BioThink E – Magazine, Vol. -3, Issue -1, January 2026
ISSN No. – 3048- 7943
www.biothink.in

RNA Interference in Plant Pathology: A Gene Silencing Approach for Sustainable Disease Management

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Introduction

RNA interference, also known as RNA-induced gene silencing, is a fundamental and highly conserved biological process found across various eukaryotes, including plants, fungi, and animals (Karimi & Innes, 2022; Rosa et al., 2018). It is a cellular defense mechanism that specifically recognizes and silences gene expression at the post-transcriptional level.(Hernández-Soto & Chacón-Cerdas, 2021; Karimi & Innes, 2022; Rosa *et al.*, 2018; Zhao *et al.*, 2024). The process is typically triggered by the presence of double-stranded RNA molecules, which subsequently results in the sequence-specific degradation or translational repression of complementary messenger RNA (Koeppe et al., 2023). RNAi technology offers a modern, eco-friendly alternative to chemical crop protection by silencing specific genes in pests and pathogens while sparing non-target organisms. It can be applied through transgenic (HIGS) or spray-based (SIGS) approaches and shows proven efficacy, such as high mortality in the diamondback moth(Qi et al., 2024). Additionally, RNAi holds promise for enhancing crop resilience and yield through selective breeding programs.

History & Background

RNA interference was formally discovered and named in 1998 by Andrew Fire and Craig Mello through their groundbreaking work with the nematode *Caenorhabditis elegans* (Hernández-Soto & Chacón-Cerdas, 2021). However, the underlying mechanisms of gene silencing had been observed and described in plants much earlier, during the early 1990s by scientist Napoli et al., when they observed an unexpected "co-suppression" effect in petunias genetically modified with an additional chalcone synthase gene(a gene involved in purple pigment production in petunia), resulting in the silencing of both the original and introduced gene copies(Karimi & Innes,

2022; Rössner et al., 2022). A similar mechanism was described in the fungus *Neurospora crassa* by different researchers like Romano and Macino, and later Cogoni et al., where the expression of certain genes (like *albino-1* or *albino-3*) led to the silencing of their endogenous counterparts, which correlated with reductions in mRNA levels(Dalakouras et al., 2024; Imran et al., 2025; Qi et al., 2024) (Karimi & Innes, 2022).

Fundamentals of RNA Interference

RNA interference is defined as a mechanism of sequence-specific gene silencing initiated by a double-stranded RNA, leading to the breakdown of complementary messenger RNA and consequently, post-transcriptional gene silencing(Chen *et al.*, 2025)(Fire *et al.*, 1998). This process is linked to eukaryotic cellular defense mechanism, involving a highly precise and complicated cascade of enzymatic reactions contributing to the degradation of target foreign mRNA.

Main Components

1. Double-stranded RNA (dsRNA)

RNAi is commenced by a double-stranded RNA (dsRNA), which may originate from viruses, transgenes, endogenous hairpin structures, or experimental application (e.g., spray-induced or host-induced RNAi). dsRNA acts as the trigger molecule that signals the silencing pathway. There are different sources of dsRNA , which are broadly classified under endogenous, exogenous and cross **kingdom** sources(Chen *et al.*, 2025; Qi *et al.*, 2024; Rosa *et al.*, 2018)

Endogenous/Natural Sources: Endogenous hairpin RNAs (hpRNAs): They are formed when RNA transcripts undergo intramolecular folding to form stem-loop structures with incomplete base pairing. These serve as precursors for miRNAs and some siRNAs.*(Chen et al., 2025; Dalakouras et al.*, 2024; Rosa *et al.*, 2018)

- **Sense/antisense transcription:** Overlapping transcripts originating from opposite DNA strands can anneal to form dsRNA.(Dalakouras *et al.*, 2024; Imran *et al.*, 2025; Rosa *et al.*, 2018)
- **Transposons and repeat-associated RNAs:** Plants can naturally synthesize dsRNA from **repetitive elements**, which helps in silencing of transposons through the RNA-directed DNA methylation (RdDM) pathway.(Chen *et al.*, 2025; Rössner *et al.*, 2022)
- **Viral infection:** Replication processes in numerous plant viruses generate double-stranded RNA intermediates, which effectively initiate RNA silencing and trigger antiviral defenses(Qi et al., 2024; Rössner et al., 2022).

Exogenous Sources: Transgenes (Host-Induced Gene Silencing): Plants can be genetically engineered to express dsRNA against virulent genes of different pathogens (fungi, nematodes, and insects). (Hernández-Soto & Chacón-Cerdas, 2021)

- **Spray-induced dsRNA (Spray-Induced Gene Silencing):** dsRNA molecules are directly sprayed to plant surfaces , following different application methods. These are taken up by plants and/or pathogens to induce gene silencing.

Cross-kingdom Sources : Pathogens and host plants have been observed to produce dsRNA molecules as part of their inter-kingdom communication and defense mechanisms, leading to cross-kingdom RNA interference (Hernández-Soto & Chacón-Cerdas, 2021).

In plants, dsRNA can be amplified by RNA-dependent RNA polymerases (RDRs) from aberrant or cleaved RNAs. RDR6, often with the cofactor SGS3, helps in conversion of degraded mRNA to secondary dsRNAs, which are further diced into secondary siRNAs, that amplifies the initial silencing and contributes to long term RNA Interference.(Rössner *et al.*, 2022; Zhao *et al.*, 2024)

2. Dicer-like (DCL) Enzymes

Dicer-like (DCL) proteins or enzymes are ubiquitous RNA III Endonucleases that cleaves or processes dsRNA(long or hairpin dsRNA) into small RNA(siRNA or miRNA)(Chen *et al.*, 2025; Rössner et al., 2022). Host plant produces a different DCL protein that specializes in processing of distinct classes of small RNAs:

- DCL1: Processes primary miRNA transcripts into mature miRNAs.(Rössner *et al.*, 2022)
- DCL2: Produces 22-nt siRNAs and has been reported to coordinate with DCL4 in antiviral defense; in scenarios where DCL4 is not present, DCL2 is capable of generating substantial quantities of viral secondary siRNAs. (Rosa *et al.*, 2018)
- DCL3: Generates 24-nt siRNAs for transcriptional gene silencing (TGS) via RNA-directed DNA methylation(RdDM), associated with silencing of transposons and repetitive elements(Rössner *et al.*, 2022) (Rosa *et al.*, 2018) .
- DCL4: Produces 21-nt siRNAs, and acts as one of the principal components in post-transcriptional gene silencing (PTGS) and antiviral immunity.*(Karimi & Innes, 2022; Qi et al*., 2024; Rössner *et al.*, 2022)

3. Small RNAs

They are small RNA particles, generated by action of DCL proteins, are 20–24 nucleotide noncoding RNAs that combine with Argonaute (AGO) to form the RISC(RNA-induced silencing complex) complex to silence specific nucleic acid targets (Rosa *et al.*, 2018) .They are further classified into -

I. Small Interfering RNAs (siRNAs):They are derived from long dsRNA cut by DCLs that silence exogenous/virulent RNAs (viruses, transgenes) and some endogenous repetitive elements or transposon. They can be further classified into :-

- 21-nt siRNAs (DCL4): Involved in PTGS and antiviral defense.
- 22-nt siRNAs (DCL2): The 22-nt siRNAs have a backup antiviral role and can trigger RDR6-mediated amplification.

24-nt siRNAs (DCL3): Guide RdDM, directed against the host's heterochromatin, including inverted repeats and transposons, and this mechanism is also used for defense against DNA viruses, leading to transcriptional gene silencing (TGS) (Rössner *et al.*, 2022)

II. MicroRNAs (miRNAs): They originate from endogenous hairpin precursors (pri-miRNAs) transcribed by RNA polymerase II, being processed by DCL1 in the nucleus with cofactors HYL1 and SE (SERRATE).They are then loaded into AGO1 protein in the RISC complex, directing cleavage or translational repression of complementary mRNAs.(Qi *et al.*, 2024; Rosa *et al.*, 2018). The microRNA pathway functions at the post-transcriptional stage and plays a vital role in various physiological and pathophysiological conditions. (Qi *et al.*, 2024).

The distinction between siRNA and miRNA lies in their biogenesis pathway, the precision of cleavage by DICER proteins and the nature of the gene or nucleic acid they target. While siRNA targets foreign genes and transposons mainly, the miRNA acts upon the endogenous host genes, contributing to the development and stress resistance of host plants.(Qi *et al.*, 2024)

4. Argonaute (AGO) proteins

Argonaute proteins are a family of highly conserved RNA-binding proteins that serves as the core of the RNA-induced silencing complex (RISC). They help binding of small RNAs and utilize them as guides to cleave target mRNA (post-transcriptional silencing) and thereby supress translation of pathogenic proteins or mediate Direct DNA methylation (transcriptional silencing)(Rössner *et al.*, 2022). Argonaute proteins feature three primary conserved domains: PAZ, MID, and PIWI domains. The N-terminal region includes a variable domain, potentially aiding in the dissociation of the small RNA-target duplex post-slicing by disrupting its structure. The PAZ domain, situated near the N-terminal domain, binds to the 3′ end of small RNAs. The C-terminal region encompasses the MID and PIWI domains, with their junction forming a binding pocket for the 5′ end of the guide RNA. (Rosa et al., 2018)

BioThink E – Magazine, Vol. -3, Issue -1, January 2026
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5. RNA-induced silencing complex (RISC)

It is the multiprotein complex containing AGO proteins and the siRNA/miRNA, processed by DCL Proteins. This complex executes gene silencing by slicing complementary mRNA by Watson-Crick base pairing, generally leading to RISC cleavage/slicing of the RNA target .Within the RISC complex, the Argonaute protein, bound to the siRNA-target duplex, utilizes its PIWI domain, characterized by an RNase-H-like fold and a conserved catalytic site, to cleave the target RNA, leading to its subsequent degradation.(Karimi & Innes, 2022; Rosa *et al.*, 2018)

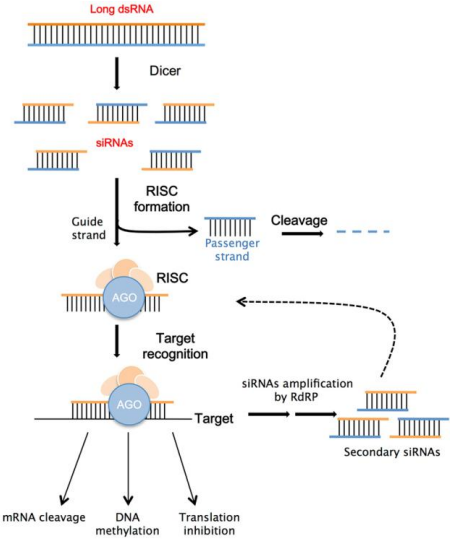

Fig 1: RNAi Mechanism (Limera et al., 2017)

6. RDRs (RNA-dependent RNA Polymerases) & accessory proteins

These enzymes convert single-stranded RNAs (ssRNAs) (usually aberrant, viral, or cleaved mRNAs) to double-stranded RNA (dsRNA) which is to be processed by Dicer-like (DCL) enzymes into secondary siRNAs, thereby reinforcing and amplifying RNAi. They are unique to plants potentially explaining the systemic spread of RNAi in plants, a phenomenon less commonly observed in many animal species. Some of the important RDRs are : RDR6 (Most important role in antiviral defense, contributing to production of secondary siRNA (phasiRNA/ta-siRNA) and playing an important role in post-transcriptional gene silencing/PTGS), RDR2 (initiates RNA-directed DNA methylation (RdDM) for transcriptional gene silencing /TGS and assists DCL3 to produce 24-nt siRNAs), RDR1(Induced by pathogen infection and serving to antiviral defense)*(Rössner et al.*, 2022; Zhao *et al.*, 2024).

Accessory proteins like SGS3 (Suppressor of Gene Silencing 3), DRBs (Double-stranded RNA-binding proteins),HEN1 (Hua Enhancer 1), HYL1 (Hyponastic Leaves 1) and SERRATE (SE) assist RDRs and DCL proteins in their functional role to ensure efficiency and long term efficacy of RNAi machinery.(Dalakouras *et al.*, 2024; Qi *et al.*, 2024; Rosa *et al.*, 2018)

RNAi Strategies for Plant Disease Resistance

A. **Host Induced Gene Silencing:** Host-induced gene silencing (HIGS) is an RNAi-mediated strategy where plants are engineered to produce double-stranded RNAs (dsRNAs) or hairpin RNAs that specifically target essential genes in invading pathogens. These dsRNAs are processed by the plant's Dicer-like (DCL) enzymes into small interfering RNAs (siRNAs), which can move into the pathogen during infection. Inside the pathogen, siRNAs associate with Argonaute (AGO) proteins to form the RNA-induced silencing

complex (RISC), leading to post-transcriptional gene silencing of the targeted pathogen mRNA(Karimi & Innes, 2022). For example, by expressing dsRNA designed to target the *CYP51* gene family in *Fusarium graminearum*, resistance was induced in barley and *Arabidopsis*, thereby mitigating fungal growth and the severity of disease symptoms.(Hernández-Soto & Chacón-Cerdas, 2021)

B. **Spray-Induced Gene Silencing:** This approach involves a non-transgenic RNAi-based crop protection strategy that delivers double-stranded RNAs (dsRNAs) or small interfering RNAs (siRNAs) externally to plant surfaces such as leaves, stems, or fruits. This method bypasses the need for genetic modification of crops, unlike Host-Induced Gene Silencing (HIGS) and employs exogenous application of RNA molecule, typically as a foliar spray or root drench, to deliver RNA molecules into plant tissues and/or invading pathogens(Chen *et al.*, 2025). These dsRNA molecules are taken up by the host plant or absorbed directly by the pathogen during colonization, thereby initiating the gene silencing pathway. For example, Exogenous application of dsRNAs targets genes essential for *Botrytis cinerea* growth (e.g., *DCL1*, *DCL2*) that has been found to significantly reduce gray mold severity on fruits and vegetables(Chen et al., 2025).

C. **Virus-Induced Gene Silencing:** VIGS utilizes the plant's natural antiviral RNAi machinery as a defense tool, thereby knocking down critical endogenous genes rapidly without creating stable transgenic lines. It is a transient, RNAi-based reverse genetic tool that exploits the plant's innate antiviral defense system to silence the target genes. In this approach, a viral vector is engineered to carry a fragment of the plant gene of interest. Upon infection, the virus replicates and expresses this inserted fragment, forming double-stranded RNA (dsRNA) intermediates that are recognized by the plant's Dicer-like (DCL) enzymes, leading to the RNAi cascade (Rössner et al., 2022; Senthil-Kumar et al., 2010). For example, BSMV-HIGS (Barley stripe mosaic virus) of three pathogenicity-related genes in the wheat rust fungus *Puccinia triticina* suppressed the disease expression in susceptable wheat phenotypes (Wheat rust) (Rössner *et al.*, 2022).

Challenges and Limitations

The application of RNAi in plant protection is restricted by instability of dsRNA molecules, inefficiency of delivery mechanism, off-target effects, regulatory barriers, and pathogen adaptation. Fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* show strong RNA uptake ability, while others like *Zymoseptoria tritici*, exhibit very limited uptake, reducing RNAi effectiveness(Wang *et al.*, 2016)(*Zhao et al.*, 2024). Species-specific RNA uptake mechanism in nematodes and insects often complicates broad-spectrum application (Zhao *et al.*, 2024). Pathogens can evolve/adapt to produce RNAi suppressor proteins, mutate target genes or silence the dsRNA uptake machinery, thereby reducing

the overall effectivity (Weiberg *et al*., 2013) (Zhao *et al*., 2024). dsRNA molecules, especially the exogenously delivered particles applied in SIGS are highly susceptible to degradation by UV radiation, temperature fluctuations and RNases in the field, limiting their persistence and protective efficacy under real agricultural conditions (Hernández-Soto & Chacón-Cerdas, 2021). Moreover, RNA particles are required to be repeatedly applied/sprayed due to their very short life in phyllosphere and soil conditions, which increases overall cost of application (Mitter *et al*., 2017)(Wang *et al*., 2023). Movement of the RNA particles can be restricted by different physiological features of host plant like cuticular barriers, cell wall thickness, and limited vascular translocation (Koch et al., 2013). Error in application or formulation design may cause off-target gene silencing, creating adverse effects on host plant or beneficial microbes/insects (Zhao *et al*., 2024). Development of Transgenic plants as in case of HIGS is a time consuming and rigorous process and often faces constraints of biosafety and GMO regulations in several countries (Nowara *et al*., 2010) (Karimi & Innes, 2022). Last but not the least, large-scale production of high-quality dsRNA based formulations for agricultural application is highly expensive due to complex synthesis and purification processes (Imran *et al*., 2025).

Conclusion

RNA interference represents a transformative step toward sustainable and precise crop protection, offering a targeted, environmentally sound alternative to traditional pesticides. By exploiting natural gene silencing pathways, RNAi allows plants to defend themselves and supports safer agricultural ecosystems. Despite current challenges in delivery, stability, and cost, rapid progress in nanotechnology, dsRNA synthesis, and molecular design is steadily overcoming these barriers. While nanocarriers such as chitosan nanoparticles and layered double hydroxide (LDH) clays are being designed to protect RNA molecules from environmental degradation, bioinformatics-based dsRNA design tools are integrated to enhance sequence specificity, minimizing off-target effects while enabling multi-gene targeting for more durable pathogen resistance(Zhao *et al*., 2024) (Imran *et al*., 2025). With continued interdisciplinary innovation, RNAi technology holds the promise to become a cornerstone of next-generation, eco-friendly crop protection strategies.

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