DOI: https://doi.org/10.54083/978-81-980121-1-1-2

CHAPTER : 02

Use of RNAi in Plant Disease Management

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Abstract

Plant diseases have posed a significant threat to agriculture for centuries. The emergence of RNA interference (RNAi) technology offers a novel and promising approach to combat these challenges. Cells naturally employ RNAi to control gene expression by silencing particular genes. RNAi can successfully manage plant diseases by targeting genes necessary for pathogen survival or virulence. To induce RNAi in plants, a number of techniques have been devised, such as topical administration of double-stranded RNA (dsRNA) and the use of transgenic plants producing RNAi constructs. While transgenic plants have demonstrated success in managing certain plant diseases, they face regulatory hurdles and public concerns associated with genetically modified organisms (GMOs). Transient RNAi, on the other hand, offers a more sustainable and environmentally friendly alternative. Despite challenges such as delivery efficiency and the potential for pathogen resistance, RNAi technology holds significant promise for the future of plant disease management. Continued research and development are crucial to refine RNAi-based strategies and ensure their widespread application in agriculture.

Keywords Plant disease management, Plant pathogens, RNAi, Transient, Transgenic

1. Introduction

Believes were that the first land plants appeared on earth nearly around 460 million years ago. Since then, plants have evolved to adapt to various environments. Since the advent of agriculture, humans have cultivated crops to sustain themselves and provide for future generations. As long as plants have existed, so too have plant diseases. Pathogens and plants are locked in a constant struggle, with each side continually evolving in this ongoing conflict and are always in a perpetual evolutionary arms race, reflecting the natural dynamics between pathogens and plants (Anderson *et al.,* 2010). These different plant diseases pose a significant threat to modern-day agriculture. Plant diseases have been a significant concern throughout history, with records of afflictions such as rusts, mildews

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In: Current Trends in the Diagnosis and Management of Plant Diseases. (Eds.) Dutta, P., Upamanya, G.K., Pandey, A.K., 2024. Biotica Publications, Tripura, India.

and blights emerging from ancient texts (Gullino, 2021). These diseases have had profound impacts on agriculture, leading to severe famines and consequential economic upheavals. Historical accounts, including biblical and other early writings, document the effects of these diseases on food security and national economies, illustrating their long-standing influence on human societies. Now, years later, our modern-day agriculture stands poised to confront one of its most significant challenges: How are we going to raise crops in an efficiently sustainable and profitable way to feed our ever-growing population projected to be about 10 billion people by 2050?

To minimize damages caused by pathogens, growers have relied heavily on synthetic chemicals since the early 1800s. Bunt was controlled in the 17th century by briming grain with saltwater and then liming (Thind, 2010). In 1882, Millardet's discovery of the Bordeaux mixture provided a breakthrough in managing downy mildew. By 1915, organo-mercurial compounds were introduced as seed treatments. The 1940s brought the discovery of Dithiocarbamates (Thiram in 1940 and Ferbam in 1943). In 1961, Mancozeb was introduced, followed by the launch of the first systemic oomycete fungicide, Metalaxyl, in 1977. Fast forward to 1996, Strobilurins were discovered, offering a broad-spectrum fungicide with a novel mode of action (Thind, 2010). The third green revolution in modern agriculture is under underway, with a primary focus on sustainable plant disease management techniques. While successfully managing plant diseases, these tactics must minimize environmental risks and detrimental effects on ecosystems and natural resources. They should also, to the greatest extent feasible, lessen the need for chemical pesticides, protecting soil health, biodiversity, and water quality. The RNAi, or RNAi, strategy is one such mechanism for the efficient management of plant diseases. In order to effectively manage plant diseases in the field, what is RNAi (RNAi) and how may it be used?

2. What is RNAi?

RNAi can be considered as one of the important mechanisms of RNA silencing which is defined by the sequence-specific degradation of mRNAs or transcripts by small interfering RNAs (siRNAs) or microRNAs (miRNAs) with 21–24 nucleotides. RNAi is one of several RNA silencing mechanisms, characterized by the degradation of mRNAs or transcripts through small interfering RNAs (siRNAs) or microRNAs (miRNAs) ranging from 21 to 24 nucleotides, in a sequence-specific manner. The mechanism was first described and experimentally validated by Fire *et al.* (1998) during their work on *Caenorhabditis elegans*, which led to the term "RNA interference." However, various scientists had already begun exploring similar concepts in the 1990s, using different terminology. In 1990, while investigating the enzyme chalcone synthase (*CHS*) responsible for the deep violet colour in petunia plants, Napoli *et al.* (1990) made a surprising discovery. They had hoped to create more vibrant flowers by overexpressing *CHS*, but interestingly ended up with complete loss of the colour and producing white petunias

instead. They term the phenomenon as "Co-Suppression". Romano and Macino (1992) noticed a similar event in *Neurospora crassa* two years later, in 1992. They called this process "quelling," in which the introduction of homologous RNA sequences caused the target gene to go silent. But the real deal only occurred in the late 1990s. The scientific world was about to be captivated by a revolutionary discovery. Andrew Fire and Craig Mello, two researchers exploring the mysteries of *C. elegans*, a tiny roundworm, stumbled upon a phenomenon that would transform our understanding of gene regulation (Fire *et al.,* 1998). They observed that introducing double-stranded RNA (dsRNA) into these worms led to the unexpected silencing of specific genes. This elegant mechanism, they termed as RNAi, revealed that genes could be turned off with remarkable precision by small RNA molecules. Their groundbreaking work, published in 1998, illuminated a new layer of genetic control and earned them the Nobel Prize in 2006, forever changing the landscape of molecular biology.

3. Mechanism of RNA interference

Imagine RNAi as a highly specialized security system of the cell, what it really does is that, when foreign genes attempt to infiltrate, RNAi steps in to neutralize the threat. So, it is believed that RNAi has evolved as an effective strategy for cells to defend itself against foreign genes, such as those from viruses or transposons. This mechanism is evolutionarily conserved and is triggered by dsRNA, which uses the gene's own DNA sequence to silence it, a process known as gene silencing. The general mechanism of RNAi involved the following four stages (Baulcombe, 2004).

a) Initiation: When the long dsRNA is delivered into the cell, the process of RNAi initiates through recognition of these long dsRNA by the enzyme Dicer and cleaves into smaller RNAs referred to as microRNA (miRNA) or small interfering RNA (siRNA).

b) Formation of RISC: These siRNA or miRNA fragments are then fitted into a protein complex called the RNA-induced silencing complex (RISC). Within this complex, the double-stranded RNA is unwound and the guide strand is retained while the passenger strand (sense strand) is degraded.

c) Targeting mRNA: The guide strand within the RISC, pairs with the complementary sequence of the target mRNA, which leads to degradation of the transcript through sequence-specific cleavage by the endonucleolytic protein, Argonaute (AGO) at the center of the siRNA:mRNA or miRNA:mRNA duplex.

d) Gene Silencing: The degradation of the target mRNA prevents it from being translated into protein, effectively silencing the gene. This can occur through two main pathways:

• Post-transcriptional gene silencing (PTGS): mRNA degradation occurs after it has been transcribed.

• Transcriptional gene silencing (TGS): Transcription is inhibited through

modifications in the DNA.

4. Uses of RNAi in Plant Disease Management

RNAi has demonstrated efficacy in managing plant virus infections involving a broad range of viruses and plant types. The outcomes of applying RNAi-based transgenic antiviral resistance in agriculture have proven astounding. The development of transgenic papaya that is resistant to one of the economically important diseases of papaya i.e., papaya ringspot virus (PRSV) is one noteworthy success story; it played a critical role in preserving Hawaii's papaya sector (Gonsalves and Ferreira, 2003). The creation of transgenic cassava in Africa that is resistant to cassava brown streak disease (CBSD) is another comparable example (Wagaba *et al.,* 2017). Another benefit of RNAi-based transgenic resistance is its capacity to target many viruses at once. However, only a small number of genetically modified virus-resistant crops have obtained the required regulatory permission for commercial agriculture, even though it is possible to design antiviral immunity even in situations where natural resistance is lacking. The high costs of intellectual property for genetic engineering, the expenditures of regulatory approval, and societal worries over genetically modified organisms (GMOs) are more likely to be to blame than a lack of demand or potential efficacy.

4.1. RNAi in management of plant viruses

In potatoes infected with potato virus Y (PVY), the first effective application of RNAi technology to create virus-resistant plants was documented. This utilized vectors that allowed the helper-component proteinase (HC-Pro) gene's sense and antisense transcripts to be expressed simultaneously (Waterhouse *et al.,* 1998). Immunity against a variety of plant viruses has been effectively induced using this approach in a variety of plant-virus systems. For example, the 35S promoter regulated the expression of the intergenic region (IR) of the mungbean yellow mosaic India virus (MYMIV) as a hairpin construct in DNA viruses such as geminiviruses. It was administered biolistically to black gram plants infected with MYMIV, and the plants recovered completely from the infection until they reached senescence (Shivaprasad *et al.,* 2003). Additionally, co-transfection with small interfering RNA (siRNA) targeting the replicase (Rep) gene of ACMV using protoplast assays decreased *Rep* transcripts and viral DNA significantly (Vanitharani *et al.,* 2004). While siRNA was ineffective against more distantly related viruses, it proved efficient against a closely related strain of ACMV. Nevertheless, plant viruses have developed counter-silencing mechanisms through the production of proteins called viral silencing suppressor of RNAi (VSR) that circumvent this resistance (Li and Ding, 2006). These suppressors frequently contribute to the development of severe symptoms, viral pathogenicity, and plant virus synergism. More than 45 VSRs have been reported from various plant viruses (Ruiz and Voinnet, 2007). Numerous of these suppressors disrupt systemic silencing signals, according to studies. According to recent studies, the turnip yellow mosaic virus's p69 protein functions as a silencing suppressor,

preventing host RNA-dependent RNA polymerase (RDR)-dependent dsRNA synthesis (Ni, 2017). According to Lu *et al.,* (2003), the citrus tristeza virus (CTV) also has a number of suppressors, such as p20 and coat protein (CP), which block silencing signals and p23, which prevent intracellular silencing. The Pns10 protein of the rice dwarf virus inhibits inverted-repeat PTGS (IR-PTGS) but not local or systemic post-transcriptional gene silencing (S-PTGS), suggesting that it targets an early stage of dsRNA production (Ren *et al.,* 2010).

4.2. RNAi in management of Phytopathogenic Bacteria

Since prokaryotes do not have the RNAi machinery that eukaryotes do, prokaryotic plant pathogens cannot be effectively targeted by traditional RNAi techniques. However, new RNAi-based tactics can occasionally be developed through further studies on the interaction between host and pathogen and its underlying mechanism of disease development. For instance, by incorporating the T-DNA of *A. tumefaciens* into the chromosomal DNA of infected plant cells, changes in the host cells could be achieved (Escobar *et al.,* 2001). The cellular machinery of the plant transcribes and translates the genes of interest eg. the pathogenicity gene, that has been incorporated in the T-DNA. In order to stop crown gall formation, Dandekar and associates (1989) used a transgenic RNAi technique to target two mRNAs transcribed from the T-DNA. Hairpin RNA constructs that target the two genes derived from the oncogenes of T-DNA, *iaaM* and *ipt* mRNAs, were transformed in plants. In contrast to non-engineered control plants, which showed a 100% gall formation rate, these engineered plants showed a 0–24% gall development rate when exposed to virulent A. tumefaciens. This showed that mRNAs generated by the transcription of the two oncogenes were silenced through RNAi processes. Long-siRNAs (lsiRNAs), a novel kind of short RNA with a length of 30 to 40 nucleotides, have recently been linked to *P. syringae* infection. Resistance to bacterial infection was found to achieved by the inhibition of the *AtRAP* transcript level which is a negative regulator of plant defense (Katiyar-Agarwal *et al.,* 2007). Furthermore, it was shown that a peptide produced from the flagellin of *Pseudomonas* bacteria caused *Arabidopsis* to accumulate miR393, overexpression of which leads to induction of bacterial resistance in the host plant through (Navarro *et al.,* 2006).

4.3. RNAi in management of Phytopathogenic Fungi

The main biotic deterrent responsible for large crop losses is fungus. A reverse genetic technique for focusing on fungal genes is RNA-mediated gene silencing, also known as RNAi. Numerous plant pathogenic fungi have been shown to exhibit gene silencing mechanism as a part of viral defense, gene regulation during developmental process or during interaction with the various organisms in the environment (Mann *et al.,* 2023). Kadotani *et al.,* (2003) made the first systematic attempt to silence genes in *Magnaporthe grisea*, the causative agent of rice blast through use of enhanced green fluorescent protein (eGFP). Without requiring DNA methylation, hairpin RNA structures

dramatically decreased the accumulation of eGFP mRNA, outperforming other RNA species in the process. A method to mute the *mpg1* and *polyketide synthase-like* genes in *M. grisea* was further developed by Nakayashiki and colleagues (2005); 70–90% of the resultant transformants displayed gene silencing, while 10-15% had a nearly "null phenotype." Additionally, a GFP reporter gene in *Colletotrichum lagenarium* was silenced using this vector. These instances demonstrate how RNAi mechanisms in fungi can be an effective means to suppressed the expression of fungal virulence genes. It was shown by Schweizer *et al.* (2000) that dsRNA can disrupt gene activity in individual cereal cells. Through particle bombardment, they introduced dsRNA targeting the dihydroflavonol-4-reductase gene into single epidermal cells of wheat, barley, and maize. This resulted in a decrease in the buildup of anthocyanin pigment in barley and maize.

4.4. RNAi in management of Phytoparasitic Nematodes

Along with other minor species, various phytoparasitic nematodes such as *Meloidogyne* spp. (root-knot nematodes), *Heterodera* spp. (cyst nematodes) etc., seriously harm important crops like legumes, vegetables, and cereals globally, thereby posing a persistent threat to agricultural production. This emphasizes the necessity of a natural, economical, and environmentally friendly approach to pest control, which is challenging to accomplish with traditional techniques. New options for shielding plants against nematode damage have been devised by exploiting RNAi mechanism, which was initially shown in experiments on *C. elegans* (Fire *et al.,* 1998). Targeting critical nematode genes and plant genes implicated in the infection process are the two primary strategies that have surfaced. The application of RNAi in phyto-parasitic nematodes started in the early 2000s (Urwin *et al.,* 2002). It was reported that by producing hpRNAs with gene sequences derived from *M. incognita*, *Heterodera glycines* and *Globodera pallida* in host plants and feeding the nematodes leads to ultimately killing them successfully due to the silencing of its vital genes. Fanelli *et al.,* (2005), showed that by soaking eggs in dsRNA derived from chitin synthase gene from *M. artiella* was able to reduce the amount of chitin in the eggshell and postpone the hatching of juveniles. Additionally, RNAi targeting cysteine proteinase transcripts did not diminish nematode populations in plants but altered their sexual destiny in favour of males (Urwin *et al.,* 2002). While dsRNA targeting a protein similar to C-type lectins in *H. glycines* decreased nematode recovery by 41%, it had no effect on sexual fate. However, neither nematode growth nor sexual fate were impacted by targeting the main sperm protein (MSP). Nonetheless, RNAi experiments targeting various genes, including dual oxidase in *M. incognita*, resulted in reduced female nematode size and egg production by up to 70%. In *Heterodera schachtii*, RNAi silencing of genes responsible for sucrose transport, essential for nematode feeding structures, significantly reduced female nematode development (Colinet *et al.,* 2010). Similarly, tobacco plants engineered to express hpRNAs against two root-knot nematode genes exhibited strong resistance and effectively protect from

nematode infection (Fairbairn *et al.,* 2007).

5. Methods to Induce RNAi in Plants

So as the saying goes, "Every perk comes with a catch"; despite the powerful potential of RNAi in controlling gene expression in plants, the biggest challenge has always been about accurately targeting the right gene to be suppressed or induced. In order to achieve the most precise gene silencing, we need to have an effective delivery mechanism of dsRNA into the plant system. This could be attained through virus-induced gene silencing (VIGS), agroinfiltration and particle bombardment methods.

5.1. Virus-Induced Gene Silencing (VIGS)

Scientists design plant viruses (either of DNA/RNA genome) to carry a piece of the gene of interest. The modified viruses can act as vectors to deliver the genetic material into plant cells. Common plant viruses exploited for VIGS are tobacco mosaic virus (TMV), tobacco rattle virus (TRV), potato virus X (PVX), *etc.* (Dhir, 2019). When this modified virus is inoculated into the plant system, replication of the virus starts and produces dsRNA from the replication intermediate of the virus. This dsRNA acts as a crucial intermediate in the gene silencing process. The plant's defense system recognizes the dsRNA as a foreign entity and triggers PTGS. The resulting cleavage of viral RNA leads to a decrease in virus levels both locally and systemically within the plant, contributing to the plant's recovery and reduced symptoms of infection. But the twist is that not all RNA virus-derived vectors are effective for gene silencing as some of them contain anti-silencing proteins that disrupt the plant's silencing mechanisms like in the case of the tobacco etch virus. Moreover, the large genome size of DNA viruses limits their movement within plants and so are not considered effective expression vectors (Robertson, 2004).

5.2. Agroinfiltration

Agroinfiltration is a technique in plant biology, that involves injecting a suspension of *Agrobacterium tumefaciens* into plant tissue to introduce foreign DNA into plant cells. Agroinfiltration also termed as agroinoculation, efficiently induces cytoplasmic RNAi (RNAi) in plants (Lee *et al.,* 2006). Specific RNAi constructs, such as short hairpin RNA (shRNA) or dsRNA, are designed and incorporated into a specific plasmid. The vector is introduced into *Agrobacterium tumefaciens and* is then cultured to high density. The prepared *Agrobacterium* is introduced into plant leaves using a syringe to inject the bacteria directly into the leaf tissue or by vacuum infiltration, where leaves are submerged in the bacterial suspension and placed under reduced pressure to help the bacteria enter the cells. Once it enters the plant cells, the *Agrobacterium* transfers the RNAi constructs. These constructs are expressed, producing RNA molecules that activate the RNAi pathway. The plant's RNAi machinery processes these RNA molecules and specifically target the transcripts with complementary sequence and effectively silenced them.

Infiltrating hairpin RNA constructs is particularly effective for gene silencing because they produce dsRNA, which is directly converted into siRNA by the plant's RNAi machinery. This process leads to efficient degradation of the target gene and robust gene silencing (Senthil-Kumar and Mysore, 2010).

5.3. Micro-Bombardment

Biolistics or micro bombardment, is a method used to introduce RNAi constructs into plant cells. Particles coated with ssRNA, dsRNA, or DNA encoding hairpin constructs can be bombarded into cells to activate the RNAi silencing pathway (Sharma *et al.,* 2013). In micro-bombardment, a circular or linear DNA template is introduced into the plant nucleus using gene gun method. Moreover, synthetic siRNAs are delivered into the plant cells *via* biolistic pressure to induce silencing of GFP expression.

6. Challenges of RNAi in Agriculture

Despite the effectiveness of transgenic plants in managing plant diseases through RNAi, this strategy faces several challenges that limit its widespread application. Some key challenges are:

6.1. Regulatory and Biosafety Concerns

• *Gene Flow*: There is potential for transgenic plants to crossbreed with wild relatives, leading to the spread of RNAi traits in natural ecosystems, which could disrupt non-target species.

• *Biosafety Regulations*: Strict regulatory approval processes for transgenic crops can slow down the deployment of RNAi technologies and public concerns about genetically modified organisms (GMOs) may limit acceptance.

6.2. Delivery Challenges

Introducing RNAi mechanisms effectively into some plant species, particularly woody perennials, can be difficult. It may require specific methods like tissue culture, which are labour-intensive.

6.3. Cost and Time-Intensive Development

Developing transgenic plants with RNAi resistance involves significant investment in research, development, testing and regulatory approval, making it expensive as well as the process of identifying target genes, developing transgenic plants and field testing can take years, slowing the commercialization process.

6.4. Public Acceptance

As RNAi plants are considered GMOs, they face resistance from consumers and policymakers in countries where GMOs are controversial or restricted. Intellectual property rights and patenting of RNAi technologies can lead to legal constraints and hinder broader adoption, particularly in developing countries.

7. Transient RNAi: A Novel and Alternative Approach for Sustainable Disease Management

Although transgenic plants have proven to be effective in managing plant diseases, this approach is not universally successful across all crops or against every target pathogen. Additionally, the development of transgenic lines involves significant initial costs and lengthy timelines for commercial release, largely due to socio-political concerns, environmental issues and low public acceptance of genetically modified (GM) crops (Bawa and Anilakumar, 2013). Recently, a novel and an alternative strategy involving the topical application of dsRNA was found highly effective in inducing RNA silencing in plants and provide resistance against plant pathogens. The first successful proof of concept of plant disease management through topical application of dsRNA was reported against three RNA viruses. This was achieved through mechanical co-inoculation into a local lesion hosts (Tennelado *et al.,* 2001). The researchers discovered that in order to produce robust viral resistance, dsRNA needed to have at least a certain length, have sequence specificity, and have the right dosage. However, protection failed when more than 24 hours elapsed between dsRNA application and virus inoculation. In vivo synthesis of dsRNA was carried out using HT115 cells, which have an IPTG-inducible T7 promoter and are deficient in the RNaseIII enzyme (Tennelado *et al.,* 2003). Subsequent research revealed that applying French press crude extracts of *E. coli* HT115 cells to plants reduced PMMoV and PPV infections in the systemic host *N. benthamiana* for a long time, from 24 hours to 5 days after the lysate was applied (Tennelado *et al.,* 2003). Following this success, dsRNA technology was further explored and shown to provide protection against various plant viruses (Sangwan *et al.,* 2023; Singh *et al.,* 2021; Gupta *et al.,* 2021; Namgial *et al.,* 2019; Konakalla *et al.,* 2019; Mitter *et al.,* 2017) and fungi (Mann *et al.,* 2023; McRae *et al.,* 2023; Sundaresha *et al.,* 2021) in plants.

8. Conclusion

RNAi (RNAi) has become a viable method for managing plant diseases, providing a potential remedy for the increasing problems caused by plant infections. By targeting specific genes involved in pathogenicity, RNAi can disrupt the life cycle of various pathogens, including viruses, bacteria, fungi and nematodes. While transgenic plants expressing RNAi constructs have shown significant success in controlling plant diseases, the associated regulatory hurdles and public concerns have limited their widespread adoption. To address these limitations, researchers have explored alternative strategies, such as transient RNAi that involves the external application of dsRNA on to the plants. This approach offers a more sustainable and environmentally friendly solution, as it avoids the need for genetically modified plants. However, challenges such as efficient delivery, stability and the potential for development of pathogen resistance remained to be explored. Despite these challenges, RNAi technology holds great promise

for the future of plant disease management. By continuing research and development, scientists can refine RNAi-based strategies to create more effective and sustainable solutions to protect our crops from the ever-evolving threat of plant pathogens.

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