academicJournals

Vol. 12(18), pp. 2303-2312, 1 May, 2013
DOI: 10.5897/AJB12.2791
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

RNA interference: A novel tool for plant disease management

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Accepted 17 April, 2013

Plant diseases pose a huge threat to crop production globally. Variations in their genomes cause selection to favor those who can survive pesticides and *Bacillus thuringiensis* (*Bt*) crops. Though plant breeding has been the classical means of manipulating the plant genome to develop resistant cultivar for controlling plant diseases, the advent of genetic engineering provides an entirely new approach being pursued to render plants resistant to fungi, bacteria, viruses and nematodes. RNA interference (RNAi) technology has emerged to be a promising therapeutic weapon to mitigate the inherent risks such as the use of a specific transgene, marker gene, or gene control sequences associated with development of traditional transgenics. Silencing specific genes by RNAi is a desirable natural solution to this problem as disease resistant transgenic plants can be produced within a regulatory framework. Recent studies have been successful in producing potent silencing effects by using target double-stranded RNAs through an effective vector system. Transgenic plants expressing RNAi vectors, as well as, dsRNA containing crop sprays have been successful for efficient control of plant pathogens affecting economically important crop species. The present paper discusses strategies and applications of this novel technology in plant disease management for sustainable agriculture production.

Key words: Plant disease, RNA interference, transgene, management.

INTRODUCTION

To offset the crop losses from pathogens, various attempts have been made in the field of disease management since inception of green revolution. Pesticides have been traditionally used on crop to prevent crop damages. Once pesticides were discovered to pollute the environment and be harmful to human health, agriculture research began to focus on alternative safer methods. In last two and half decades, much attention has been paid on integrated disease management practices which make disease control inexpensive and safe (Mandal et al., 2012). Plant breeding has been the classical means of manipulating the plant genome to develop resistant cultivar for controlling plant diseases. Further study of genetic host resistance fulfils this requirement but is a continuous endeavor as the boom and bust cycle goes on in the process of co-evolution, though therapeutic tools based on current molecular biology hold the key after the

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exploitation of traditional breeding and biotechnological methods likely use molecular markers for identification, mapping, cloning of pest and disease resistant genes, and their utilization by introgression, pyramiding, and development of transgenics (Mann et al., 2008, Sanghera et al., 2011).

The inherent risks associated with traditional transgenics can be mitigated by new and innovative strategies, and transgenic plants can be produced within a regulatory framework. RNA interference is a natural process, which silences specific genes before being translated. RNAi inducers, in the form of transgenic plants or a crop spray, have the potential to effectively silence specific genes (Baum et al., 2007; Mao et al., 2007). During the last decade our knowledge repertoire of RNA-mediated functions has hugely increased with the discovery of small non-coding RNAs, which play a central part in a process called RNA silencing. Ironically the very important phenomenon of co-suppression has recently been recognized as a manifestation of RNA interference (RNAi), an endogenous pathway for negative post-transcriptional regulation. RNAi has revolutionized the possibilities for creating custom “knock-downs” of gene activity. RNAi operates in both plants and animals and uses double stranded (dsRNA) as a trigger that targets homologous mRNAs for degradation or inhibiting its transcription or translation (DeBakker et al., 2002; Almeida and Allshire, 2005) whereby susceptible genes can be silenced. This RNA-mediated gene control technology has provided new platforms for developing eco-friendly molecular tools for crop improvement by suppressing the genes responsible for various stresses and improving novel traits in plants including disease resistance. Also, it will be a promising future therapeutic agent to combat plant invaders.

Thus, posttranscriptional gene silencing by RNAi has emerged as a method of choice for gene targeting in fungi (Nakayashiki, 2005), viruses (Baulcombe, 2004), bacteria (Escobar et al., 2001), and plants (Brodersen and Voinnet, 2006), as it allows the study of the function of hundreds of thousands of genes (Godge et al., 2008). With this technology it is possible to silence a gene throughout an organism or in specific tissues, offering the versatility to partially silence or completely turn off genes in both cultured cells and whole organisms, and can selectively silence genes at particular stages of the organism’s life cycle. Transgenic plants would be cost-effective by producing RNAi inducers throughout a plant’s life constantly silencing different pathogen genes. The applications of this technology in the improvement of plants with special reference to disease management are discussed below.

RNAI IN CONTEXT TO HOST-PATHOGEN SYSTEM

The evolutionary story of RNAi began in the early 1990s with Napoli and colleagues who tried to deepen the purple colour by introducing a *chalcone synthase* gene in *Petunia* under a strong promoter. Contrary to their expectations, the pigmentation in the flowers of transformed plants was not enhanced. Instead, the flowers were de-pigmented and endogenous gene mRNA transcript levels were greatly reduced (Napoli et al., 1990). Based on the fact that both the transgene and the endogenous gene were suppressed, the observed phenomenon was termed “co-suppression”. The mechanistic aspect of this phenomenon remained unknown at that time, since post-transcriptional gene silencing (PTGS) was not the most accepted explanation (Napoli et al., 1990; Jorgensen et al., 1996; Cogoni and Macino, 2000). This phenomenon of suppression of an endogenous gene by transformation with homologous sequences was also observed in the fungus *Neurospora crassa* where it was termed quelling (Romano and Macino, 1992).

However, the significance of these observations went unnoticed for several years until the mystery was solved in 1998, when it was demonstrated that dsRNA is even more effective in silencing gene expression than antisense RNA, the phenomenon was termed as RNAi (Fire et al., 1998). Although such gene silencing can occur at the transcriptional level, it was recognized that a major mechanism of gene suppression occurs post-transcriptionally and that a major mechanism of this PTGS is RNAi, the selective degradation of mRNAs targeted by siRNA (van Blokland et al., 1994). This mechanism was later on developed as a virus-induced gene silencing (VIGS) system based on sequence homology studies between a virus and either a transgene or an endogenous gene that would cause PTGS (Lindbo et al., 1993; Kumagai et al., 1995).

In this system, a virus vector carrying a copy of the gene to be silenced is introduced into the cell, the cellular machinery recognizes the viral threat and releases a protective defense to destroy not only viral genes but also any extra-gene being carried by the viral vector, affecting any native or transgenic homologous transcripts (Ruiz et al., 1998; Waterhouse et al., 2001). Such PTGS via RNAi can occur rapidly with proteins for many genes, being decreased within hours and completely absent within 24 h (Jagtap et al., 2011). Based upon these and other findings initially made in studies of plants, it seems very likely that RNAi evolved as a mechanism to defend plant cells against fungal, bacterial, viral and nematode infections (Mann et al., 2008).

METHODS TO INDUCE RNAI IN PLANTS

In RNAi research field, one of the biggest challenges is the delivery of the active molecules that will trigger the RNAi pathway in plants. In this system, a number of methods for delivery of dsRNA or siRNA into different cells and tissues include transformation with dsRNA-forming vectors for selected gene(s) by *Agrobacterium*-

mediated transformations (Chuang and Meyerowtiz, 2000; Waterhouse et al., 2001); delivery cognate dsRNA of uidA GUS (β-glucuronidase) and TaGLP2a:GFP (green fluorescent protein) reporter genes into single epidermal cells of maize, barley and wheat by particle bombardment (Schweizer et al., 2000), introducing a tobacco rattle virus (TRV)-based vector in tomato plants by infiltration (Liu et al., 2002); delivery of dsRNA into tobacco suspension cells by cationic oligopeptide polyarginine-siRNA complex; infecting plants with viral vectors that produce dsRNA (Dalmay et al., 2000), and delivery of siRNA into cultured plant cells of rice, cotton and slash pine for gene silencing by nanosense pulsed laser-induced stress wave (LISW) (Tang et al., 2006). Among these, the most reliable and commonly used approaches for delivery of dsRNA to plant cells are agroinfiltration, micro-bombardment and virus-induced gene silencing.

AGROINFILTRATION

Agroinfiltration is a powerful method to study processes connected with RNAi. The injection of Agrobacterium carrying similar DNA constructs into the intracellular spaces of leaves for triggering RNA silencing is known as agroinoculation or agroinfiltration (Hily and Liu, 2007). In plants, cytoplasmic RNAi can be induced efficiently by agroinfiltration, similar to a strategy for transient expression of T-DNA vectors after delivery by *Agrobacterium tumefaciens*. The transiently expressed DNA encodes either an ss- or dsRNA, which is typically a hairpin (hp) RNA. The infiltration of hairpin constructs are especially effective, because their dsRNA can be processed directly to siRNAs, while the constructs expressing asRNA can also be useful to induce silencing (Johansen and Carrington, 2001; Voinnet, 2001; Mlotshwa et al., 2002; Tenllado et al., 2003), and for dissecting the mechanism of gene silencing, especially concerned with its suppressors, systemic silencing signal, and also for simple protein purification (Johansen and Carrington, 2001; Voinnet, 2001; Mlotshwa et al., 2002; Tenllado et al., 2003). Besides, they provide a rapid, versatile and convenient way for achieving a very high level of gene expression viz. iaaM and ipt responsible for inducing resistance to crown gall disease in apple (Dunoyer et al., 2006), hpGUS in different transgenic lettuce lines (Wroblewski et al., 2007), OsGEN-L-green fluorescent (GFP) fusion protein in rice (Moritoh et al., 2005) etc.

MICRO-BOMBARDMENT

In this method, a linear or circular template is transferred into the nucleus by micro-bombardment. Synthetic siRNAs have been delivered into plants by biolistic pressure to cause silencing of GFP expression (Liu et al., 2002; Nakayashiki). Bombarding cells with particles coated with dsRNA, siRNA or DNA that encode hairpin constructs, as well as, sense or antisense RNA, activate the RNAi pathway. The silencing effect of RNAi is occasionally detected as early as a day after bombardment for GFP gene, and it continues up to three to four days post bombardment. Systemic spread of the GFP gene silencing occurred after two weeks and RNA blot hybridization with systemic leaves indicates that the biolistically delivered siRNAs induced de novo formation of siRNAs, which accumulated to cause silencing (Klahre et al., 2002).

VIRUS INDUCED GENE SILENCING (VIGS)

Modified viruses as RNA silencing triggers are used as a mean for inducing RNAi in plants. Different RNA and DNA viruses have been modified to serve as vectors for gene expression (Timmermans et al., 1994; Pogue et al., 2002). Some viruses, such as tobacco mosaic virus (TMV), potato virus X (PVX) and tobacco rattle virus (TRV), can be used for both protein expression and gene silencing (Kumagai et al., 1995; Angell and Baulcombe, 1999; MacFarlane and Popovich, 2000; Mallory et al. 2002). All RNA virus-derived expression vectors are not useful as silencing vectors because they contain potent anti-silencing proteins such as tobacco etch virus (TEV), that directly interfere with the host silencing machinery (Kumagai et al., 1995; Palmer and Rybicki, 2001). Similarly, DNA viruses have not been used extensively as expression vectors due to their size constraints for movement (Kjemtrup et al., 1998). However, a non-mobile Maize streak Virus (MSV)-derived vector has been successfully used for long-term production of protein in maize cell cultures (Kumagai et al. 1995).

Using viral vectors to silence endogenous plant genes requires cloning of homologous gene fragments into the virus without compromising viral replication and movement. This was first demonstrated in RNA viruses by inserting sequences into the TMV (Dallwitz and Zurcher 1996), and then for DNA viruses by replacing the coat protein gene with a homologous sequence (Kjemtrup et al. 1998). These reports used visible markers for silencing phytoene desaturase(PDS) and chalcone synthase (CHS), providing a measure of tissue specificity of silencing as these have been involved in the carotenoid metabolic pathway. The PDS gene acts on the antenna complex of the thylakoid membranes, and protects the chlorophyll from photo oxidation. By silencing this gene, a drastic decrease in leaf carotene content results in the appearance of the photobleaching symptom (Liu et al., 2002; Turnage et al., 2002). Similarly, over expression of the CHS gene caused an albino phenotype, instead of producing the anticipated deep orange color (Cogoni et al., 1994).

As a result, their action as a phenotypic marker helps the understanding of the mechanism of gene silencing.

Most viruses are plus-strand RNA viruses or satellites, whereas, the tomato golden mosaic virus (TGMV) and the cabbage leaf curl virus (CaLCuV) are DNA viruses. Though RNA viruses replicate in the cytoplasm, DNA viruses replicate in plant nuclei using the host DNA replication machinery. Both types of viruses induce diffusible, homology-dependent systemic silencing of endogenous genes. However, the extent of silencing spread and the severity of viral symptoms can vary significantly in different host plants and host/virus combinations. With the variety of viruses and the diversity of infection patterns, transmission vectors, and plant defenses it is not surprising that viruses differ with respect to silencing (Teycheney and Tepfer 2001). Because the continuing development of virus-based silencing vectors can extend VIGS to economically important plants, it is useful to consider some of the characteristics of successful VIGS vectors.

RNAI STRATEGIES IN PLANT DISEASE MANAGEMENT

Despite substantial advances in plant disease management strategies, our global food supply is still threatened by a multitude of pathogens and pests. This changed scenario warrants us to respond more efficiently and effectively to this problem. The situation demands judicious blending of conventional, unconventional and frontier technologies. In this sense, RNAi technology has emerged as one of the most potential and promising strategies for enhancing the building of resistance in plants to combat various fungal, bacterial, viral and nematode diseases causing huge losses in important agricultural crops (Mann et al., 2008). The nature of this biological phenomenon has been evaluated in a number of host-pathogen systems and effectively used to silence the action of pathogens. Many of the examples listed below illustrate the possibilities for commercial exploitation of this inherent biological mechanism to generate disease-resistant plants in the future by taking advantage of this approach.

MANAGEMENT OF PLANT PATHOGENIC FUNGI

RNA-mediated gene silencing (RNA silencing) is used as a reverse tool for gene targeting in fungi. Homology-based gene silencing induced by transgenes (co-suppression), antisense, or dsRNA has been demonstrated in many plant pathogenic fungi, including *Cladosporium fulvum* (Hamada and Spanu 1998), *Magnaporthae oryzae* (Kadotani et al., 2003), *Venturia inaequalis* (Fitzgerald et al., 2004), *Neurospora crassa* (Goldoni et al., 2004), *Aspergillus nidulans* (Hammond and Keller, 2005), and *Fusarium graminearum* (Nakayashiki, 2005), whether it is suitable for large-scale mutagenesis in fungal pathogens remains to be tested. The hypermorphic mechanism of RNA interference implies that this technique can also be applicable to all those plant pathogenic fungi, which are polyploid and polykaryotic in nature. And also offers a solution to the problem of frequent lack of multiple marker genes in fungi. Simultaneous silencing of several unrelated genes by introducing a single chimeric construct has been demonstrated in the case of *Venturia inaequalis* (Fitzgerald et al., 2004).

HCf-1, a gene that codes for a hydrophobin of the tomato pathogen *C. fulvum* (Spanu, 1997), was co-suppressed by ectopic integration of homologous transgenes. Transformation of *Cladosporium fulvum* with DNA containing a truncated copy of the hydrophobin gene *HCf-1* caused co-suppression of hydrophobin synthesis in 30% of the transformants. The co-suppressed isolates had a hydrophilic phenotype, lower levels of *HCf-1* mRNA than wild type and contain multiple copies of the plasmid integrated as tandem repeats at ectopic sites in the genome (Hamada and Spanu, 1998). The transcription rate of *HCf-1* in the co-suppressed isolates was higher than in the untransformed strains, suggesting that silencing acted at the post-transcriptional level (Hamada and Spanu, 1998). This was due to ectopic integration of the transgene next to promoters which initiate transcription to form antisense RNA, and that this in turn determined the down-regulation of HCf-1. Gene silencing was not associated with DNA cytosine methylation (Hamada and Spanu, 1998).

Similarly, the silencing of *cgl1* and *cgl2* genes using the cgl2 hairpin construct in *Cladosporium fulvum* has also been reported (Segers *et al*. 1999), though the effect was possibly restricted to highly homologous genes (exons of *cgl 1* and *cgl 2* are 87% identical). However, the less homologous *cgl 3* (53% overall identity to *cgl 2*) was not affected as the target specificity always depends upon the actual sequence alignment and more over, short regions of high density that led to unwanted off-target effects. Such a strategy could be exploited for protecting the consumable products of vegetables and fruit crops from the post harvest diseases caused by different plant pathogens in the future.

Fitzgerald and colleagues (2004), using the hairpin vector technology, have been able to trigger simultaneous high frequency silencing of a green fluorescent protein (*GFP*) transgene and an endogenous trihydroxynaphthalene reductase gene (*THN*) in *V. inaequalis*. *GFP* transgene, acting as an easily detectable visible marker while the trihydroxynaphthalene reductase gene (*THN*) playing a role in melanin biosynthesis. High frequency gene silencing was achieved using hairpin constructs for the *GFP* or the *THN* genes transferred by *Agrobacterium* (71 and 61%, respectively). *THN*-silenced transformants exhibited a distinctive light brown phenotype and maintained the ability to infect apple. Silencing of both genes with this construct occurred at a

frequency of 51% of all the transformants. All 125 colonies silenced for the GFP gene were also silenced for THN (Fitzgerald et al., 2004). Similarly, multiple gene silencing has been achieved in *Cryptococcus neoformans* using chimeric hairpin constructs (Liu et al., 2002) and in plants using partial sense constructs (Abbott et al., 2002).

The first effort towards the systematic silencing of *Magnaporthe grisea*, a causal organism of rice blast was carried out in by Kadotani et al. (2003) by using the enhanced green florescent protein gene as a model. To assess the ability of RNA species induce silencing in fungus, plasmid construct expressing sense, antisense and hairpin RNA were introduced into an e*GFP*-expressing transformants. The fluorescence of *eGFP* in the transformants was silenced much more efficiently by hairpin RNA of *eGFP* than by other RNA species. In the silenced transformants, the accumulation of *eGFP* mRNA was drastically reduced, but not methylation of coding or promoter regions was involved. The small interfering RNA molecules of 19-23 nucleotides were observed in both sense and antisense strands of *eGFP* gene (Kadotani et al., 2003). Later on Nakayashiki and colleagues (2005) developed a protocol for silencing the *mpg1* and polyketide synthase-like genes. *mpg1* gene is a hydrophobin gene, which is essential for pathogenicity as it acts as a cellular relay for adhesion and trigger for the development of appressorium (Talbot et al., 1996). Their work on this host-pathogen system revealed that they were successfully able to silence the above mentioned genes at varying degrees by pSilent-1-based vectors in 70 to 90% of the resulting transformants. Ten to fifteen percent of the silenced transformants exhibited almost ‘‘null phenotype’’. This vector was also efficiently applicable to silence a *GFP* reporter in another ascomycete fungus *Colletotrichum lagenarium* (Nakayashiki 2005).

MANAGEMENT OF PLANT PATHOGENIC BACTERIA

One of the striking examples of bacterial disease management where RNAi showed a remarkable type of gene regulation was documented by Escobar et al. (2001). They developed a crown gall disease management strategy that targets the process of tumourogensis (gall formation) by initiating RNAi of the *iaaM* and *ipt* oncogenes. Expression of these genes is a prerequisite for wild type tumor formation. Transgenic *Arabidopsis thaliana* and *Lycopersicon esculentum* transformed with RNAi constructs, targeting *iaaM* and *ipt* gene(s) show resistance to crown gall disease. Transgenic plants generated through this technology contained a modified version of these two bacterial gene(s) required to cause the disease and was the first report to manage a major bacterial disease through RNAi. The extra genes recognize and effectively shut down the expression of the corresponding bacterial gene during infection, thus preventing the spread of infection. The incoming bacteria could not make the hormones needed to cause tumors, and plants deficient in silencing were hyper-susceptible to *A tumefaciens* (Dunoyer et al., 2007).

Successful infection relied on a potent anti-silencing state established in tumors whereby siRNA synthesis is specifically inhibited. The procedure can be exploited to develop broad-spectrum resistance in ornamental and horticultural plants which are susceptible to crown gall tumorigenesis. This approach can be advocated for the effective management of those pathogens which multiply very rapid, and results in tumor formation such as *Albugo candida*, *Synchytrium endobioticum*, and *Erwinia amylovora* among others. The natsiRNA (nat-siRNAATGB2) was strongly induced in *Arabidopsis* upon infection by *Pseudomonas syringae pv tomato* and down-regulates a PPRL gene that encodes a negative regulator of the RPS2 disease resistance pathway. As a result, the induction of nat-siRNAATGB2 increases the RPS2-mediated race-specific resistance against *P. syringae* pv *tomato* in *Arabidopsis* (Katiyar-Agarwal et al., 2007). Recently, the accumulation of a new class of sRNA, 30 to 40 nucleotides in length, termed long-siRNAs (lsiRNAs), was found associated with *P. syringae* infection. One of these lsiRNAs, AtlsiRNA-1, contributes to plant bacterial resistance by silencing AtRAP, a negative regulator of plant defense (Katiyar- Agarwal et al., 2007). A *Pseudomonas* bacterial flagellin-derived peptide is found to induce the accumulation of miR393 in *Arabidopsis*. miR393 negatively regulates mRNAs of F-box auxin receptors, resulting in increased resistance to the bacterium (*P. syringae*), and the overexpression of miR393 was shown to reduce the plant’s bacterial titer by five-fold (Navarro et al., 2006).

MANAGEMENT OF PLANT PATHOGENIC VIRUSES

Antiviral RNAi technology has been used for viral disease management in human cell lines (Bitko and Barik, 2001; Gitlin et al., 2002; Jacque et al., 2002; Novina et al., 2002). Such silencing mechanisms (RNAi) can also be exploited to protect and manage viral infections in plants (Waterhouse et al., 2001; Ullu et al., 2002). The effectiveness of the technology in generating virus-resistant plants was first reported to PVY in potato, harboring vectors for simultaneous expression of both sense and antisense transcripts of the helper-component *proteinase* (*HC-Pro*) gene (Waterhouse et al., 1998). The *P1/HC-Pro* suppressors from the potyvirus inhibited silencing at a step down stream of dsRNA processing, possibly by preventing the unwinding of duplex siRNAs, or the incorporation into RISC or both (Chapman et al., 2004). The utilization of RNAi technology has resulted in inducing immunity reactions against several other viruses in different plant-virus systems (Wani and Sanghera

2010).

In phyto-pathogenic DNA viruses like geminiviruses, the non-coding intergenic region of *Mungbean yellow mosaic India virus* (MYMIV) was expressed as hairpin construct under the control of the 35S promoter, and used to biolistically inoculate MYMIV-infected black gram plants, showing a complete recovery from infection, which lasted until senescence (Pooggin et al., 2003). RNAi-mediated silencing of geminiviruses using transient protoplast assay where protoplasts were co-transferred with a siRNA designed to the replicase (Rep)-coding sequence of *African cassava mosaic virus* (ACMV), and the genomic DNA of ACMV, resulted in 99% reduction of Rep transcripts and 66% reduction of viral DNA (Vanitharani et al., 2003). It was observed that siRNA was able to silence a closely related strain of ACMV but not a more distantly related virus.

More than 40 viral suppressors have been identified in plant viruses (Ruiz and Voinnet 2007). Results from some of the well-studied virus suppressors indicated that they interfere with systemic signaling for silencing (Mlotshwas et al., 2002). During the last few years, the *p69* encoded by *Turnip yellow mosaic virus* has been identified as a silencing suppressor preventing host RDR-dependent secondary dsRNA synthesis (Chen et al., 2004). *P14* protein encoded by Aureus viruses suppressed both virus and transgene-induced silencing by sequestering both long dsRNA and siRNA without size specificity (Merai et al., 2005). Multiple suppressors have been reported in the *Citrus tristeza virus,* where *p20* and coat protein (*CP*) play important roles in suppression of the silencing signal, and *p23* inhibited intracellular silencing (Lu et al., 2004). Multiple viral components, viral RNAs and putative RNA replicase proteins were reported for the silencing or suppression of the *Red clover necrotic mosaic virus* (Takeda et al., 2005). In this case, the RNA silencing machinery deprived of DICER-like enzymes by the viral replication complexes appeared to be the cause of the suppression. *Pns10* encoded by *Rice dwarf virus* suppressed local and systemic S-PTGS but not IR-PTGS suggesting that *Pns10* also targets an upstream step of dsRNA formation in the silencing pathway (Cao et al., 2005).

Niu and colleagues (2006) used a 273-bp (base pair) sequence of the *Arabidopsis* miR159 a pre-miRNA transcript expressing amiRNAs against the viral suppressor genes *P69* and *HC-Pro* to provide resistance against *Turnip yellow mosaic virus* and *Turnip mosaic virus* infections, respectively. In addition, a dimeric construct harbouring two unique amiRNAs against both viral suppressors conferred resistance against these two viruses in inoculated *Arabidopsis* plants. Similarly, Qu et al. (2007) used a different amiRNA vectors to target the 2 b viral suppressor of the *Cucumber mosaic virus* (CMV), a suppressor that interacted with and blocked the slicer activity of *AGO1* had also shown to confer resistance to CMV infection in transgenic tobacco. A strong correlation between virus resistance and the expression level of the 2 b-specific amiRNA was shown for individual plant lines. It is evident from the above-mentioned reports that the RNA components, such as single strand template RNA, dsRNA and/or siRNA of the silencing pathways are the preferred targets of most viral suppressors. However, plant viruses are known to have evolved a counter-silencing mechanism by encoding proteins that can overcome such resistance (Li and Ding, 2006; Díaz-Pendón and Ding, 2008). These suppressors of gene silencing are often involved in viral pathogenicity, mediate synergism among plant viruses and result in the induction of more severe disease. Simultaneous silencing of such diverse plant viruses can be achieved by designing hairpin structures that can target different viruses in a single construct (Díaz-Pendón and Ding, 2008).

Contrarily, the RNAi system may cause an increase in the severity of viral pathogenesis and/or encode proteins, which can inactivate essential genes in the RNAi machinery (Elbashir et al., 2001; Li et al., 2002) allowing their replication in the host genome (Hannon, 2002).

MANAGEMENT OF PLANT PARASITIC NEMATODES

Several major plant parasitic nematodes such as the root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* spp.) along with other minor nematodes cause significant damage to important crops like legumes, vegetables, and cereals in most parts of the world, and continue to threaten these agricultural crops. So a natural, eco-friendly defense strategy that delivers a cost-effective control of plant parasitic nematodes is needed but is difficult to achieve through conventional approaches. However, the birth of RNAi technology from classical *Caenorhabditis elegans* studies has shown the ways and means to explore the possibilities of this mechanism for protecting plants from nematode damage. In this context, two approaches have been advocated, one of them relies on targeting plant genes that are involved with the infection process, and the second approach targets essential genes within the nematode. RNAi can be induced in *C. elegans* by feeding it dsRNA, so it was reasoned that expressing hpRNAs-containing sequences of vital nematode genes in the host plant might deliver dsRNA to a feeding nematode to incapacitate or kill it.

After the demonstration of gene silencing using siRNA duplexes in the nematode (Fire et al., 1998), the use of RNAi has rapidly emerged as the technique of choice for plant nematologists, especially for nematode management in agriculture. RNAi-mediated suppression of a gene plays an indispensable role in hampering the nematode development and may affect the progression of pathogenesis in direct or indirect ways. There are accumulating evidences for the efficacy of RNAi in plant parasitic nematode management and a wide range of genes have been targeted for silencing in cyst and root-

knot nematode species (Mann et al., 2008,).

RNAi in the context of phyto-parasitic nematodes was used as early as the beginning of this century, when stimulation of oral ingestion by second-stage juveniles of cyst nematodes *Heterodera glycines*, *G. pallida* (Urwin et al., 2002) and root-knot nematode *M. incognita* (Bakhetia et al., 2007) was achieved by using octopamine. Later on, resorcinol- and serotonin-inducing dsRNA uptake by second stage juvenile of *M. incognita* was found to be more effective than octopimine (Rosso et al., 2005). The genes targeted by RNAi to date are expressed in a range of different tissues and cell types.

The ingested dsRNA can silence genes in the intestine (Urwin et al., 2002; Shingles et al., 2007), female reproductive system (Lilley et al., 2005), sperm (Urwin et al., 2002; Steeves et al., 2006), and both subventral and dorsal oesophageal glands (Chen et al., 2005; Rosso et al., 2005; Huang et al., 2006; Bakhetia et al., 2007). Uptake of dsRNA from the gut is a proven route to systemic RNAi in *C. elegans*. The systemic nature of RNAi in plant parasitic nematodes following ingestion of dsRNA suggests that they share similar uptake and dispersal pathways.

However, RNAi of a *chitin synthase* gene expressed in the eggs of *Meloidogyne artiella* was achieved by soaking intact eggs contained within their gelatinous matrix in a solution containing dsRNA (Fanelli et al., 2005). The enzyme plays a role in the synthesis of the chitinous layer in the eggshell. Depletion of its transcript by RNAi led to a reduction of stainable chitin in eggshells and a delay in hatching of juveniles from treated eggs. Similarly, RNAi targeting for *cysteine proteinase* transcripts did not reduce parasitic population of established nematodes on plants but result into the alteration of their sexual fate in favour of males at 14 days after invasion (Urwin et al., 2002).

On the other hand, *H. glycines* exposed to dsRNA corresponding to a protein with homology to C-type lectins did not affect sexual fate, but 41% fewer nematodes were recovered from the plants (Urwin et al., 2002). However, treatment with dsRNA corresponding to the major sperm protein (MSP) had no effect on nematode development or sexual fate 14 days after treatment. In addition to this, reduction in transcript abundance for targeted mRNAs in the infective juvenile and for MSP transcripts when males reached sexual maturity and sperm are produced was observed (Urwin et al., 2002).

Further extension of such types of experiments show efficient FITC uptake by soaking *M. incognita*, 90 to 95% of individuals swallowed the dye when the target was a dual oxidase (an enzyme comprised with a peroxidase domain EF-hands and NADPH oxidase domain and potentially involved in extracellular matrix development). The effect of RNAi was observed when root knot nematode (RKN) juveniles were fed on dual oxidase-derived dsRNA, the reduction in the number and size of established females at 14 and 35 days post infection with an overall reduction of 70% in egg production (Bakhetia et al., 2005). RNAi has also been induced for a *chitin synthase* gene that is expressed in the eggshells of *M. artiella* after soaking its developing eggs in a dsRNA (Fanelli et al., 2005).

Heterodera schachtii induces syncytial feeding structures in the roots of host plants, and this requires the up-regulation of *Suc* transporter genes to facilitate increased nutrient flow to the developing structure. Targeting these genes and down-regulating them with RNAi resulted in a significant reduction of female nematode development (Hoffman et al., 2008). Indeed, tobacco plants transformed with hpRNA constructs against two root-knot nematode genes have shown such an effect: the target mRNAs in the plant parasitic nematodes were dramatically reduced, and the plants showed effective resistance against the parasite (Fairbairn et al., 2007).

CONCLUSION

The field of RNAi is moving at an impressive pace and generating exciting results associated with RNAi, transgene silencing and transposon mobilization. This technology can be considered an eco-friendly, biosafe and ever green technology as it eliminates even certain risks associated with development of transgenic plants carrying first generation constructs (binary vectors and sense and antisense genes). As witnessed from earlier strategies for obtaining viral resistant plants, the expression of protein products from the transgene of interest risked hetero-encapsidation through protein-protein interactions between target and non-target viral gene product, resulted in the development of a non-aphid transmissible strain of Zucchini yellow mosaic virus to aphid-transmissible strain from a transgene expressing a plum pox capsid protein (Lecoq et al. 1993).

To facilitate silence gene expression, timespecific and inducible promoters active in the target tissues which could, when required, minimize “off-target” effects. Conventional transgenic technologies generally need the expression of whole genes, which are in contrast to comparatively small size of the RNAi transgene required for silencing, permitting multiple genes to be targeted in a single construct. For changing stages in a particular metabolic pathway or resisting multiple pathogen attack, this would assist to lessen the amount of manipulation and time required to accomplish the desired traits. In future opportunities, RNAi may even hold guarantee for development of gene-specific therapeutics or a complete understanding of genomics.

With the methodical research in RNAi mechanisms and understanding the entire development of RNAi technology, it would be feasible to create a new biological science offering massive economic and social spin-offs.

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