*Plant Archives* Vol. 25, No. 1, 2025 pp. 27-37 e-ISSN:2581-6063 (online), ISSN:0972-5210

# Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2025.v25.no.1.005

# HARNESSING RNA-MEDIATED RESISTANCE TO COMBAT YELLOW VEIN MOSAIC VIRUS IN OKRA

**G.J. Abhishek[1], D.D. Deepika[1], M.S. Jagadeesh[1], N. Nandkumar[1], G. Praveen Kumar[1], T. Danakumara[1], V. Celia Chalam[2] and Rajneesh Kumar[3]**

[1]Graduate school, Indian Agricultural Research Institute, New Delhi -110012, India.
[2]Division of Plant Quarantine, ICAR-National Bureau of Plant Genetic Resources, New Delhi -110012, India.
[3]Division of Genetics and Plant Breeding, Faculty of Agriculture (FoA), Sher-e-Kashmir University of Agricultural Sciences and Technology (SKUAST–K), Wadura- 193201, J & K, India..
*Corresponding authorE-mail: celia.chalam@icar.gov.in
(Date of Receiving-29-06-2024; Date of Acceptance-01- 09-2024)

**ABSTRACT**

Okra (*Abelmoschus esculentus* L.) is a significant vegetable crop cultivated globally, yet its production is frequently compromised by viral pathogens, particularly the yellow vein mosaic virus (YVMV). YVMV induces severe symptoms that lead to substantial yield losses in okra crops. Traditional methods of disease management, such as chemical treatments and breeding for resistance, have their limitations. However, recent advancements in biotechnology have paved the way for novel strategies to combat YVMV. One promising approach is RNA-mediated resistance, especially RNA interference (RNAi), which provides targeted and specific control over viral infections. This review article presents an overview of the molecular mechanisms underlying YVMV infection in okra, examines the potential of RNA-mediated resistance, and discusses recent advancements and challenges in utilizing this technology for effective YVMV management in okra cultivation.

***Key words*:** *Abelmoschus esculentus*, Yellow vein mosaic virus, *Bemisiatabaci*, RNA interference.

## Introduction

Okra (*Abelmoschus esculentus* L.), commonly known as lady's finger or gumbo, is a warm-season vegetable crop valued for its tender pods and versatile culinary applications. Originated from Africa, okra is now cultivated globally in tropical and subtropical regions due to its adaptability to diverse climates and soil types. This crop plays a crucial role in global food security and economic livelihoods, particularly in areas where it is a dietary staple. Despite its significance, okra cultivation faces several challenges, including pest and disease pressures. Among the various pathogens that affect okra, the Yellow Vein Mosaic Virus (YVMV) is a major concern. YVMV belongs to the genus *Begomovirus* within the family *Geminiviridae*, a group known for causing severe diseases in many crop plants. Transmitted by whiteflies (*Bemisia tabaci*), YVMV infects okra plants, leading to distinct symptoms such as leaf yellowing and curling, vein yellowing, stunted growth, and reduced fruit quality and yield.

The impact of YVMV on okra cultivation is substantial, leading to significant economic losses for farmers and threatening food security in affected regions. Traditional management strategies, such as chemical treatments and breeding for resistance, have been employed to control YVMV infections. However, these methods often face limitations regarding efficacy, sustainability, and long-term viability. Therefore, there is an urgent need for sustainable and effective management strategies to combat YVMV and ensure the productivity and resilience of okra crops. Recent advancements in biotechnology have opened new avenues for developing innovative solutions to address viral diseases in crops. One promising approach is RNA-mediated resistance, particularly RNA interference (RNAi), which provides targeted and specific control over viral infections.

In this review article, we provide a comprehensive overview of the molecular mechanisms underlying YVMV infection in okra, examine traditional management strategies, and discuss the potential of RNA-mediated resistance as a novel and sustainable approach to combat YVMV in okra cultivation. By synthesizing current knowledge and identifying future research directions, this review aims to contribute to the development of effective strategies for managing YVMV and ensuring the continued success of okra production globally.

## YVMV: Symptoms, Transmission, and Economic Impact

### Yellow vein mosaic disease

Yellow Vein Mosaic Virus (YVMV) has long been a serious issue in okra cultivation, causing yield losses ranging from 20% to 50%. In cases of early infection, yield loss can increase up to 90%. YVMV is classified under the genus *Begomovirus*, which can have either monopartite or bipartite genomic organization. Bipartite *Begomovirus* genomes consist of two single-stranded DNA (ssDNA) molecules, approximately 2.6 kb in size, designated as DNA-A and DNA-B, each serving different functions during infection (Jose and Usha, 2003). According to Lazarowitz (1992), the genes present on DNA-A are responsible for encapsidation and replication, while the genes on DNA-B are involved in systemic movement, host range determination, and symptom expression within the host plant. In monopartite *Begomoviruses*, in addition to DNA-A, some satellite DNA molecules, known as DNA-β or beta satellites (approximately 1,350 bp), are also reported (Briddon *et al*., 2003). These beta satellites depend on DNA-A for encapsidation, replication, and insect-borne transmission. YVMV is a monopartite *Begomovirus* belonging to the *Geminiviridae* family (Stanley, 2006; Brown *et al*., 2012).

### Symptom of YVMV

The symptoms of Yellow YVMV infection are characterized by a homogenous, interwoven network of yellow mosaic patterns that enclose islands of green tissue in the leaf blades. As the virus load increases, infected leaves become more yellow, plants become stunted, and fruits are small and pale yellow in color (Sanwal *et al*., 2014; Khaskheli *et al*., 2017). The severity of symptoms varies depending on the timing of infection. During the late rainy season, symptoms are less severe compared to the summer season due to a lower vector population during the rainy season (Sanwal *et al*., 2016).

### Transmission of YVMV

YVMV is primarily transmitted among plants by the whitefly (*Bemisia tabaci* Genn.) (Venkataravanappa *et al*., 2014). Sanwal *et al*., (2014) observed that female whiteflies are more effective at transmitting the virus than male whiteflies. Chattopadhyay *et al*., (2011) reported that the whitefly population increases from February to August, peaking in June and July. YVMV occurrence is higher under optimal temperatures, bright sunshine, and lower relative humidity (Dhankar, 2012).

### Economic Impact of YVMV

Yellow Vein Mosaic Virus (YVMV) is a severe disease in okra, causing yield losses ranging from 20% to 80%, and in some cases, up to 100%, depending on the stage of infection. Losses are particularly severe when the infection occurs within 35-50 days after sowing (Sanwal *et al*., 2014). Khaskheli *et al*., (2017) observed a significant reduction in the height of infected plants (48.67 cm) compared to healthy plants (62.96 cm). The damage is not limited to plant biomass but also affects fruit quality parameters. A survey conducted by Venkataravanappa (2008) revealed that YVMV disease incidence ranged from 42.45% to 75.64% in Kerala, 23% to 85.64% in Maharashtra, 35.76% to 57% in Uttar Pradesh, 45.89% to 56.78% in Andhra Pradesh, 23% to 67.67% in Karnataka, 24.85% to 65.78% in Haryana, 67.78% in Chandigarh, 23% to 75.64% in Tamil Nadu, 45.89% to 66.78% in Rajasthan, and 45.45% in Delhi.

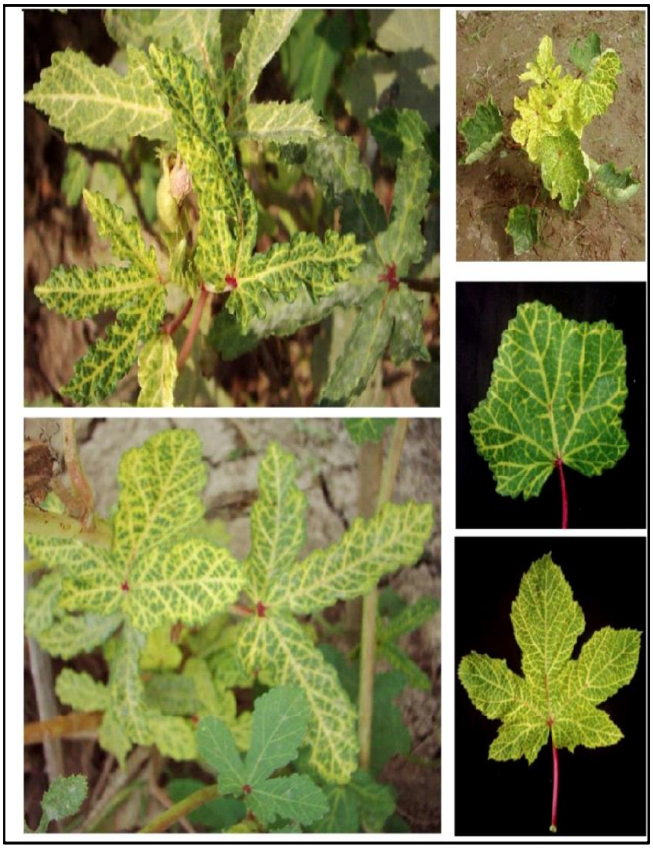

**Fig. 1:** Symptoms observed in okra due to Okra Yellow Vein Mosaic Disease (Khaskheli *et al*., 2017).

### Management of YVMV

Managing whiteflies is key to controlling YVMV since whiteflies have a wide range of host plants, making them difficult to control throughout the crop growth period. Several strategies for virus control are discussed below.

### Cultural practices

Field cleaning and removal of infected plants are common practices for reducing the vector-borne spread of the disease.

### Biological Control

Plant growth-promoting rhizobacteria (PGPR) can be used to control the population of whiteflies. Genes encoding enzymes such as chitinase, beta-1, 3-glucanase, peroxidase, and PALase are activated by rhizobacteria, which enhances systemic defence mechanisms and reduces YVMV incidence by up to 86.6% (Patil *et al*., 2011). Additionally, the fungal biocontrol agent *Verticillium lecanii* is effective in managing YVMV in okra (Alavo, 2015).

### Management using organic compounds

Patil *et al*., (2011) observed that the use of biological products, such as azadirachtin applied at 15-day intervals, effectively controls the whitefly population by up to 79.2%. The application of Crozophera oil at 1.0 ml/L, followed by Palmrosa oil at 1.0 ml/L, has also been found effective in controlling whitefly populations and ultimately reducing disease incidence (Biswas *et al*., 2008). Additionally, soap solution (0.5%) and neem oil spray are commonly used to manage YVMV in okra (Ansar *et al*., 2014). Singh *et al*., (2010) attempted to reduce YVMV incidence under natural conditions using a 4% root extract of *Boerhaaviadiffusa* at 7-day intervals, starting from the seedling stage. Regular spraying was more effective, reducing YVMV incidence by 80% and minimizing crop losses.

### Chemical control

Imidacloprid, Acetamiprid, and Trizophos are the most commonly recommended chemicals for managing whiteflies (Gowdar *et al*., 2007). Alam *et al*., (2010) observed that two applications of Acetamiprid 20SP at a rate of 40 g ai/ha effectively reduce the incidence of YVMV. Imidacloprid, a neonicotinoid systemic insecticide, reduces the pest population by up to 90.2% (Ansar *et al*., 2014).

### Legal control

Virus infection may occur through infected planting material, insect vectors, or common agricultural practices (Fereres and Moreno, 2009). Novy *et al*., (2007) suggested that using certified planting material is essential to restrict virus transmission through planting material. Vector control and adherence to good agricultural practices are also effective in managing virus transmission (Castle *et al*., 2009; Fereres and Moreno, 2009).

## Approaches to induce resistance against viral disease

### Breeding in okra for YVMV resistance

Food security is a major goal for developing countries, and viral diseases of crop plants are a significant constraint in achieving this goal. Yield losses due to viruses can be mitigated through the development of resistant varieties via plant breeding. However, genetic resources in okra with resistance to YVMV have not yet been reported (Dhankhar *et al*., 2005). While wild species such as *A. manihot*, *A. crinitus*, *A. angulosus*, and *A. tetraphyllus*ssp. *tetraphyllus* are known to harbour resistance genes and some varieties have been bred using these species, the genetic distances among different species limit the success of hybridization (Singh *et al*., 2007). The variety Parbhani Kranti (a hybrid of *A. esculentus* × *A. manihot*), which was released as a resistant variety (Jambhale and Nerkar, 1986), lost its resistance after two decades due to mutations in the virus genome. Small changes or mutations in the virus genome can lead to the emergence of new strains with higher infectivity (Jones, 2009).

### Crop protection based on engineered resistance

The development of resistance in plants can be achieved by either introducing resistance genes against the virus or by introducing genes that confer resistance to the vector (Groen *et al*., 2017). Manipulating the host-pathogen interaction is an effective approach for developing plant resistance (Rodrigues *et al*., 2009). Virus entry into plant cells, which involves disrupting the plasma membrane, is known as inoculation or infection (Rodrigues *et al*., 2009). The effectiveness of a virus within a host plant depends on its compatibility with host cell components, including plasmodesmata (PD) and vascular bundles, which play crucial roles in cell interaction (Taliansky *et al*., 2008). Internal immunity in the host plant triggers resistance mechanisms, leading to reduced viral replication (Ascencio-Ibanez *et al*., 2008). The movement of the virus within the plant cell is regulated by virus-encoded movement proteins (MPs), which attach to nucleic acids and target sites (Lucas, 2006).

Ma *et al*., (2004) suggested that developing cultivars with high levels of resistance is the only effective way to reduce crop losses due to virus infection. Research on the biochemistry of virus infection by Tenllado *et al*.,

(2003) has shown that advanced techniques such as RNA silencing hold significant potential for developing virus-resistant plants. Recombinant DNA technologies and the standardization of transformation protocols have facilitated biotechnological approaches for enhancing virus resistance in plants (Lapidot and Friedmann, 2002). For example, transgenic okra lines resistant to shoot and fruit borer have been developed by Narendran *et al*., (2013). Additionally, replication protein and movement protein genes are considered promising targets for transgenesis aimed at achieving virus resistance in plants.

## RNAinterference (RNAi)

### Mechanisms of RNA-Induced Gene Silencing

RNA interference (RNAi) is a conserved regulatory mechanism of gene expression observed in eukaryotic organisms (Bartel, 2004). This mechanism involves gene silencing through sequence-specific degradation of complementary mRNA, which is triggered by double-stranded RNA (dsRNA). RNAi is categorized based on the stage of gene silencing into two types: post-transcriptional gene silencing (Vaucheret *et al*., 2001) and transcriptional gene silencing (induced by DNA methylation) (Castel and Martienssen, 2013). Wang and Chekanova, (2016) have shown that RNA silencing plays a crucial role in the cellular mechanisms of eukaryotic organisms, regulating nearly all cellular processes, including growth and physiological activities, through small non-coding RNAs (sRNAs). RNAi is also associated with the regulation of genome stability, viral infection, epigenetic modification, suppression of transposons, and regulation of heterochromatin (Castel and Martienssen, 2013).

Double-stranded RNA (dsRNA) acts as a trigger for gene silencing by RNA-dependent RNA polymerases (RDRPs) in plant-infecting RNA viruses (Ruiz-Ferrer and Voinnet, 2009). The RNAi mechanism is initiated with the incorporation of dsRNA into the RNA-induced silencing complex (RISC), which contains an Argonaute (AGO) protein as an sRNA-binding domain and an RNase III-like enzyme called Dicer, which has cleavage activity on target sequences (Qiu *et al*., 2007; Ketting, 2011). This mechanism specifically relies on guide RNA (dsRNA) and RISC, with the reaction being ATP-dependent (Liu and Paroo, 2010).

The term "RNAi" was first coined by Fire *et al*., (1998) during studies on gene silencing in the nematode *Caenorhabditis elegans*. The mechanism was rapidly and widely adopted for gene silencing in plants due to its high specificity, accuracy, and heritability (Baulcombe, 2004). Napoli *et al*., (1990) accidentally discovered RNAi while attempting to overexpress the chalcone synthase (CHS) gene in pigmented petunia petals. Unexpectedly, the introduced gene led to a block in anthocyanin biosynthesis, resulting in entirely white flowers or flowers with white or pale non-clonal sectors on a wild-type pigmented background. This phenomenon, later identified as RNAi, involves both dsRNA, which triggers RNAi, and siRNA, which directly interacts with the RISC complex for cleavage (Hannon and Zamore, 2003).

Earlier studies have reported that DNA methylation is a key factor inducing gene silencing at the transcriptional level (Mette *et al*., 2000). RNA-induced gene silencing occurs through small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Bartel, 2004). In the case of siRNA, double-stranded RNA (dsRNA) originates from single-stranded viral intermediate replication, while miRNA is involved in the negative regulation of gene expression (Meister *et al*., 2004).

At the application level, RNAi is utilized in various fields of agriculture to enhance nutritional content and protect crop plants. RNAi has been successfully employed in many crops to develop lines with desirable traits, such as cottonseeds with high stearic and high oleic acids (Tang *et al*., 2005), soybeans with increased oil stability at high temperatures (Flores *et al*., 2008), tomatoes with increased carotenoid and flavonoid content (Davuluri *et al*., 2005), corn with high lysine content (Houmard *et al*., 2007), rapeseed with increased flower production (Byzova *et al*., 2004), and roses with blue petals (Katsumoto *et al*., 2007).

## RNAi for virus resistance

### Applications in Tomato

RNA interference (RNAi) has demonstrated effectiveness in virus gene suppression in plants due to its sequence-specific nature. For instance, Zrachya *et al*., (2007) used siRNA derived from an intron-hairpin RNA (ihpRNA) construct targeting the coat protein gene to generate tomato lines resistant to Tomato yellow leaf curl virus (TYLCV). Some of the transformed tomato plants did not show disease symptoms even after seven weeks of inoculation. Similarly, Chen *et al*., (2016) developed transgenic tomato plants targeting six segments of the monopartite genome of Tomato leaf curl Taiwan virus (ToLCTWV), achieving resistance against multiple Begomovirus strains.

### Applications in Other crops

BBTV-resistant banana (cv. Rasthali) plants were developed through RNAi strategies (Shekhawat *et al*., 2012). Leksmi *et al*., (2020) developed resistance in banana plants against Banana bract mosaic virus by

transferring an ihpRNA cassette targeting the viral replicase gene. An RNAi construct was developed by fusing sections of the C1, C3 (encoding proteins responsible for virus replication), and C2 (regulating gene expression) genes. The resistance to Mungbean yellow mosaic India virus (MYMIV) in cowpea was achieved by targeting the AC2, AC4, and a combination of AC2 and AC4 (AC2+AC4) genes (Kumar *et al*., 2017). Resistance in soybean was developed by targeting the conserved region of the AC2 open reading frame (ORF) of MYMIV (Ramesh *et al*., 2019).

Translation Initiation Factors TaeIF(iso)4E and TaeIF4G are crucial for the translation process in wheat potyviruses, such as Wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV). Transgenic wheat lines resistant to these viruses were developed through RNA interference targeting these genes (Rupp *et al*., 2019). Tatineni *et al*., (2020) developed resistant transgenic lines by targeting the replicase gene of WSMV and TriMV.

RNAi was also used to develop Cotton Leaf Curl (CLC) virus resistance in tobacco (*Nicotiana tabacum*) by targeting the BC1 region of Cotton leaf curl *multanbetasatellites* (CLCuMuB), with resistance observed in transgenic plants even after 90 days of virus inoculation (Akhtar *et al*., 2017). Hashmi *et al*., (2011) developed CLCuMuB resistance in cotton by targeting the truncated Rep gene of the virus through RNA interference. RNAi is a powerful tool for developing cotton varieties resistant to Cotton leaf curl disease (CLCuD) (Sattar *et al*., 2013).

### Challenges and Future Directions

Despite its success, RNAi faces challenges, such as viral defense mechanisms targeting the RISC complex of plants (Ding, 2010). Nonetheless, RNAi remains a leading strategy in virus resistance, employing hairpin RNA-expressing vectors and virus-induced gene silencing (VIGS) (Burch-Smith *et al*., 2004). Given that whiteflies are phloem feeders and most Begomovirus strains are phloem-limited, RNAi mechanisms for Begomovirus resistance in okra should target genes involved in related pathways (Thakur *et al*., 2014; Zaidi *et al*., 2017).

## RNA-Mediated Resistance: An Innovative Approach

### Overview of RNA interference (RNAi) as a mechanism for antiviral defence

RNA interference (RNAi) is a conserved cellular process that plays a crucial role in regulating gene expression and defending against viral infections in plants. The RNAi pathway involves the sequence-specific degradation of target RNA molecules, mediated by small RNA molecules known as small interfering RNAs (siRNAs). These siRNAs are generated from double-stranded RNA (dsRNA) precursors by the enzyme Dicer and incorporated into the RNA-induced silencing complex (RISC), where they guide the cleavage of complementary target RNAs. In the context of antiviral defense, plants can harness the RNAi pathway to recognize and degrade viral RNA molecules, thereby inhibiting viral replication and spread. This process involves the production of virus-derived siRNAs (vsiRNAs) from viral double-stranded RNA intermediates, followed by their incorporation into RISC for specific targeting of viral RNA transcripts. The efficiency of RNAi-mediated antiviral defense depends on various factors, including the abundance and accessibility of viral dsRNA, the activity of Dicer and RISC components, and the specificity of vsiRNA-target interactions.

### Application of RNAi in combating viral infections in plants

RNAi has emerged as a powerful tool for engineering viral resistance in plants by exploiting the endogenous RNAi pathway. By generating transgenic plants expressing hairpin RNA (hpRNA) or artificial micro RNA (amiRNA) constructs targeting viral genes, researchers can induce RNAi-mediated silencing of viral transcripts and confer resistance against specific viral pathogens. This approach has been successfully applied to various plant-virus systems, including geminiviruses, potyviruses, and tobamoviruses, demonstrating its broad applicability and efficacy in conferring resistance against diverse viral pathogens.

### Potential advantages of RNA-mediated resistance for controlling YVMV in okra

The application of RNA-mediated resistance offers several potential advantages for controlling YVMV in okra cultivation:

1. **Specificity**: RNAi-mediated resistance can be precisely targeted to inhibit the expression of viral genes essential for infection and replication, minimizing off-target effects and preserving the integrity of host plant genes.
2. **Flexibility**: RNAi technology allows for the rapid design and optimization of RNAi constructs targeting specific YVMV genes, enabling tailored resistance strategies tailored to the genetic diversity of viral strains and the host plant.
3. **Durability**: RNAi-mediated resistance can confer durable protection against viral infections by targeting conserved viral genes or essential viral functions, reducing the likelihood of viral

escape mutants and the development of resistance-breaking strains.

4. **Compatibility**: RNAi-mediated resistance can be combined with other management strategies, such as breeding for host plant resistance or integrated pest management approaches, to achieve synergistic effects and enhance overall disease control efficacy.

## Exploiting RNA-Mediated Resistance against YVMV in Okra

### Target identification and validation for RNAi-mediated silencing of YVMV genes;

The first step in exploiting RNA-mediated resistance against YVMV in okra is to identify and validate suitable target genes for RNAi-mediated silencing. This process involves the characterization of YVMV genes essential for viral replication, transcription, movement, and pathogenesis, as well as the assessment of their conservation and specificity across different YVMV strains. Candidate target genes can be selected based on their critical roles in the viral lifecycle and their potential vulnerability to RNAi-mediated degradation. Once candidate target genes have been identified, their efficacy as RNAi targets can be validated through *in vitro* and *in vivo* assays, such as transient expression studies in model plant systems or protoplasts, to assess the efficiency of gene silencing and its impact on viral replication and spread. Target validation experiments may also involve the generation and characterization of transgenic plants expressing RNAi constructs targeting specific YVMV genes, followed by challenge inoculation with YVMV to evaluate the extent of viral resistance conferred by RNAi-mediated silencing.

### Delivery methods for introducing RNAi constructs into okra plants

The successful implementation of RNAi-mediated resistance against YVMV in okra requires efficient delivery methods for introducing RNAi constructs into okra plants. Several approaches can be employed for the delivery of RNAi constructs, including Agrobacterium-mediated transformation, particle bombardment (biolistics), and viral vectors. Agrobacterium-mediated transformation is a widely used method for generating transgenic plants expressing RNAi constructs. This approach involves the introduction of T-DNA containing RNAi expression cassettes into okra plants via Agrobacterium tumefaciens-mediated transformation, followed by selection and regeneration of transgenic plants expressing the desired RNAi constructs. Particle bombardment offers an alternative method for delivering RNAi constructs into okra plants by physically bombarding plant tissues with DNA-coated microprojectiles, enabling the direct transformation of plant cells without the need for bacterial infection.

Viral vectors represent another promising delivery strategy for RNAi-mediated resistance against YVMV in okra. Recombinant viral vectors can be engineered to express RNAi constructs targeting specific YVMV genes and delivered into okra plants via viral infection. This approach exploits the natural ability of viruses to replicate and spread within plant tissues, facilitating the systemic delivery of RNAi constructs and the induction of RNAi-mediated resistance against YVMV.

### Strategies for enhancing the efficacy and stability of RNAi-mediated resistance

To maximize the efficacy and stability of RNAi-mediated resistance against YVMV in okra, several strategies can be employed to optimize RNAi construct design, delivery, and expression:

1. **Selection of target genes**: Careful selection of target genes for RNAi-mediated silencing is critical to ensure efficient inhibition of viral replication and spread. Target genes should be essential for viral infection and highly conserved among different YVMV strains to minimize the risk of resistance development.
2. **Optimization of RNAi construct design:** RNAi constructs should be designed to maximize target specificity and efficacy while minimizing off-target effects and unintended silencing of host plant genes. Optimization of hairpin RNA (hpRNA) or artificial microRNA (amiRNA) structures can enhance RNAi-mediated gene silencing and improve overall resistance efficacy.
3. **Promoter selection:** The choice of promoter driving RNAi construct expression can significantly impact the efficacy and stability of RNAi-mediated resistance. Strong and constitutive promoters, such as the Cauliflower Mosaic Virus 35S promoter, can ensure high-level expression of RNAi constructs throughout the plant tissues, enhancing resistance against YVMV infection.
4. **Integration and stability:** Integration of RNAi constructs into the plant genome and stable inheritance of transgenic traits are essential for long-term resistance efficacy and durability. Integration of RNAi constructs at single-copy loci and the use of transformation techniques that promote stable transgene expression can improve

**Table 1:** Functions of different genes in Okra.

| Gene Name | Function | Example |
|---|---|---|
| CP (Coat Protein) | Encapsidates viral genome | Transgenic okra expressing CP-RNAi constructs |
| Rep (Replication-associated protein) | Facilitates viral replication | Transgenic okra expressing Rep-RNAi constructs |
| AC4 (Viral Movement Protein) | Facilitates viral movement | Transgenic okra expressing AC4-RNAi constructs |

the stability and heritability of RNAi-mediated resistance in okra plants.

5. **Stacking with other resistance genes:** RNAi-mediated resistance can be stacked with other resistance genes or traits to confer broad-spectrum and durable protection against YVMV and other viral pathogens. Combinatorial approaches that harness multiple resistance mechanisms can enhance overall disease control efficacy and reduce the risk of resistance development.

By employing these strategies, researchers can optimize the design and delivery of RNAi constructs and enhance the efficacy and stability of RNAi-mediated resistance against YVMV in okra, paving the way for the development of sustainable and effective management strategies for controlling this devastating viral pathogen.

## Recent Advancements in RNA-Mediated Resistance Against YVMV in Okra

### Case studies and experimental evidence demonstrating the effectiveness of RNAi against YVMV in okra

Several studies have demonstrated the effectiveness of RNAi-mediated resistance against YVMV in okra through case studies and experimental evidence. For example, researchers have successfully engineered transgenic okra plants expressing hairpin RNA (hpRNA) or artificial microRNA (amiRNA) constructs targeting key YVMV genes, such as the coat protein (CP) or replication-associated protein (Rep). These transgenic plants exhibited reduced symptoms of YVMV infection, including leaf yellowing, vein clearing, and stunted growth, compared to non-transgenic control plants. Molecular analyses confirmed the presence of YVMV-specific small interfering RNAs (siRNAs) in transgenic plants, indicative of RNAi-mediated gene silencing and suppression of viral replication. These findings highlight the potential of RNAi technology as a powerful tool for conferring resistance against YVMV in okra and provide proof-of-concept evidence for its effectiveness under controlled laboratory conditions.

### Genetic engineering approaches for enhancing RNAi efficiency and durability in okra plants

Recent advancements in genetic engineering have focused on enhancing the efficiency and durability of RNAi-mediated resistance against YVMV in okra plants. Strategies for improving RNAi efficiency include optimizing RNAi construct design, such as the selection of highly conserved target sequences and the incorporation of viral-derived sequences known to trigger strong RNAi responses. Additionally, researchers have explored the use of viral vectors and RNAi enhancer sequences to enhance RNAi efficacy and systemic spread of RNAi signals within okra plants, thereby conferring broader and more durable resistance against YVMV infection. Furthermore, efforts have been made to engineer transgenic okra plants with enhanced RNAi machinery, such as overexpression of Dicer-like (DCL) proteins or RNA-dependent RNA polymerases (RDRs), to boost the production and amplification of small interfering RNAs (siRNAs) and improve overall RNAi efficiency and specificity. These genetic engineering approaches hold promise for developing okra cultivars with enhanced resistance to YVMV and other viral pathogens, contributing to sustainable and resilient okra production systems.

### Field trials and practical applications of RNAi-mediated resistance in okra cultivation

Field trials and practical applications of RNAi-mediated resistance against YVMV in okra cultivation are underway to evaluate the performance and efficacy of transgenic plants under real-world growing conditions. These field trials involve the deployment of RNAi-transgenic okra lines in YVMV-endemic regions and monitoring their performance in terms of disease incidence, symptom severity, yield, and quality compared to conventional okra varieties. Preliminary results from field trials have shown promising outcomes, with RNAi-transgenic okra plants exhibiting reduced susceptibility to YVMV infection and improved agronomic traits under field conditions. These findings underscore the potential of RNAi-mediated resistance as a practical and effective strategy for managing YVMV in commercial okra production and provide valuable insights into the feasibility and scalability of deploying transgenic crops in agricultural settings.

## Challenges and Future Directions

### Off-target effects and unintended consequences of RNAi technology

One of the major challenges associated with RNAi-mediated resistance is the potential for off-target effects

and unintended consequences, including unintended silencing of host plant genes or the emergence of viral escape mutants. Off-target effects can arise due to sequence homology between target and non-target genes, leading to unintended gene silencing and physiological abnormalities in transgenic plants. To mitigate off-target effects, researchers are exploring strategies for improving the specificity and selectivity of RNAi constructs, such as bioinformatics-assisted target prediction, target site engineering, and the use of tissue-specific or inducible promoters to restrict RNAi activity to specific tissues or developmental stages. Additionally, comprehensive risk assessments and molecular characterization of transgenic plants are essential for identifying and minimizing potential off-target effects before their commercial release.

**Regulatory considerations and public acceptance of genetically modified okra varieties**

The regulatory approval and public acceptance of genetically modified (GM) okra varieties expressing RNAi constructs pose significant challenges to the widespread adoption of RNAi-mediated resistance in okra cultivation. Regulatory agencies require rigorous safety assessments and environmental impact evaluations to ensure the safety and efficacy of GM crops before their commercialization. Public perception and acceptance of GM okra varieties may vary depending on cultural, social, and economic factors, as well as concerns related to food safety, environmental sustainability, and socio-economic impacts. Effective communication and engagement with stakeholders, including farmers, consumers, policymakers, and non-governmental organizations (NGOs), are essential for fostering transparent dialogue and building trust in the regulatory process and the potential benefits of RNAi-mediated resistance for okra production.

**Strategies for overcoming technical and logistical challenges in implementing RNAi-mediated resistance in okra cultivation**

Technical and logistical challenges in implementing RNAi-mediated resistance in okra cultivation include optimizing transformation protocols, scaling up production of transgenic plants, ensuring trait stability and heritability, and integrating RNAi technology into existing breeding programs. Collaboration between academia, industry, and government agencies is critical for developing standardized protocols, sharing resources and expertise, and establishing infrastructure for the production and distribution of RNAi-transgenic okra seeds. Capacity building and technology transfer initiatives can facilitate the adoption of RNAi-mediated resistance by smallholder farmers and promote sustainable okra production practices in resource-limited settings. Furthermore, research efforts should focus on developing alternative delivery methods for RNAi constructs, such as nanoparticle-based delivery systems or foliar sprays, to overcome barriers associated with genetic transformation and facilitate the rapid deployment of RNAi technology in okra cultivation.

**Future prospects and potential synergies with other biotechnological approaches for managing YVMV in okra**

Looking ahead, the future of RNAi-mediated resistance against YVMV in okra holds promise for addressing the challenges of viral disease management and ensuring the sustainability of okra production systems. Continued research efforts are needed to further optimize RNAi technology, improve understanding of host-pathogen interactions, and develop innovative solutions for controlling YVMV and other viral pathogens in okra. Furthermore, synergies with other biotechnological approaches, such as genome editing, marker-assisted breeding, and biostimulant treatments, offer opportunities for enhancing the resilience and productivity of okra crops in the face of emerging threats and changing environmental conditions. By leveraging the collective strengths of different biotechnological tools and approaches, researchers can develop integrated and holistic strategies for managing YVMV and promoting sustainable okra production systems that are resilient to viral diseases and other stresses.

## Conclusion

In conclusion, recent advancements in RNA-mediated resistance against YVMV in okra have demonstrated the potential of RNAi technology as a promising and sustainable strategy for controlling viral diseases in agricultural crops. Case studies and experimental evidence have provided proof-of-concept for the effectiveness of RNAi-mediated resistance against YVMV in okra, while genetic engineering approaches have enabled the optimization of RNAi efficiency and durability in transgenic plants. Field trials and practical applications of RNAi-mediated resistance have shown promising outcomes in terms of disease control and agronomic performance under real-world growing conditions. However, challenges remain, including off-target effects, regulatory considerations, and technical hurdles, which must be addressed to realize the full potential of RNAi technology for okra cultivation. By overcoming these challenges and embracing collaborative and interdisciplinary approaches, researchers can unlock new opportunities for enhancing the resilience and sustainability of okra production systems and ensuring food security for future generations.

## References


Akhtar S., Akmal M., and Khan J. (2017). Resistance to cotton leaf curl disease in transgenic tobacco expressing βC1 gene derived intron hairpin RNA. *Indian J. Biotechnol.* **16**, 56-62.

Alam, M.M., Hoque M.Z., Khalequzzaman K.M., Humayun M.R. and Akter R. (2010). Eco-friendly management agents of Okra yellow vein clearing mosaic virus of okra (*Abelmoschusesculentus* L. Moench). *Bangladesh J. Agric.* **35(1)**, 11-16.

Alavo, T.B.C. (2015). The insect pathogenic fungus *Verticilliumlecanii* (zimm.) viegas and its use for pests control, a review. *J. Exp. Biol. Agric. Sci.* **3(4)**, 337- 345.

Ali Dhankhar, S.K., Dhankhar B.S. and Yadava R.K. (2005). Inheritance of resistance to Yellow vein mosaic virus in an interspecific cross of okra (*Abelmoschusesculentus*). *Indian J. Agric. Sci.* **75**, 87-89.

Ansar, M., Saha T., Sarkhel S. and Bhagat A.P. (2014). Epidemiology of okra yellow vein mosaic disease and its interaction with insecticide modules. *Trends Biosci.* **7(24)**, 4157-4160.

Ascencio-Ibanez, J.T., Sozzani R., Lee T.J., Chu T.M., Wolfinger R.D., Cella R. and Hanley-Bowdoin L. (2008). Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during Geminivirus infection. *Plant Physiol.* **148**, 436-454.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* **116(2)**, 281-297.

Baulcombe, D. (2004). RNA silencing in plants. *Nature*. **431**, 356-363.

Biswas, N.K., Nath P.S., Srikanta D. and De B.K. (2008). Management of yellow vein mosaic virus disease of okra (*Abelmoschusesculentus* L. Moench) through different plant oils. *Res. Crops*. **9(2)**, 345-347.

Briddon, R.W. and Stanley J. (2006). Subviral agents associated with plant single stranded DNA viruses. *Virology.* **344**, 198-210.

Briddon, R.W., Bull S.E., Amin I., Idris A.M., Mansoor S., Bedford I.D., Dhawan P., Rishi N., Siwatch S.S., Abdel-Salam A.M., Brown J.K., Zafar Y. and Markham P.G. (2003). Diversity of DNA β, a satellite molecule associated with some monopartite Begomoviruses. *Virology*. **(312)**, 106-121.

Brown, J.K., Fauquet C.M., Briddon R.W., Zerbini M., Moriones E. and NavasCastillo J. (2012). Geminiviridae. In, King A.M.Q., Adams M.J., Carstens E.B. and Lefkowitz E.J. (eds), Virus Taxonomy- Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, 351-373.

Burch Smith, T.M., Anderson J.C., Martin G.B. and Dinesh Kumar S.P. (2004). Applications and advantages of virus induced gene silencing for gene function studies in plants. *Plant J*., **39**, 734-746.

Byzova, M., Verduyn C., De Brouwer D. and De Block M. (2004). Transforming petals into sepaloid organs in Arabidopsis and oilseed rape, implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner. *Planta*, **218(3)**, 379-387.

Castel, S.E. and Martienssen R.A. (2013). RNA interference in the nucleus: Roles for small RNAs in transcription, epigenetics and beyond. *Nat. Rev. Genet.* **14**, 100-112.

Castle, S., Palumbo J. and Prabhakar N. (2009). Newer insecticides for plant virus disease management. *Virus Res*. **141**, 131-139.

Chattopadhyay, A., Dutta S. and Shatterjee S. (2011). Seed yield and quality of okra as influenced by sowing dates. *African J. Biotechnol.* **28(5)**, 461-467.

Chen, H.M., Lin C.Y., Tsai W.S., Kenyon L., Chan M.T., Yen J.Y., Chang S.Y., de la Pena R. and Schafleitner R. (2016). Resistance to viral yellow leaf curl in tomato through RNAi targeting two Begomovirus species strains. *J. Plant Biochem. Biotechnol.* **25(2)**, 199-207.

Davuluri, G.R., Van Tuinen A., Fraser P.D., Manfredonia A., Newman R., Burgess D., Brummell D.A., King S.R., Palys J., Uhlig J. and Bramley P.M. (2005). Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol.* **23**, 890-895.

Dhankar, S.K. (2012). Genetic improvement of adopted okra cultivars for YVMV disease resistance involving wild relatives in genus Abelmoschus. In: Holmer,R., Linwattana, G. and Keatinge, J. D. H. (eds), High Value Vegetables in Southeast Asia: Production, Supply and Demand. Proceedings of the Regional Symposium on High Value Vegetables in Southeast Asia: Production, Supply and Demand (SEAVEG2012), Thailand, 56-60.

Ding, S.W. (2010). RNA-based antiviral immunity. Nat. Rev. *Immunol*. **10**, 632-644.

Fereres, A. and Moreno A. (2009). Behavioral aspects influencing plant virus transmission by homopteran insects. *Virus Res.* **141**, 158-168.

Fire, A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E. and Mello C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature, **39**, 806-811.

Flores, T., Karpova O., Su X., Zeng P., Bilyeu K., Sleper D.A., Nguyen H.T. and Zhang Z.J. (2008). Silencing of GmFAD3 gene by siRNA leads to low α- linolenic acids (18: 3) of fad3-mutant phenotype in soybean *Glycinemax* (Merr.). *Transgenic Res*. **17**, 839-850.

Gowdar, S.B., Ramesh Babu H.N. and Aswathanarayana R.N. (2007). Efficacy of insecticides on Okra yellow vein mosaic virus and Whitefly Vector, *Bemisiatabaci* (Guenn.). *Ann. Plant Prot. Sci.* **15(1)**, 116-119.

Groen, S.C., Wamonje F.O., Murphy A.M. and Carr J.P. (2017). Engineering resistance to virus transmission. *Curr. Opinion Virol.* **26**, 20-27.

Hannon, G. and Zamore, P.D. (2003). Small RNAs, Big Biology, Biochemical Studies of RNA Interference. In: Hannon, G.

(ed.), RNAi: A Guide to Gene Silencing. Cold Spring Harbor Laboratory, United States, 436.

Hashmi, J.A., Zafar Y., Arshad M., Mansoor S. and Asad S. (2011). Engineering cotton (*Gossypiumhirsutum* L.) for resistance to cotton leaf curl disease using viral truncated AC1 DNA sequences. *Virus Genes*. **42**, 286-296.

Houmard, N.M., Mainville J.L., Bonin C.P., Huang S., Luethy M.H. and Malvar T.M. (2007). High lysine corn generated by endosperm specific suppression of lysine catabolism using RNAi. *Plant Biotechnol. J.* **5(5)**, 605-614.

Jambhale, N.D. and Nerkar Y.S. (1986). "Parbhani Kranti", a yellow vein mosaic resistant okra. *Hortic. Sci.* **21**, 1470–1471.

Jones, R.A. (2009). Plant virus emergence and evolution, Origin, new encounter scenarios, factors driving emergence, effects of changing world conditions and prospectus for control. *Virus Res.* **141(2)**, 113-130.

Jose, J. and Usha R. (2003). Bhendi yellow vein mosaic disease in India caused by association of a DNA-β satellite with a Begomovirus. *Virology.* **305**, 310-317

Katsumoto, Y., Fukuchi-Mizutani M., Fukui Y., Brugliera F., Holton T.A., Karan M., Nakamura N., Yonekura-Sakakibara K., Togami J., Pigeaire A., Tao G.Q., Nehra N.S., Lu C.Y., Dyson B.K., Tsuda S., Ashikari T., Kusumi T., Mason J.G. and Tanaka Y. (2007). Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol. **48(11)**, 1589-600.

Ketting, R.F. (2011). The many faces of RNAi. *Dev. Cell.* **20**, 148-161.

Khaskheli, M.I., Jiskani M.M., Goswami S.P., Poussio G.B. and Khaskheli M.A. (2017). Effect of Okra yellow vein mosaic virus (OYVMV) on plant growth and yield. *J. Basic Appl. Sci*. **13**, 1-7.

Kumar, S., Tanti B., Patil B.L., Mukherjee S.K. and Sahoo L. (2017). RNAi- derived transgenic resistance to Mungbean yellow mosaic India virus in cowpea. *PloSOne*. **12(10)**, e0186786.

Lapidot, M. and Friedmann M. (2002). Breeding for resistance to whitefly-transmitted Geminiviruses. *Ann. Appl. Biol.* **140**, 109-127.

Lazarowitz, S.G. (1992). Geminiviruses, genome and structure and gene function. *Rev. Plant Sci.* **11**, 327-349

Lekshmi, R.S., Harshitha C.K., Soni K.B. and Swapna A. (2020). Transgenic banana plants carrying ihpRNA cassette targeting viral Replicase gene show resistance against Banana bract mosaic virus. *J. Hortic. Sci. Biotechnol.* **96(3)**, 324-329.

Liu, Q. and Paroo Z. (2010). Biochemical principles of small RNA pathways. *Annu. Rev. Biochem.* **79**, 295-319.

Lucas, W.J. (2006). Plant viral movement proteins, agents for cell-to-cell trafficking of viral genomes. *Virology*. **344**, 169-184.

Ma, G., Chen P., Buss G.R. and Tolin S.A. (2004). Genetics of resistance to two strains of soybean mosaic virus in differential soybean genotypes. *J. Hered.*, **95**, 322-326.

Meister, G. and Tuschl T. (2004). Mechanisms of gene silencing by double stranded RNA. *Nature*. **431**, 343-349.

Mette, M.F., Aufsatz W., Van der Winden J., Matzke M.A., and Matzke A.J.M. (2000). Transcriptional silencing and promoter methylation triggered by double stranded RNA. *EMBO J.* **19(19)**, 5194-5201.

Napoli, C., Lemieux C. and Jorgensen R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell*. **2**, 279-289.

Narendran, M., Deole S.G., Harkude S., Shirale D., Nanote A., Bihani P., Parimi S., Char B.R. and Zehr U.B. (2013). Efficient genetic transformation of okra (*Abelmoschusesculentus* (L.) Moench) and generation of insect-resistant transgenic plants expressing the Cry1Ac gene. *Plant Cell Rep.* **32**, 1191-1198.

Novy, R.G., Gillen A.M. and Whitworth J.L. (2007). Characterization of the expression and inheritance of Potato leafroll virus (PLRV) and Potato virus Y(PVY) resistance in three generations of germplasm derived from Solanum tuberosum. *Theor. Appl. Genet.* **114**, 1161-1172.

Patil, M.N., Jagadeesh K.S., Krishnaraj P.U., Patil M.S. and Vastrad A.S. (2011). Plant growth promoting rhizobacteria (PGPR) mediated protection in bhindi against yellow vein mosaic virus. *Indian J. Plant Prot.* **39(1)**, 48-53.

Qiu, C.X., Xie F.L., Zhu Y.Y., Guo K., Huang S.Q., Nie L. and Yang Z.M. (2007). Computational identification of microRNAs and their targets in *Gossypiumhirsutum* expressed sequence tags. *Gene*. **395(1-2)**, 49-61.

Ramesh, S.V., Shivakumar M., Praveen S., Chouhan B.S. and Chand S. (2019). Expression of short hairpin RNA (shRNA) targeting AC2 gene of Mungbean yellow mosaic India virus (MYMIV) reduces the viral titre in soybean. *3Biotech.* **9(9)**, 334-340.

Rodrigues, S.P., Andrade J.S., Ventura J.A., Lindsey G.G. and Fernandes P.M.B. (2009). Papaya meleira virus is neither transmitted by infection at wound site nor by the whitefly Trialeurodes variabilis. *J. Plant Pathol.* **91**, 87-91.

Ruiz-Ferrer, V. and Voinnet O. (2009). Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.* **60**, 485-510.

Rupp, J.S., Cruz L., Trick H.N. and Fellers J.P. (2019). RNAi-mediated silencing of endogenous wheat genes EIF (Iso) 4E-2 and EIF4G induce resistance to multiple RNA viruses in transgenic wheat. *Crop Sci.* **59(6)**, 2642-2651.

Sanwal, S.K., Venkataravanappa V. and Singh A. (2016). Resistance to okra yellow Xviii mosaic disease: A review. *Indian J. Agric. Sci.* **6(7)**, 835-843.

Sanwal, S.K., Singh M., Singh B. and Naik P.S. (2014). Resistance to yellow vein mosaic virus and okra enation leaf curl virus, challenges and future strategies. *Curr. Sci.* **106**, 1470-1471.

Sattar, M.N., Kvarnheden A., Saeed M. and Briddon R.W. (2013). Cotton leaf curl disease-an emerging threat to cotton production worldwide. *J. Gen. Virol.***94**, 695-710.

Shekhawat, U.K.S., Ganapathi T.R. and Hadapad A.B. (2012). Transgenic banana plants expressing siRNAs targeted against viral replication initiation gene display high-level resistance to Banana bunchy top virus infection. *J. Gen. Virol.* **93(8)**, 1804-1813.

Singh, A.K., Najam A., Verma H.N. and Awasthi L.P. (2010). Control of natural virus infection on okra (*Abelmoschusesculentus*) by root extract of *Boerhaaviadiffusa*. *Int. J. Plant Prot.* **2(2)**, 195-198.

Singh, B., Rai M., Kalloo G., Satpathy S. and Pandey K.K. (2007). Wild taxa of okra (*Abelmoschus* species), reservoir of genes for resistance to biotic stresses. *Acta Hortic*. **752**, 323-328.

Taliansky, M., Torrance L. and Kalinina N.O. (2008). Role of plant virus movement proteins. *Methods Mol. Biol.* **451**, 33-54.

Tang, G.Q., Novitzky W.P., Carol Griffin H., Huber S.C. and Dewey R.E. (2005). Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. *Plant J.* **44**, 433-446.

Tatineni, S., Sato S., Nersesian N., Alexander J., Quach T., Graybosch R.A. and Clemente T.E. (2020). Transgenic wheat harboring an RNAi element confers dual resistance against synergistically interacting Wheat streak mosaic virus and Triticum mosaic virus. *Mol. Plant-Microbe Interact.* **33(1)**, 108-122.

Tenllado, F., Barajas D., Vargas M., Atencio F.A., Gonzalez-Jara P. and DiazRuiz J.R. (2003). Transient expression of homologous hairpin RNA causes interference with plant virus infection and is overcome by a virus encoded suppressor of gene silencing. Mol. *Plant-Microbe Interact.* **(16)**, 149-158.

Thakur, N., Upadhyay S.K., Verma P.C., Chandrashekar K., Tuli R. and Singh P.K. (2014). Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of v-ATPase A gene. *PLoS One.* **9**, e87235.

Vaucheret, H. and Fagard M. (2001). Transcriptional gene silencing in plants: Targets,inducers and regulators. *Trends Genet.* **17**, 29-35.

Venkataravanappa, V. (2008). Molecular characterization of okra yellow vein mosaic virus. Ph. D. thesis, University of Agricultural Sciences, Bengaluru, 368.

Venkataravanappa, V., Prasanna H.C., Reddy C.N.L. and Reddy M.K. (2014). Evidence for two predominant viral lineages, recombination and subpopulation structure in Begomoviruses associated with yellow vein mosaic disease of okra in India. *Plant Pathol.* **64**, 508-518.

Wang, H.L. and Chekanova J.A. (2016). Small RNAs, Essential regulators of gene expression and defenses against environmental stresses in plants. *RNA.* **(7)**, 356-381.

Zaidi, S.S.A., Briddon R.W. and Mansoor S. (2017). Engineering dual Begomovirus- Bemisia tabaci resistance in plants. *Trends Plant Sci*. **22**, 6-8.

Zrachya, A., Kumar P.P., Ramakrishnan U., Levy Y., Loyter A., Arazi T., Lapidot M. and Gafni Y. (2007). Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. *Transgenic Res*. **16**, 385-398.