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Review Article

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RNAi: A Promising Approach to Develop Transgenic Plants Against Geminiviruses and Insects

Abhinav Kumar[1]* and Neera Bhalla Sarin[1]

Abstract

Viruses, especially geminiviruses and insects infect a wide range of economically important crops across the globe. To reduce the losses caused by them, scientists have adopted several genetic engineering methods to develop resistant plants. Among them, RNA silencing based resistance has proven to be a reliable approach. In this review, we focus on RNAi mediated gene silencing approaches against this class of virus and insects. RNA silencing is a complex and conserved defence mechanism utilized by plants and other eukaryotes to protect themselves from aberrant nucleic acids. The use of RNAi provides an environmental friendly approach to generate plants resistant to viruses and insects, which is not possible otherwise.

Introduction

Plants are consistently attacked by a number of microorganisms such as viruses and bacteria, and plant parasites such as nematodes, herbivorous insects, weeds, fungus and several other pathogens. Most of them cause significant economic loss to agricultural countries such as India. Geminiviruses are one such group of viruses, due to which agricultural economy of India and its neighbour countries suffers major setback every year. These viruses grouped in family *Geminiviridae,* are widely distributed plant viruses infecting a wide range of plants from monocots such as maize to dicots such as cassava and tomato (Figure 1) [1]. They all shares two distinctive features [2] the geminate morphology of the virion particle, ~18–30 nm in size, whose detailed electron microscopic-derived structure has been reported, and [3] the nature of their genetic material that consists of one or two single-stranded DNA (ssDNA) molecules (2.5–3.0 kb in length), depending on the group. Differences in the genetic organization of their genomes as well as their host range and insect vectors serve as distinguishing criteria to recognize four different genera [4]. At present, the family *Geminiviridae* consisting of four genera viz. *Mastrevirus, Begomovirus, Curtovirus* and *Topocuvirus*. A mastrevirus is transmitted by leafhopper, generally infects monocots and has monopartite genome (Type species: *Maize streak virus*). A curtovirus is leafhopper-transmitted, infects dicots and has monopartite genome (Type species: *Beet curly top virus*). Begomoviruses are whitefly-transmitted, infect dicots and have either monopartite or bipartite genome (Type species: *Bean golden mosaic virus-Puerto Rico*). The members of genus *Topocuvirus* are transmitted by treehoppers infect dicots and have monopartite genome (Type species: *Tomato pseudo curly top virus*).

Several conventional methods have been employed in the past such as crop rotation, breeding cross protection etc. Cultural practices such as crop-free periods, altering dates, crop rotation, and weed and crop residue disposal, high planting densities, floating row cover, mulches, trap crop, or living barriers performed well. It has been long observed that plants infected by mild strain can be protected against infection by more severe strain of the related virus, a biological term called cross protection. The cross-protection test has been previously regarded as an important means for identifying the same strain or a distinct species of plant virus [5].

Over the years, chemical or pesticide control has been adopted to counter the pests, but only partial success was felt. Use of excessive chemicals may change the texture of the soil, and there is also fear of eradication of natural enemy. There are however several drawbacks in the above mentioned conventional methods. These methods require good knowledge and skill, are time consuming and expensive. A serious limitation of breeding program is the availability of resistance traits. To overcome these difficulties and limitations, non-conventational methods of genetic engineering has now been practiced to develop resistant transgenic plants [6,2]. Initially coat protein mediated resistance was shown against *Tobacco mosaic virus* (TMV) from where concept of pathogen derived resistance or parasite derived resistance (PDR) was introduced [6]. On the other hand downregulation of the expression of specific genes in insects through RNAi relies on the feeding or injection of dsRNA (Figure 2). Development of a robust dsRNA feeding methodology in insects that mimics the result obtainable with *C. elegans* is a prerequisite for utilization of RNAi for crop protection against insect pests [6]. Here in this review we focus on the potential of RNAi to develop transgenic plants against geminiviruses and insects.

Figure 1: Figure showing Gemini virus infection in different plants. A: Leaves of cotton; B and C: leaves of papaya; D: Single leaf of okra. All the leaves are showing characteristic geminivirus symptoms such as leaf curling, vein thickening, mosaics etc.

***Corresponding author:** Abhinav Kumar, School of Life Sciences, Jawahar Lal Nehru University, New Delhi-110067, India, Tel: +91-11-2670-4523; Fax: +91-11-2618-7338; E-mail: abhibiot2k4@gmail.com

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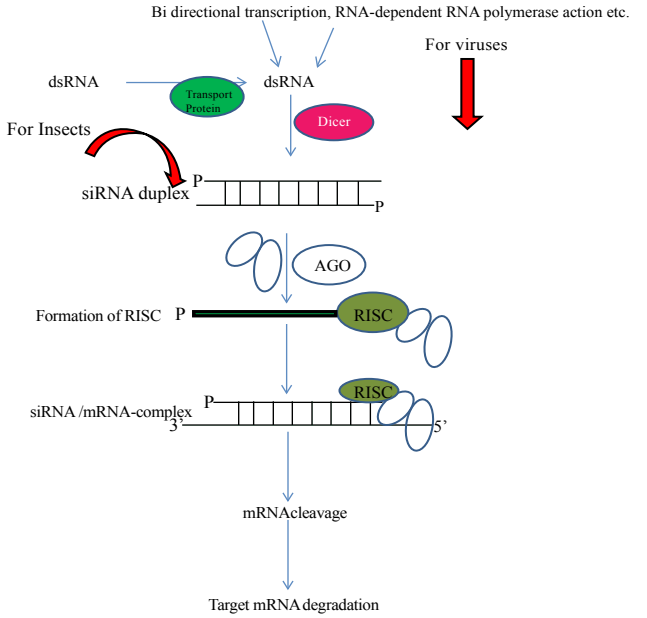

Figure 2: Functional stages of gene silencing with dsRNA in cells of insects and viruses. The figure shows steps involved in systemic gene silencing and the molecules involved in them. Exogenous dsRNA is imported into cells (for insects), processed by dicer into small interfering (siRNA) and loaded into RNA induced silencing complex (RISC) with the help of argonaute protein (AGO). This RISC complex targets and degrades specific mRNAs based on siRNA sequence. For viruses perfectly base-paired dsRNA is produced from bidirectional transcription, RNA dependent RNA polymerase (RdRP) action etc. processed by dicer into siRNAs and loaded into AGO containing RISC complex. This siRNA-RISC complex further target homologous mRNAs for translational arrest.

Small RNA in Antiviral Defence and Insect Genetics

RNA silencing involves suppression of gene expression by sequence specific degradation of mRNA in diverse eukaryotes. The RNA silencing phenomena was first discovered and termed post transcription gene silencing (PTGS) in plants [7], quelling in fungi [8] and RNA interference (RNAi) in animals [9,10].

Key molecules involved in the RNA silencing pathways are ribonuclease Dicer (RNA-dependent RNA polymerase, RDR), and Argonaute (AGO). The RNA silencing machinery in plants are more evolved than in fungal and animal systems. The *Arabidopsis* genome encodes four Dicer like (DCL) enzymes, six RDRs and ten AGO proteins [11].

There are three different pathways in the gene silencing mechanism (i) cytoplasmic short interfering (siRNA) silencing, (ii) silencing of endogenous mRNAs by micro RNAs (miRNAs) (iii) DNA methylation and suppression by transcription [12]. siRNA silencing is actually post transcriptional gene silencing (PTGS) which was first identified by Bisaro in 2006 [13]. This mechanism results in the production of 21-25 nucleotide siRNA which are generated by inducing dsRNA [14] leading to the degradation of mRNA.

Broadly defined, RNA interference is a collection of processes that uses short RNAs (20-30 nt) to recognize, manipulate and manage the complementary nucleic acids.

Besides the regulatory roles in plant development and stress related functions, the siRNA-mediated RNA silencing also functions as a natural antiviral defence mechanism, a process named virus-induced gene silencing (VIGS) [15,16]. Host RNA silencing machinery targets and processes the virus derived dsRNA into small viral siRNAs, which are then used to target destruction of additional viral RNA molecules. The viral siRNAs are then loaded to host RNA induced silencing complex (RISC), which targets and inhibits gene expression and protein translation in the viral genome. Nearly one and a half decade has gone for these discoveries which showed antiviral defense mechanism [17,18]. There are four more classes of small RNAs besides siRNAs, which includes microRNAs (miRNAs), transacting siRNAs (ta-siRNAs) natural antisense siRNAs (nat-siRNAs) and Piwi-interacting RNAs [19,20]. These endogenous small RNAs have important regulatory roles in gene development and programming. Downregulation of the expression of specific genes through RNAi technology has been widely used for genetic research in insects. This method follows the injection of long dsRNA (synthesised *in vitro*) into the insect haemoceol [21]. Feeding and/or injection method is necessary to induce RNAi effect, because complete genome sequencing of insects (including *D. melanogaster*) has shown absence of RNA-dependent DNA polymerase (RdRP), which is a key enzyme for systemic RNAi effect [6]. Absence of this key enzyme suggests that effects of RNAi are limited to cell/cells which uptake dsRNA in continuous manner [22].

Pathogen-derived resistance

Concept of pathogen derived resistance or parasite derived resistance was originally developed by Sanford and Johnston in 1985. Since then several attempts were made to develop transgenic plants using virus derived genes or genome fragments [23-26]. While many of these attempts have been successful and led to the development of virus- resistant plants, in some cases durable geminivirus resistance has not been achieved [27].

Coat protein

The viral coat protein (*CP*) gene was the first and one of the most widely used genes to confer PDR against plant viruses [28,29]. Virus resistance has been achieved by transforming the plants with viral *CP* gene which ultimately showed resistance against infection by the homologous virus. Limited success has been observed in tobacco showing resistance to *Tobacco mosaic virus* (TMV), [30] and papaya resistant to Papaya ring spot virus . It was later proved that resistance was mediated by the RNAs of the *CP* transgene, rather than the protein, as an inverse correlation between resistance and the accumulation levels of mRNAs of *CP* transgene were observed, indicating that a PTGS mechanism is likely to be involved in the *CP* RNA-mediated protection [31]. In recent report by Amudha et al. [32], transgenic cotton transformed with antisense coat protein showed considerable resistance against CLCuV.

Movement protein

The movement protein (MP) is required by Geminiviruses for movement and spread into the host. Some researchers tried to engineer pathogen-derived resistance with dominant negative mutant of viral gene. This strategy was successfully demonstrated in the development of transgenics using dysfunctional MP [33,34]. In another experiment, transgenic tobacco plants that expressed a gene encoding a defective TMV movement protein (TMV-MP) showed

resistance to *Tobacco rattle tobovirus, Tobacco ringspot nepovirus, Alfalfa mosaic alfamovirus* and *Cucumber mosaic virus* [35].

However, transgenic expression of a functional MP has no effect on virus infection or increased susceptibility [36]. Resistance conferred by transgenic expression of a dysfunctional TMV MP is likely due to compete for plasmodesmatal binding sites between the mutant MP and the wild-type MP of the inoculated virus [33].

Replication associated protein mediated resistance

The engineering of geminivirus resistance using *Rep* gene has been achieved in model host species against begomoviruses. Expression of full-length or a truncated N-terminal portion of *Rep* gene of *African cassava mosaic virus* (ACMV) inhibits replication of ACMV in *Nicotiana tabacum* protoplasts. A modest degree of ACMV resistance was achieved by the expression of full-length *Rep* gene in experimental plant tobacco. None of these transgenic tobacco plants was resistant to distantly related viruses Tomato golden mosaic virus (TGMV) and Beet curly top virus (BCTV) (sharing 60% Rep amino acid sequence identity with ACMV) [37]. The experiment thus suggested that resistance was probably ACMV specific or to its closely related viruses [38].

Other approaches which manipulated *Rep* gene to engineer resistance against *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in *N. benthamiana* [39] and tomato [40] were proved successful. In another approach using manipulated *Rep* gene against cotton leaf curl disease [41] in experimental plant tobacco was very successful. Even after 120 days of continuous exposure of viruleferous whiteflies, no observable symptoms were observed [41].

Gene Silencing

The processes of PDR mentioned above involve resistance generated through expression of transgenic proteins. However, RNAi allows silencing of viral gene without protein expression. Gene silencing is a ubiquitous form of genetic regulation used by plants and other eukaryotes to tightly control the expression of certain proteins [42-48]. The gene silencing phenomena follows either at transcriptional level i.e. transcriptional gene silencing (TGS) or post transcriptional gene silencing (PTGS). The transcriptional gene silencing is usually induced by DNA methylation [49-51].

PTGS can be induced in the transgenic plants by the expression of dsRNAs homologous to viral sequences. These can be generated mainly by two approaches-first one is through transgene in the sense/antisense orientation. The mechanism resembles co-suppression where the expression of the transgene is silenced together with the homologous viral gene. The major drawback described above is that the resistance is unstable and it has been reported that some groups of viruses including potyviruses, cucumoviruses and tobamoviruses are able to counteract these mechanisms by inhibiting this type of PTGS [48,52]. The next approach is by dsRNA delivery vehicles, i.e. hairpin vectors, in which virus-derived sequence is cloned in sense and antisense orientations and separated by an intron that is post-transcriptionally spliced to create dsRNA with a hairpin structure [53-55]. This strategy is useful in attaining resistance in most of the cases, some examples are: development of resistance against ACMV, Mungbeen yellow mosaic virus, ToLCV, TYLCV, SriLankan cassava mosaic virus and East African cassava mosaic virus [37] Cotton leaf curl disease [41].

However, resistance efficacy may vary for different strategies and also for different viruses. Although the exact mechanism is unclear, it is believed that methylation in the incoming virus sequence may not allow virus particle to accumulate and hence result in resistance. However, mutation of over 10-20% in the homologous viruses breaks resistance and results in infection, as demonstrated by Haan et al. [56]. To overcome this problem, transgenic plants can be raised with multiple hpRNA construct targeting different regions and different viral source, or alternatively, transgenic plants can be developed with minimal sized chimeric constructs [57].

A second silencing pathway is microRNA (miRNA) mediated pathway in which endogenous production of 21-22 nucleotide takes place. The miRNA is processed by one key enzyme DCL-1, which processes pri-miRNA to pre-miRNA from larger miRNA precursors specified by non-protein-coding genes [58,59]. The miRNA negatively regulate their target mRNAs, either by inhibiting translation (primarily in animal systems) or by degradation. In plants, miRNAs are usually perfectly complementary to their target mRNAs and direct RISC cleavage in essentially the same manner as siRNAs [60]. In animal miRNA maturation takes place with the processing of pre-miRNA which yields miRNA/miRNA* duplex, in which the miRNA is selectively loaded into RISC. The presence of miRNA/miRNA* duplex is supported by the isolation of certain miRNA* sequences from small RNA cloning efforts [55] and the detection of several miRNA* by filter hybridization [3,61]

Expression of artificial microRNAs (amiRNAs) in plants can target and degrade the invading viral RNA, consequently conferring virus resistance. Earlier RNA silencing technique was developed based on DCL4-dependent siRNAs generated from perfectly base-paired dsRNAs [62]. Recently developed silencing technique using amiRNA has been developed based on miRNA. The amiRNA utilizes miRNA precursor as a backbone and introduces mutations in several base pair to gain new targeting ability [63]. Utilization of amiRNA has several possible advantages over the pathogen derived sequence to engineer virus resistant plants the first, is highly specific nature of amiRNA towards the target and second and the most important is miRNA is not the direct target of the virus-encoded suppressors, thus the amiRNA-mediated resistance could be more stable than the resistance induced by siRNAs generated from long viral sequence.

Another gene silencing method widely adopted worldwide is antisense RNA. Antisense RNA which is complementary to target RNA (mRNA) has a capability to hybridize the messenger RNA, a tool to natural and artificial gene regulation by silencing the suppression of the corresponding gene [64]. The targeted gene inactivation by interfering and inhibiting the translation of RNA has now become a prominent tool in applied molecular biology. Antisense RNA acts in transcript level and is effective against multiple gene copy number and hence can be an ideal tool against viral infection [65]. The duplex RNA formation due to sense- antisense interaction also led to the formation of siRNA [41]. This strategy has been successfully applied in the development of transgenic plants against many Geminiviruses [37].

The idea of using RNAi approach to protect plants against insects depends on the downregulation of certain essential gene(s) function which ultimately leads to the death of that insect. This idea was first demonstrated by using dsRNA expressing corn and tobacco [66]. Engineered tobacco has been developed targeting cytochrome *P450* monoxygenase gene (*CYPAE*14) of the cotton bollworm (*Helicoverpa*

armigera) which inhibits expression levels of *CYPAE*14 in this pest. Also, the inhibition of *CYPAE*14 expression leads to their increased sensitivity to the natural defense compound, gossypol, produced by the plant [67]. The engineered corn plant expressing dsRNA directed against the vacuolar ATPase of the western corn rootworm, *Diabrotica virgifera* showed resistance against the feeding damage caused by the pest [66].

Both the injection and feeding of dsRNA have proven to be effective for a variety of insect species. However oral delivery of *in vitro* synthesized dsRNA is technically less difficult. Several other proteins such as protinase inhibitors, lectins, cholesterol oxidase, avidin, *Bacillus thuringiensis* (*Bt*) insecticidal toxins were also employed to develop transgenic plants [6]. Among them Bt toxins has been widely used in recent years, but in some cases resistant populations have evolved. So, some other strategies are needed for durable pest control.

Problems and Prospects

The application of RNAi holds immense potential to manage the problem of viruses and pests and to limit their capacity of economical damage. Once dsRNA production takes place from transgenic plant and delivered to pest pathogens, the endogenous cognate transcripts would be degraded. The RNAi mediated approach against pests and pathogens offers several advantages over conventional method and the resistance is more durable. The other most important thing is that there is no protein production in RNAi approach, so there is less or no chance of interfering with plant proteins or mutation. One of the main challenges of RNAi based crop protection strategy is identification of target. The target is generally specific, but "off-target" effects cannot be ignored. Sometimes partial identity of internal transcripts with introduced dsRNA sequence may knockdown the unintended genes. This phenomenon may result in changed phenotype of plants or some unexpected mutant phenotype in addition to the target gene [68].

The use of RNAi approach to counter insects is still in infancy and it needs further investigation. A major problem using RNAi approach against insects is that adequate amount of dsRNA is needed to effectively block the targeted expression, since dsRNA itself cannot replicate inside the insects.

The strategies reviewed in this paper have the potential to protect against viruses and insects either partially or fully. But most of strategies certainly deserve further investigation as most of them have been applied and proved to be effective in model system only.

Conclusion

In this paper the feasibility of using RNAi approach in the protection of crops against geminiviruses and insects has been discussed. There is a fine and balanced battle occurring between plants and pathogens, which consistently attack on them. The role of genetic engineering strategies should tip the balance in favour of plant. The RNAi approach certainly holds great promise for the future because it enjoys a wide range of targets of insects and viruses.

In conclusion, engineering plant resistance against pathogens and insects using RNAi approach bring high expectations for the future, although further research is needed to evaluate its potential in natural field conditions. Moreover RNAi approach, if applied responsibly, is a powerful and safe means for combating plant pathogens which cannot be controlled otherwise.

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Author Affiliation

Top

[1]*School of Life Sciences, Jawahar Lal Nehru University, New Delhi-110067, India*