

# APPLICATIONS OF MIRACULOUS RNA PATHOGENS IN BIOTECHNOLOGY

## Abstract

Viroids are the smallest infectious agents currently known. They are small, circular, single-stranded RNA molecules without the ability to code for proteins. Viroids were initially identified as the primary culprits in a number of plant illnesses, but they are now showing promise in a number of biotechnological uses. This book chapter explores the various applications of viroid in biotechnology, including gene regulation, plant protection, agricultural improvement, and RNA-based therapeutics. We examine the viroid research landscape, highlight their special qualities, and discuss about the difficulties and probable future directions of viroid applications in biotechnological development.

**Keywords:** Pathogens, RNA molecules, Viroid

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## I. INTRODUCTION

- 1. Definition and Characteristics of Viroids:** The process that led to the discovery of viroids began in the late 1960s with studies that focused on elucidating the causative agent of potato spindle tuber disease [1], the existence of a new type of pathogens, similar but different from viruses and RNA satellites, was proposed. T.O. Diener coined the term “viroid” (virus-like) to describe these RNAs. Viroids are single-stranded, covalently closed circular RNA molecules, small size (~250–400 nt), do not encode any protein and lack a capsid [2]. Currently, 33 different viroid species and several sequence variants have been biologically and molecularly characterized. These species are broadly classified into two families, the *Pospiviroidae* and the *Avsunviroidae* [3]. The members of the family *Pospiviroidae*, (the type species being potato spindle tuber viroid (PSTVd)), contain five structural/functional domains, a central conserved region and replicate in the nucleus of infected cells through an asymmetric rolling circle mechanism. In contrast, the members of the family *Avsunviroidae* (the type species being avocado sunblotch viroid (ASBVd)), do not have any structural/functional domains, nor a central conserved region. However, they do exhibit self-cleavage activity through a hammerhead ribozyme, and they replicate via a symmetric rolling circle mechanism in the chloroplasts of infected cells [4]. They infect several crop plants vegetables, field and ornamental crops, fruit and palm trees, and grapevine, causing symptoms of differential severity, up to deformation, necrosis or chlorosis and severe stunting [5] and can cause disease leading to significant agricultural damage [5].
- 2. Historical Perspective: Discovery and Early Studies:** The field of virology emerged in the late 19th and early 20th centuries as scientists began to investigate the cause of infectious diseases in plants and animals [6]. The discovery of tobacco mosaic virus (TMV) by Dmitri Ivanovsky and Martinus Beijerinck in the late 1800s laid the foundation for virology as a distinct scientific discipline. In the 1920s and 1930s, plant pathologists observed certain diseases, such as the potato spindle tuber disease, that did not exhibit any evidence of the presence of conventional viruses [7]. These observations raised questions about the potential existence of unconventional infectious agents. In the 1940s, a significant breakthrough occurred when the American plant pathologist Theodor O. Diener investigated the potato spindle tuber disease. Diener noticed that the disease could be transmitted to healthy potato plants through sap, but the causal agent could not be detected using conventional methods like electron microscopy [1].
- 3. The Discovery of Viroids:** The discovery of viroids was similar in that it took some time before it was realized how much of an impact it was having on agricultural and fruit crops. Werner in Nebraska was working on a degenerative disease of the potato (perhaps potato spindle tuber) about a century ago (1917) [8]. Martin, however, is given credit for the disease's first report and the adoption of the term "spindle tuber" [1]. Schultz and Folsom proved the transmissibility of what they called "spindling tuber" disease without being aware of Martin's nomenclature of the illness [9]. In Canada's New Brunswick, this condition was investigated by MacLeod as early as 1925 [10].

In 1971, after several decades of research and experimentation, Theodor O. Diener successfully isolated and characterized the causal agent responsible for the potato spindle tuber disease. He named these novel infectious entities "viroids" to distinguish them from

viruses. His groundbreaking discovery was published in the Proceedings of the National Academy of Sciences. Diener's research revealed that viroids were significantly smaller and simpler than viruses. They consisted solely of a short, single-stranded RNA molecule without any protein coat, unlike viruses, which have both RNA and DNA and protein components [2].

Following the discovery of the potato spindle tuber viroid (PSTVd), researchers began to investigate other plant diseases and found more viroids associated with various crops, such as citrus and avocado [11]. These findings expanded the understanding of viroids as pathogens in plants. The discovery and early studies of viroids marked a pivotal moment in the field of virology and provided valuable insights into the diversity of infectious agents. The subsequent research on viroids has contributed to our understanding of RNA biology and has opened up new avenues for exploring their potential roles in biotechnology and other scientific applications [12-14].

## II. VIROIDS AS MOLECULAR TOOLS IN GENE REGULATION

Viroids, being small infectious RNA molecules, have attracted attention in recent years as potential molecular tools in gene regulation [15,16]. Although viroids were initially studied in the context of causing plant diseases, researchers have started exploring their unique properties for applications beyond pathogenesis. Here's how viroids can be used as molecular tools in gene regulation:

- 1. RNA Interference (RNAi):** RNA interference is a natural cellular mechanism that regulates gene expression by degrading specific RNA molecules [17]. Viroids can act as triggers for RNAi, leading to the degradation of target messenger RNA (mRNA) molecules [18-20]. By designing viroid-derived RNA molecules that target specific genes, researchers can induce targeted gene silencing in plants [21,22]. This approach, known as "virogenic RNAi," has potential applications in crop improvement, allowing the suppression of genes involved in disease susceptibility or enhancing stress tolerance in plants [23,24].
- 2. Post-Transcriptional Gene Silencing (PTGS):** Viroids can induce post-transcriptional gene silencing, a process where double-stranded RNA molecules trigger the degradation of homologous mRNA sequences. In this manner, viroids can down regulate the expression of target genes and modulate specific biological processes [25-27].
- 3. Epigenetic Modifications:** Some viroids are associated with epigenetic modifications in plants. Epigenetic changes can alter gene expression without altering the DNA sequence. Viroid infection causes the hypo-methylation of the promoter region of the ribosomal genes in cucumber plants, according to research using the hop stunt viroid (HSVd) as a pathogenic model [28]. The main cytosines in the CG context were impacted by this change in the methylation pattern, which was linked to enhanced transcription and an accumulation of ribosomal RNA (rRNA) precursors. Similar outcomes were discovered when transgenic *N.benthamiana* plants were examined and found to be constitutively producing HSVd dimeric transcripts [29]. The examination of HSVd effects in the host male gametophyte of infected cucumber plants also revealed that this viroid caused a dynamic demethylation of repetitive DNA regions (rRNA genes and TEs) in cucumber

gametophytic cells [30]. Overall, these findings lend credence to the idea that the plant epigenetic modifications brought on by HSVd infection are a regulatory phenomenon that are not specific to a particular host-viroid interaction (cucumber or *N. benthamiana*) or plant tissue (vegetative or reproductive), and that these modifications have an impact on various types of repetitive sequences (rRNA and TEs). By interacting with the plant's epigenetic machinery, viroids influence the expression of genes involved in growth, development, and stress responses [31,32].

- 4. Engineered Viroids for Gene Regulation:** Researchers are exploring the possibility of engineering viroids to target specific genes of interest. By modifying the sequence of viroids or incorporating additional RNA motifs, it becomes feasible to design viroids that selectively regulate particular genes in plants [33,34]. In spite of the relative simplicity of viroid genomes, they can trigger complex host responses. PSTVd and CEVd infections alter host metabolism and markedly change the levels of various host proteins [35,36]. A comprehensive analysis of the differential gene expression patterns of tomato plants at various stages of infection by mild and severe strains of PSTVd revealed that both of these strains altered expression of genes encoding products involved in defense/stress response, cell wall structure, chloroplast function, protein metabolism, hormone signaling, and other diverse functions [37,38]. Viroids used as molecular tools in gene regulation represent a promising area of biotechnology, with potential applications in agriculture, functional genomics, and synthetic biology [40].

### III. VIROIDS IN CROP IMPROVEMENT

- 1. Viroids as Vectors for RNA-Based Crop Engineering:** Viroids can serve as vectors for RNA-based crop engineering, presenting a unique and innovative approach to introduce desired traits into plants. This application involves utilizing viroids as vehicles to deliver specific RNA molecules to the plant cells, which can then influence gene expression and confer beneficial traits [40]. Here's how viroids can be used as vectors for RNA-based crop engineering: It can be used for RNA delivery, epigenetic modifications and gene silencing [42].
- 2. Enhancing Stress Tolerance and Disease Resistance in Crops:** By targeting essential genes in plant pathogens, viroids can trigger RNAi-mediated degradation of the pathogen's RNA, providing resistance against specific diseases that negatively impact crop yield and quality [43,44]. Utilizing various populations of short RNAs, RNA silencing controls a wide range of crucial functions [45]. MicroRNAs (miRNAs) are one of them, and it is known that they play crucial roles in organism development and adaptability to environmental challenges [46]. These hairpin-like initial transcripts are converted into these 21-nt RNAs by particular RNase III-type enzymes (Drosha + Dicer in mammals and DCL1 in plants). Viroids can also be used to confer tolerance to abiotic stresses such as drought, salinity, and extreme temperatures [47].
- 3. Exploiting Viroids for Improved Crop Yield and Quality:** Exploiting viroids for improved crop yield and quality is an exciting area of research in agricultural biotechnology. Viroids can influence flowering time and fruit development in plants by modulating the expression of key regulatory genes. Precise control over these processes can lead to optimized flowering patterns, improved fruit size, and enhanced fruit quality.

For instance it can delay senescence. Adkar-Purushothama et al recently reported the function of PSTVd-derived sRNAs in early flowering in infected tomato plants and showed that a PSTVd vd-sRNA derived from the left terminal region targets an mRNA encoding FRIGIDA-LIKE protein 3, one of two genes that regulate flowering behavior in Arabidopsis [48]. Viroids can be engineered to slow down the aging process (senescence) in plants. Delaying senescence can extend the productive lifespan of crops, resulting in prolonged yield and maintaining quality attributes for a more extended period [48]. It can also enhance nutrient uptake by regulating genes involved in nutrient uptake and metabolism, viroids can improve the efficiency with which crops absorb and utilize essential nutrients. This can lead to increased crop yields and enhanced nutritional quality. Also manipulate metabolic pathway as in viroids can be used to target specific genes in metabolic pathways, leading to altered levels of metabolites in plants. This approach can be harnessed to increase the production of beneficial compounds, such as vitamins, antioxidants, or flavor-enhancing molecules. Viroids are known to reduce undesirable traits and can be employed to down regulate genes responsible for undesirable traits in crops, such as bitterness or astringency in fruits or vegetables. By fine-tuning gene expression, viroids can help improve the sensory attributes of crops, making them more appealing to consumers [49].

#### IV. VIROIDS AS DIAGNOSTIC TOOLS

While viroids can cause plant diseases, they can also serve as diagnostic tools in specific contexts. The use of viroids as diagnostic tools involves their engineered or modified forms, rather than the natural viroids causing diseases in plants. Researchers have explored the use of engineered viroids for diagnostic purposes due to their unique properties, which can be exploited in various ways:

**Viroid-Based Indicators:** Modified viroids can be designed to act as indicators of specific plant diseases. By inserting specific functional elements into the viroid sequence, researchers can create viroids that exhibit visible changes (e.g., color changes) or fluorescence in response to the presence of target pathogens. These viroids can be introduced into plants and used to detect the pathogens they were engineered to respond to.

**Signal Amplification:** Viroids can be used as amplification tools in diagnostic assays. By engineering viroids to replicate efficiently in response to the presence of target nucleic acids (e.g., from viruses or other pathogens), researchers can achieve signal amplification, making the detection of low concentrations of pathogens more sensitive.

It's essential to emphasize that using viroids as diagnostic tools or for gene manipulation in plants requires careful and controlled laboratory procedures.

Viroid-based diagnostic tools are a promising area of research, offering the potential for rapid and specific detection of plant pathogens and providing valuable insights into plant biology and disease resistance mechanisms.

**1. Viroid-Based Detection of Plant Diseases:** Viroid-based detection of plant diseases involves using techniques like Reverse Transcription Polymerase Chain Reaction (RT-PCR), Northern Blotting, or other nucleic acid-based methods to amplify and identify

viroid-specific RNA sequences present in infected plant samples. These detection methods help in the early diagnosis of viroid infections, allowing for timely disease management and control measures to protect agricultural crops [50]. It can offer Sensitivity and specificity: Viroids are highly specific to certain plant species or plant families. Viroid infections can be detected at early stages of the disease, even before visible symptoms manifest in the plants. This early detection capability can help implement timely measures to prevent further spread and minimize crop losses. Viroids can induce RNA interference (RNAi), a natural defense mechanism, through the production of viroid-derived small RNAs (vd-sRNAs). These vd-sRNAs can target and degrade complementary sequences in the plant's mRNA, leading to the silencing of specific genes [51].

- 2. RNA Nanotechnology:** RNA nanotechnology, on the other hand, is a field that involves the design and manipulation of RNA molecules to create functional nanoscale structures. It is an extension of nanotechnology that takes advantage of RNA's unique properties, such as its ability to fold into intricate secondary and tertiary structures.

RNA nanotechnology has diverse applications in biotechnology and medicine. Some key areas of interest include:

- **Drug Delivery:** RNA nanoparticles can be engineered to deliver therapeutic agents, such as small interfering RNAs (siRNAs) or drugs, to specific cells or tissues. These nanoparticles can protect the payload from degradation and improve the targeted delivery of therapeutics.
- **Gene Editing and Regulation:** RNA nanotechnology is used to design and deliver components of gene editing systems, like CRISPR-Cas9, for precise genome modifications. RNA-based gene regulation elements, such as riboswitches, allow for the control of gene expression in response to specific signals.
- **Biosensors and Diagnostics:** RNA-based biosensors can be designed to detect specific molecules, such as nucleic acids or proteins, offering sensitive and specific diagnostic tools.
- **Vaccine Development:** RNA nanotechnology has been pivotal in the development of mRNA-based vaccines, such as those used in the COVID-19 pandemic response. These vaccines use synthetic RNA to encode viral antigens and stimulate an immune response.

Viroids are primarily known for causing diseases in plants and have not been definitively shown to infect humans or animals. However, there have been some speculative hypotheses and limited studies exploring potential viroid-like sequences or viroid-related elements in the context of human health and biomedical research. Let's explore these aspects further:

- 3. Viroid-Like Sequences in Human Genome:** There have been suggestions that small RNA sequences resembling viroid-like structures or viroid-related elements might be present in the human genome. These sequences are typically short and may share some structural similarities with viroids. However, their functional significance and whether they have any active role in human health remain subjects of ongoing research and debate.

**4. Neurological Disorders and Viroids:** Some studies have proposed potential associations between viroids and certain neurological disorders, such as Alzheimer's disease and Parkinson's disease. Viroid-specific small-subunit RNA (ssRNA) is the only component of viroids, which are simple plant diseases that have many characteristics with miRNAs, including their manner of production, processing, structure, and function, mobility, and capacity to spread illness inside the host (Hill et al., 2009; Hill and Lukiw, 2014; Pogue et al., 2014). miRNA dysregulation and AD are now clearly linked by research, and miRNAs constitute a novel class of biomarkers that could be used to diagnose AD (Alexandrov et al., 2012; Danborg et al., 2014; Maffioletti et al., 2014). What is already known about plant viroids and their capacity to transmit systemic degenerative illness (Navarro et al., 2012; Hill et al., 2014; Pogue et al., 2016) may be able to provide us with insight into the mechanism of AD neuropathology driven by miRNA. However, these studies are primarily based on the detection of small RNA molecules resembling viroid-like structures in the brains of affected individuals. The functional relevance of these viroid-like sequences and their potential role in neurodegenerative diseases are areas of ongoing investigation and remain speculative.

## V. POTENTIAL NON-CODING REGULATORY FUNCTIONS

Viroid's unique structural properties and ability to self-replicate have sparked interest in studying their potential regulatory roles in gene expression. Some researchers have explored whether viroid-like elements in the human genome might have non-coding regulatory functions. However, any specific regulatory functions and their significance in human health have not been firmly established.

## VI. VIROID RESEARCH AND ITS SIGNIFICANCE FOR RNA TECHNOLOGY AND BASIC BIOCHEMISTRY

At the onset viroid research was a biologically very attractive subject for structure-function studies. Viroids were an unprecedented phenomenon of a medium-sized, capsid-free RNA molecule with the complex biology of a virus. Improvements of RNA technology developed for the study of viroid biology were applied to other nucleic acid research projects. The characterization of metastable viroid structures or the classification of hydrogen bonding as a partially covalent bond significantly contributed to knowledge in basic molecular biology. The study of viroids has contributed to advancing our understanding of RNA biology and the principles of RNA nanotechnology. Lessons learned from viroid replication, RNA folding, and self-cleavage mechanisms have inspired researchers to develop RNA-based nanotechnologies with potential applications in biomedicine, including drug delivery and gene regulation.

It's crucial to stress that viroids are distinct from viruses and do not encode proteins or possess the complex structures required for infecting animal cells. Therefore, any potential link between viroids and human health would be indirect and complicated. The field of viroids in biomedical research is still in its infancy, and findings in this area require rigorous verification and replication.

For the latest developments on viroids and their potential relevance in biomedical research, it is essential to consult recent scientific literature and seek information from reputable sources. The scientific community continues to investigate the potential roles of

viroid-like sequences and viroid-related elements in human health and disease to gain a comprehensive understanding of their impact, if any, in biomedical research.

## **VII. CONCLUSION**

In conclusion, viroids offer exciting opportunities for advancing biotechnological applications. Their unique properties, particularly their ability to induce RNAi and regulate gene expression, hold great promise in agriculture, gene therapy, synthetic biology, and basic research. However, careful research, safety evaluation, and ethical considerations are essential as we explore and harness the potential of viroids for the benefit of society.