійні неінфекційні позитивні контролі. ***Методи.*** Ми синтезували неінфекційну кільцеву РНК HLVd за допомогою самосплайсингової рибозимної системи групи І. Продукт був охарактеризований за допомогою розщеплення РНКази R, RT-PCR та секвенування для підтвердження циклюляризації та послідовності з'єднання, отриманої з рибозиму. ***Результати.*** Структурний аналіз показав, що синтетична РНК зберігає високу стабільність (ΔG = –90,4 ккал/моль) порівняно з нативним HLVd (ΔG = –93,8 ккал/моль), із введеними мутаціями, що запобігають реплікації. Молекула залишалася стабільною при температурі від –20 до 37 °C протягом 10 днів і працювала як позитивний контроль ПЛР. Висновок. Синтетична circRNA HLVd забезпечує безпечний, стабільний позитивний контроль для діагностики та дослідження вірусів, усуваючи ключову прогалину в сучасних методах виявлення.

***Ключові слова*:** латентний віроїд хмелю, канабіс, пермутований інтрон групи І, транскрипція *in vitro*, circRNA.