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RNA interference- a novel approach for plant disease management

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Abstract: RNA interference (RNAi) is an incredible revolution in the field of functional genomics, a breakthrough in plant molecular genetics. This technology will generate enormous potential for engineering control of gene expression. The success of managing biotic stress using RNAi technology will prove to be biologically and environmentally safe. It is therapeutic in approach as the resistance induced by RNAi is triggered by ds RNA that results in silencing of specific genes before being translated in a homology dependent manner. Over the time, RNAi is significantly proving it as one of the most promiscent management strategy which eliminates certain risks associated with the development of transgenic plants. This review gives an insight into the probability of management of plant diseases caused by various biotic agents *viz*. fungi, bacteria and viruses using RNA interference technique and host-pathogen related targeted sites.

Keywords: Diseases, Functional genomics, Gene Expression, Management, RNAi

INTRODUCTION

RNA interference (RNAi) is a conserved regulatory mechanism of gene expression that has been recently characterized in plants and invertebrates. It is a homology-dependent nucleotide sequence specific process of mRNA degradation or translation inhibition at the post -transcriptional level or epigenetic modification at the transcriptional level depended on RNA-directed DNA methylation (RdDM in plants) (Duan *et al*., 2012). It refers collectively to diverse RNA based processes that result in sequence specific inhibition of gene expression at the transcriptional or translational level (Fagwalawd *et al*., 2013). Once RNAi is triggered in a certain cell, a mobile signal is produced and spread through the whole plant causing the entire plant to be silenced (Broglie *et al*., 1991). RNAi has rapidly gained importance as a reverse genetics tool to knock down expression of targeted genes in plants, lower animals and micro-organisms (Senthil-Kumar and Mysore, 2010).This technology has been widely applied to alter the gene expression in plants with an aim to achieve desirable traits. RNAi has been used for enhancing the crop yield and productivity by manipulating the gene involved in biomass production, grain yield and enhanced shelf life of fruits and vegetables and nutritional improvements of crops (International service for acquisition of agri-biotech applications, Personal communication,2012).Besides, being a key regulator of important plant processes like growth, development and response to various stresses (Singh, 2005), it has also been applied for developing resistance against various biotic (bacteria, fungi, viruses, nematodes, insects) (Singh, 2005) and abiotic stresses (drought, salinity, cold etc.) (Khraiwesh *et al*., 2012). Mann *et al*., 2008, reviewed the technology as an eco-friendly approach for plant disease management. Further study of genetic host though therapeutic tools based on current molecular biology and biotechnological methods are exploited and their utilization by introgression, pyramiding, and development of transgenics have been well understood (Sanghera *et al*., 2011). This review discusses the mechanism, approaches to induce RNAi and the wide prospects of RNAi and limitations of the technology in managing plant pathogens.

Bio-chemical core of RNAi –small RNA, Dicers and Argonautes: The diverse RNA- based process that all results in degradation of target homologous mRNA based on complementary base pairing, share the common bio-chemical features. The substrate for RNAi mainly double-stranded (ds) RNA can be generated and single-stranded (ss) RNA can be processed through a diverse pathways resulting in transcriptional cleavage or regulation in post-transcriptional regulation. The pathway is built over the Dicer- Argonaute core that executes a diverse set of single stranded (ss) RNA- directed biological functions in higher plants and all the diverse mechanisms have a common bio-chemical core (Brodersen and Voinnet, 2006) as mention below:

Presence of a double-stranded dsRNA; this can be an introduced transgene, a rogue genetic element or a viral intruder that triggers the RNAi pathway of cell.

Processing of dsRNA to small(s) 20-25nt dsRNAs with staggered ends; cleaving of the dsRNA into short, 20-26 basepairs long, fragments, called small interfering RNA (siRNA).

Inhibitory action of a selected ssRNA strand within effector complexes acting on partially or fully complementary RNA or DNA; identification between the two siRNA strands as either sense or antisense and degradation of the sense strands (with exactly the same sequence as the target gene).

Mechanism of RNA interference: RNA silencing is triggered by ds RNA that results in silencing of specific genes before being translated in a homology dependent manner. The entry of long dsRNA such as an introduced transgene, a rogue genetic element or a viral intruder is needed to trigger the RNA silencing. This recruits host ribonuclease –III like enzyme called Dicer with distinctive ds RNA binding, RNA helicase, RNase III and PAZ (Piwi/ Argonaute/ Zwille) domains (Brodersen and Voinnet, 2006). Dicer binds to long dsRNA and then cleaved into small interfering RNAs (si RNAs) of 21-25 nucleotides (nt) in length (Zrachya *et al*., 2007). Small interfering RNAs (siRNAs) mediate the selective degradation of target RNA by guiding a multi-component RNAse (Pandolfini *et al*., 2003), RNA-induced silencing complex (RISCs). It is a multicomplex protein consisting of a member of Argonaute (Ago) protein family. Ago protein contains two domains: PAZ domain- siRNA binding domain and PIWI domain- that provides endonucleolytic (slicer) activity to those RISCs programmed to cleave target RNAs (Brodersen and Voinnet, 2006) . RISCs distinguish the siRNA strands as either sense / antisense. Finally, RISC target homologous mRNA for cleavage and degradation of sense strand based on complementary base pairing (Baulcombe, 2004). The antisense strands in the RISC guided to target mRNA in a sequence specific manner. The activated RISC thus participates repeatedly in mRNA degradation inhibiting protein synthesis (Brodersen and Voinnet, 2006). The Dicer-Ago core executes a diverse set of ssRNA directed biological function in higher plants (Brodersen and Voinnet, 2006).

Approaches to induce RNAi: Resistance through RNAi in plants has been achieved by expressing hpRNA that folds back to create a dsRNA *in vivo* (Wesley *et al*., 2001), delivering artificial micro (mi) RNA (Schwab *et al*., 2006) or by directly delivering RNA silencing molecules into plant tissues (Tenllado and Diaz-Ruiz, 2001).The hairpin inverse repeat sequences from viral genomes can be small hpRNA, self-complementary hpRNA, and intron-spliced hpRNA. Among these methods, self complementary hpRNAs separated by an intron likely elicit post- transcriptional gene silencing (PTGS) with highest efficiency (Smith *et al*., 2000 and Wesley *et al*., 2001). For delivering artificial micro-RNAs (miRNAs), they are designed to mimic the intact secondary structure of endogenous miRNA precursors and processed *in vivo* to target genes of interest (Duan *et al*., 2012).

Another approach is the mechanical inoculation of *in vitro* synthesized dsRNA or the Agrobacterium-mediated transient expression of dsRNA in plants, and effective resistance to sequence homologous viruses (Tenllado and Diaz-Ruiz., 2001). However, major challenge for researchers is to deliver specific short sequence of target gene that will trigger the RNAi pathway in plants. A number of methods have been used to deliver the active molecules into the plant. Among them, the most reliable and commonly exploited approaches are:

Virus–induced gene silencing (VIGS): Homology-based defense mechanisms triggered by incoming viruses to target individual genes for silencing in a process called virus-induced gene silencing (VIGS). But in the present era VIGS is synonymous to a virus based functional genomics tool to knockdown the host gene for understanding its function. Maloy (2005) defined it as 'When dsRNA molecules produced during viral replication trigger gene silencing, the process is called VIGS'. VIGS has been reported as the most popular approach for inducing genetic activity in plants. In this method, either RNA/ DNA viruses have been modified as vectors to silence endogenous plant genes. For the process, cloning homologous gene fragments into the virus without compromising viral replication and movement is aberrant / transgene coded RNAs required (Wani *et al*., 2010) .The viral replication in plants generates dsRNA molecules that triggers PTGS response in plants. This results in reduction of virus titres in local and distant leaves and plant recovery phenotype. VIGS has been first demonstrated in RNA viruses by inserting sequence into TMV (Brunt *et al*., 1996). But it will be wrong to say that all RNA virus-derived expression vectors to be as silencing vectors because many have potent anti-silencing proteins such as TEV, that directly interfere with host silencing machinery (Palmer and Rybicki, 2001; Kumagai *et al*., 1995). Similarly, DNA viruses have not been used extensively as expression vectors due to their size constraints for movement (Kjemtrup *et al*., 1998). But the most promising vector *is Maize streak virus* (MSV) - derived vector which has been successfully used for long –term production of protein in maize cell cultures (Kumagai *et al*., 1995). In the area of functional genomics VIGS are the new tools. They can be exploited to study the different processes i.e plant development, host- pathogen interaction, host- nematode interaction, host- insect interactions. Scofield *et al*. (2005) have successfully exploited the VIGS- BSMV based system for the first time in monocots to study the functional analysis of Lr21 mediated leaf rust resistance pathway (Chuang and Meyerowtiz, 2000). This VIGS strategy can confer high resistance but the efficacy varies from

Table 1. Targeted region of RNAi in some fungal plant pathogens.

Pathogen	Targeted region	References
Magnaporthe oryzae	*eGFP*	Kadotani *et al*.(2003)
Cladosporium falvum	*cgl1 and cgl2*	Segers *et al*.(1999)
F. oxysporum f. sp. conglutinans	*FOW2, FRP1 and OPR*	Zongli *et al*. (2015)
Blumeria graminis f.sp. tritici	*Rnr*	Dimitar *et al*. (2014)
Blumeria graminis	*Mlo*	Schweizer *et al*. (2000)
Venturia inaequalis	*Multiple inverted repeats*	Fitzgerald *et al*. (2004)

Table 2. Targeted region of RNAi in various plant-virus systems.

Virus	Plant host system	Targeted Region	References
African cassava mosaic virus	*N. benthamiana*, *M. esculenta*	*pds*, *su*, *cyp79d2*	Fofana *et al*., 2004
Tobacco mosaic virus	*N. benthamiana*, *N. tabacum*	*pds*, *psy*	Kumagai *et al*, 1995
Tomato bushy shunt virus	*N. benthamiana*	*gfp*	Hou and W. Qiu, 2003
Potato virus X	*N. benthamiana*, *S. tuberosum*	*pds*, *gfp*	Ruiz *et al*., 1998 Faivre-Rampant *et al*., 2004
Poplar mosaic virus	*N. benthamiana*	*gfp*	Naylor *et al*., 2005
Aquilegia Tobacco rattle virus Rar1, *EDS1*, *NPR1/NIM1*	*N. benthamiana*, *Arabidopsis*, tomato, *Solanu m*species, Chilli pepper, opium poppy	*pds*, *rbcS*, *gfp*	Ratcliff *et al*., 2001; Gould and Kramer, 2007
Tomato bushy shunt virus	*N. benthamiana*	*gfp*	*Hou et al., 2003*
Tomato yellow leaf curl China virus-associated b DNA satellite	*N. benthamiana*, *Lycopersicon esculentum* *N. glutinosa*,*N. tabacum*	*pcna*, *pds*, *su*, *gfp* [	*Tao et al.,* 2004
Tomato golden mosaic virus	*N. benthamiana*	*su*, *luc*	*Peele et al.,*2001
Satellite tobacco mosaic virus]	*Nicotiana Tabacum*	*Several genes*	Gossele *et al*.,2002
Brome mosaic virus	Barley, rice, Maize	*pds*, *actin 1*, *rubisco activase*	*Ding et al.,* 2006
Pea early browning virus	*P. sativum*	*pspds*, *uni*, *kor*	Constantin *et al*., 2004
Barley stripe mosaic virus	Barley, wheat	*Pds*	Cakir and Tor, 2010
Bean pod mottle virus	Soybean	*Pds Actin*	Zhang and Ghabrial, 2006
Cabbage leaf curl virus	*Arabidopsis*	*gfp*, *CH42*, *pds*	Turnage, 2002

virus to virus, delayed infection recovery from infection or low resistance (Chuang and Meyerowtiz, 2000; Liu *et al*., 2002). It has been reported that homologous viruses with sequence mutation rates of over approximately 10-20% in comparision to the transgene source virus will overwhelm the resistance mechanism and result in the infection (Dalmay *et al*., 2000). At the same time, the plant is invaded by a complex of diverse pathogen sources in the field. One motive of high resistance should not be against a single virus but to the multiple viruses. So, that multiple viruses can simultaneously be tangled and achieved a broader resistance. For this purpose, transgenic plants with multiple hpRNA constructs form multiple viral sources or a single hpRNA construct pyramided with different viral sequence need to be created (Wani *et al*., 2010).

Agro-infiltration: Agro-infiltration is a strategy to induce a transient RNA silencing system to directly deliver the RNA silencing molecules into plant tissues (Duan *et al*., 2012). This will overcome the growing bio-safety issues due to transgenic plants created for RNA silencing. The injection of *Agrobacterium* carrying similar DNA constructs into the intracellular spaces of leaves for triggering RNA silencing is known as agro-inoculation or agro-infiltration (Hily and Liu, 2007). The simple mechanism for inducing RNAi is similar to the strategy for transient expression of T-DNA vectors after delivery by *Agrobacterium tumefaciens* (Zrachya *et al*., 2007). The transient plants were then transformed with *A. tumefaciens* genes harboring

the gene of interest for inducing RNAi as in the case of Tomato plants cv. Micro-Tom transformed with *A. tumefaciens* Gv3101/ PMP90kk harbouring plasmid pMon RNAi CP (Fillati *et al*., 1987; Singh, 2005). They provide an efficient way for achieving a down-regulation of green fluorescent protein (GFP) expression in *Nicotiana benthaniana*. This results from agro-infiltration of the coat protein (CP) silencing construct followed by infiltration of a fused GFP-CP (Fagwalawd *et al*., 2013). Besides, it has been successfully used for triggering the gene iaaM and ipt responsible for inducing resistance to crown gall disease in apple (Dunoyer *et al*., 2007), OSGEN-1-green fluorescent fusion protein in rice (Moritoh *et al.,*2005) and hpGUS in different transgenic lettuce lines (Wroblewski *et al*., 2007). Agro-infiltration assay was successfully used for screening candidate signaling components required for the activation of R-gene mediated disease resistance in *N. benthamiana* and tomato (Persengiev *et al*., 2004; Yuan *et al*., 2004). There have been reports of transient silencing of the grapevine gene VvPGIPI encoding a polygalacturonase inhibiting protein (PGIP) by agroinfiltration with a construct for RNAi (Bertazzon *et al*., 2012).

Micro-bombardment: Direct introduction of a plasmid producing hpRNA by particle bombardment method has been used for transient gene silencing of glutathione synthetase (GSHS) in somatic embryos of *Camellia sinensis*-L (Mohanpuria *et al*., 2008). The technique involved the mechanical inoculation of *in vitro* synthesized ds RNA which mimicks the intact secondary structureof endogenous miRNA precursors. In this strategy, biolistic pressure is used to deliver particles coated with dsRNA /siRNA or DNA encoding hairpin sense /antisense RNA construct into plants. This triggers the silencing of GFP expression (Wani *et al*., 2010) thus activating RNAi pathway.

An easy way of delivering the resistance through RNAi needs to be standardized. Some researchers has formulated a new approach which involved the utilization of the bacterial system to biosynthesize dsRNA *in vivo* and crude extracts by inoculating into plants via spraying (Yin *et al*., 2010 and Gan *et al*., 2010). This approach could be a cost-effective and will require less skill but needs to be standardized for adoption of the technique widely.

RNAi in plant disease management: RNAi technology has emerged as one of the promising strategy for enhancing resistance in plants to tackle the biotic stresses caused by fungal, bacterial, viral and nematode diseases that causes huge losses in important agricultural crops (Singh, 2005). Some remarkable and plausible examples of the application of this techniquein disease management have been discussed below:

RNA silencing-mediated resistance to plant fungal pathogens: Most plant fungal pathogens interact with their corresponding host through a highly specialized cell called a haustorium. This structure is surrounded by extra-haustorial matrix bordered by plants and fungal membranes on either side. Besides facilitating signal exchange and nutrient uptake, this also points to the possibility of trafficking of dsRNA or siRNA from host plants into the fungal pathogens to trigger RNA silencing mediated resistance (Duan *et al*., 2012). If silencing signals generated in the host cell can cross this exchange and barrier, gene silencing may trigger in haustorial cells and possibly interferes with pathogenesis or other metabolic process (Yin *et al*., 2011). This type of gene silencing is known as Host-induced gene silencing (HIGS). HIGS has been reported in barley powdery mildew fungi *Blumeria graminis***(**Nowara *et al***.,** 2010**).** Development of host induced RNAi system has also been reported in wheat stripe rust fungus (*Puccinia striiformis* f.sp. *tritici*) where gene fragments from the rust fungi *Puccinia striiformis* f.sp. *tritici* or *P.graminis* f.sp. *tritici* were delivered to plant cells through *Barley stripe mosaic virus* system and some reduced the expression of the corresponding genes in the rust fungus. This is associated with fungal gene expression patterns (Yin *et al*., 2011). This reduction was detected in transcripts with relatively high levels of expression in fungal haustoria. The results proven in fungi indicated that RNAi approach can be used as tool for functional genomics, aimed at modification of gene expression. Homology based gene silencing induced by transgenes (co-suppression), antisense or dsRNA has been demonstrated in many plant pathogenic fungi including *Cladosporium fulvum* (Segers *et al*., 1999; Schweizer *et al*., 2000). *Venturia inaequalis* (Fitzgerald *et al*., 2004), *Magnaporthe oryzae* (Kadotani *et al*., 2003), *Neurospora crassa* (Romano *et al*.,1992)and *Puccinia striiformis* (Yin *et al*., 2011). The targeted region for gene-silencing has been described in table 1.

RNA silencing-mediated resistance to plant pathogenic bacteria: Escobar *et al*. (2001) for the first time documented RNAi application for engineering resistance in plant against bacterial pathogen causing crown gall disease. In the particular disease, iaaM and ipt oncogenes are responsible for tumourogensis (gall formation) and a pre-requisite for tumour formation. The management strategy of the disease targets these oncogenes.,With the help of RNAi technology, they showed that transgenic plants (*Arabidopsis thaliana* and *Lycopersicon esculentum*) containing modified construct of these two bacterial genes (s) showed resistance against crown gall. The transgenic genes shut down the expression of iaaM and ipt oncogenes of the incoming bacterial pathogen, thereby disturbing the hormonal production and ultimately, tumourogenesis process after infection. Dunoyer *et al*., 2007 also reported that plants lacking the modified oncogenes were

hyper-susceptible to *A.tumefaciens*. Another example is the RNAi-mediated enhanced resistance to *Xanthomonas oryzae*, the leaf blight bacterium due to successful knockdown of a rice homolog of OsSSI2 (Jiang *et al*., 2009). Zhai *et al*., 2011 and Li *et al*., 2012 studied the function of several miRNA families target genes of plant innate immune receptors (NBS-LRR) in Legumes and Solanaceae, respectively. They gave a new insight into viral and bacterial infection in plants that suppresses miR482- mediated silencing of *R* genes. Considering the findings from different researchers (Jiang *et al*., 2009; Zhai *et al*., 2011 and Li *et al*., 2012), a general understanding can be drawn that miRNA can either act as up-or down- regulators of the bacterial invasion. The pathogen responsive miRNA effects the gene expression either by suppression of negative regulators or up regulation of the positive factors required for immune responses. Identification and characterization of pathogen responsive miRNAs that induced positive regulators of bacterial resistance, will open a flood gate to enhancement of transgenic plants that will involve the constitutive over- expression of miRNA or a miRNA.

RNA silencing-mediated resistance to plant virus: Waterhouse *et al*., 1998 first demonstrated the effectiveness of RNAi technology for generating virus resistance in potato against *Potato virusY*, harbouring vectors for simultaneous expression of both sense and antisense transcripts of the helper-component proteinase (HC-Pro) gene. RNA components of the silencing pathways such as single strand template RNA, dsRNA and / or siRNA are the preferred targets of most viral suppressors. However, Wani *et al*.**,** 2010 reported counter-silencing mechanism in viruses against plant viruses by encoding proteins that can overcome such posed resistance. Pandolfini *et al* (2003) observed the expression of self-complementary hairpin RNAs within two complementary regions separated by an intron elicits PTGS with high efficiency as in case of *Plum pox virus*. The hairpin construct *ihprolC-pp*197 gene of PPV RNA genome (a 197 bp sequence) is controlled by the *rol C* promoter. The transgenic *N. benthamiana* plants for *ihprol C-pp197* contain siRNAs homologous to the 197bp sequence and its progenies were resistant to *Plum pox virus* infection (Pandolfini *et al*., 2003). Against DNA viruses, the phenomenon is rarely reported as compared to the RNA viruses. Infection with the homologous geminivirus could be silenced by a geminiviral promoter the DNA virus group (Seemanpillai *et al*., 2003). Bombardment with a *hpRNA* construct containing the promoter sequence of geminivirus *Vigna mungo yellow mosaic virus* (VMYMV) in black gram (*Vigna mungo*) leaves under the control of the *35 S* promoter, showed the recovery of plants completely from the VMYMV infection (Pooggin *et al*., 2003), suggesting the effectiveness of RNA silencing strategy by engineering resistance to DNA viruses. *Bean golden mosaic virus* (BGMV) can also suppressed by the expression of a *hpRNA* transgenic derived from a *replicase* coding sequence *ACI* (Aragao and Faria, 2009). Table 2 shows some other reports of RNAi in various plant-virus systems.

Conclusion

Application of RNAi in management of biotic stress will be proved to be an incredible revolution in the field of functional genomics and a breakthrough in plant molecular genetics. If RNAi technology is developed successfully and employed for management of major diseases on commercial scale, they can prove to be an eco-friendly and biologically safe technology. Moreover, this technique eliminates the risk associated with development of transgenics and it will also generate enormous potential for engineering control of gene expression. An agronomically superior cultivar can be engineered for additional plant fitness by using RNAi technology. However, selection of targeting sequence and deliver of siRNA is a major challenge for plant molecular biologists. More understanding and exploration in the field of RNAi promoting resistance is needed. Therefore, further molecular research is needed to unfurl the factors affecting RNAi-mediated resistance and solved all the challenges in delivering the siRNA to the host system and identifying the targeted region to effectively overcome the pathogen and promote crop improvement.

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