RNA interference in Plant disease management:

a non-transgenic approach

By

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(2018-11-016)

SEMINAR REPORT

Submitted in the partial fulfilment of the requirement for the course

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DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF HORTICULTURE

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CERTIFICATE

This is to certify that the seminar report entitled **"**RNA interference in Plant disease management: a non-transgenic approach**"** has been solely prepared by **KARTHIKA MOHAN (2018-11-016)**, under my guidance and has not been copied from seminar reports of any seniors, juniors or fellow students.

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DECLARATION

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25-01-2020

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CERTIFICATE

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1. INTRODUCTION

Access to safe, healthy and sustainable food source is one of the challenges of our time. As per UN, by the year 2050, we will be requiring an additional 200 billion calories which will be equating to about 100 – 110 % increase in crop production. Current practices to protect crop plant rely on chemical treatments which are hazardous to the environment as well have deleterious effect on human health. Recently, RNA interference, a conservative eukaryotic mechanism has been emerged as a potential strategy against plant pathogens. RNA interference is a mechanism that inhibits gene expression at the stage of translation or by hindering the transcription of specific genes. It is the process by which dsRNA silences gene expression by the degradation of mRNA or by translation inhibition. dsRNA rather than single- stranded antisense RNA is the interfering agent.

RNAi has been recognized as a sequence – specific defence mechanism that is based on the recognition and cleavage of ds RNA molecules and the subsequent massive production of small interfering RNAs. The conventional approaches in RNAi are Host Induced Gene Silencing (HIGS) and Virus Induced Gene Silencing (VIGS). The most exploited one is HIGS, that is by the development of transgenic plants which will produce dsRNA molecules complementary to the pathogen mRNA. But we know that the community acceptance of transgenic plants are low. It also has several other drawbacks like; high cost of production, long time for development, lack of transformation protocols, concerns regarding environmental impact and regulations of use.

Taking these into consideration, and to tackle this issue, new non transgenic RNAi approaches need to be developed that will enable the activation of RNAi through the direct exogenous delivery of RNA molecules (dsRNAs) in plants. Here we are delivering the dsRNA as a spray instead of making a GM plant i.e. Spray Induced Gene Silencing (SIGS).

2. Components of RNAi

The RNA interference machinery in eukaryotic cells include the following components;

- Dicer or dicer like protein
- siRNAs and miRNAs
- RNA Induced Silencing Complex
- RNA dependent RNA polymerase (RdRp)

DICER is the enzyme involved in the initiation of RNAi.It is Rnase-III- like ds RNA- specific ribonuclease of 200 KDa.It is an ATP –dependent nucleases. It acts as a dimer i.e a mixture of two enzymes. Dicer homologs exist in many organisms including *C. elegans*, Drosophila, yeast, humans and plants. Dicer is a highly conserved protein. It is having an Amino – terminal helicase domain, dual RNAse III motifs in the carboxyl terminal segment, a dsRNA binding domain and a PAZ domain (110 – 130 amino-acid domain present in protein like Argo, Piwi…) which is thought to be important for protein – protein interaction.

Short interfering RNAs (siRNAs) is also a dsRNA molecule but it is having only 21-22 nucleotides in length. It is produced by the *in vivo* cleavage of dsRNA molecules by the enzyme DICER. It is habing a 5' Phosphate group and 3' Hydroxyl group with 2 – 3 nucleotide overhangings at both the ends.

RNA – Induced Silencing Complex (RISC): First discovered in Drosophila, by Hammond et al. (2000). Nuclease complex composed of proteins and siRNA. It is going to target and destroy endogenous mRNAs complementary to the siRNA. RISC consists of both protein and RNA. The activities associated with RISC are; Slicing and Homology seeking /RNA binding.

RNA dependent RNA polymerase (RdRp) enzyme helps in the amplification of siRNAs produced by the cleavage of dsRNA molecule

3. Mechanisms of RNAi

The steps involved in the mechanism can be summarized as foloows;

1. DIGESTION: dsRNAs are chopped into short interfering RNAs by DICER
2. RISC Activation: The siRNA-dicer compex recruits additional components to form an RNA-induced silencing complex.
3. UNWINDING: The sense strand of the siRNA unwinds
4. BINDING: The unwound siRNA base pairs with complimentary mRNA thus guiding the RNAi machinery to the target mRNA
5. DEGRADATION: The target mRNA is effectively cleaved and subsequently degraded – resulting in gene silencing

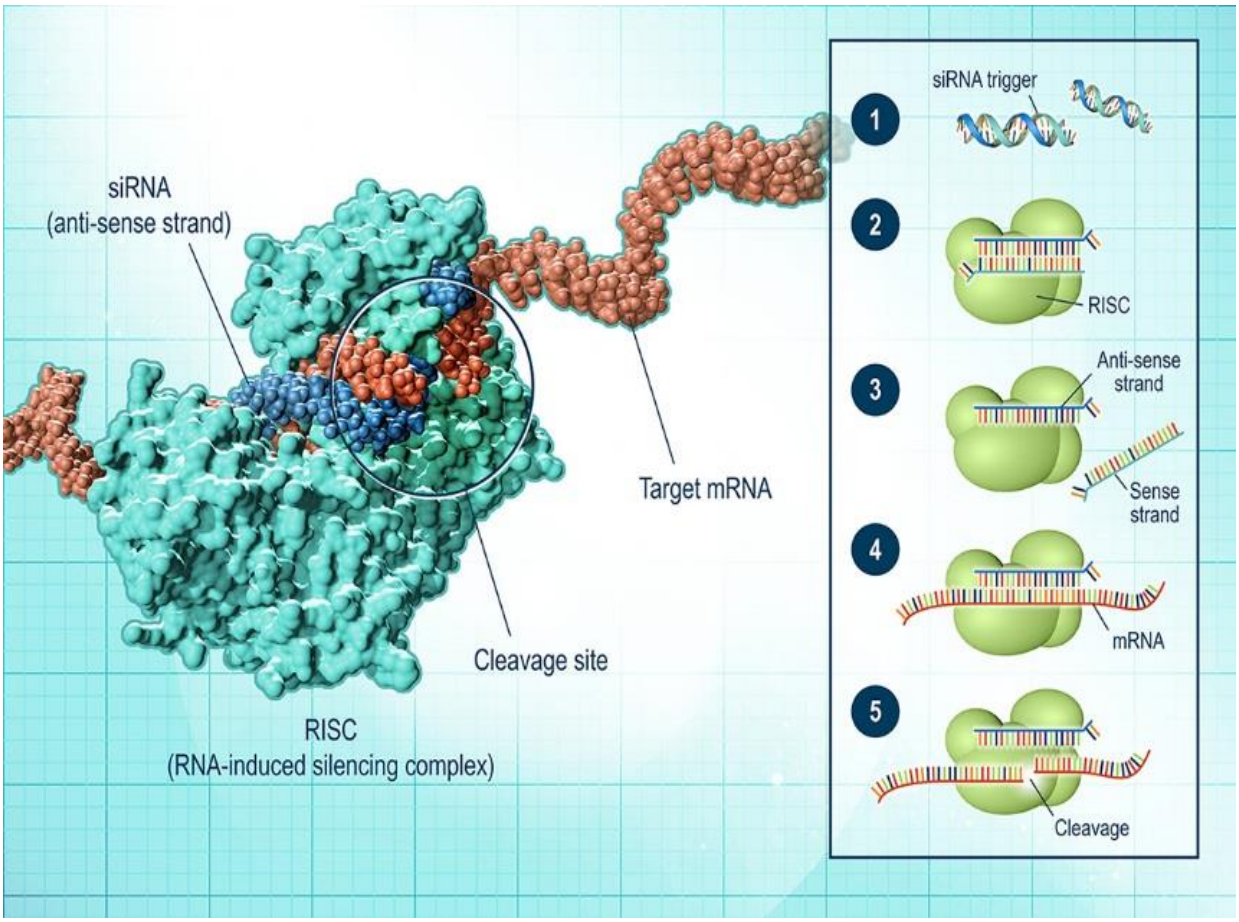


FIG 1: Mechanism of RNA interference

4. First Report

The first report where in exogenous RNA application into plants triggered RNAi of a plant gene was described in a 2011 Monsanto patent; *Nicotiana benthamiana* plants pre-treated with Silwet L-77 surfactant and sprayed (2.5 bar) with in vitro transcribed 685 bp dsRNA and/or chemically synthesized 21-nt sRNAs targeting the endogenous phytoene desaturase (PDS) mRNA displayed extensive PDS RNAi (Sammons *et al.*, 2011)

Table 1: Similar Line of Work

Sl. no.	Host	Target	Protection period	Reference
1	Tobacco Cowpea	BCMV	20 dpi	Worrall *et al.*, 2019
2	Tobacco	TMV	14 dpi	Neihl *et al.*, 2018
3	Cucumber Watermelon Squash	ZYMV	20 dpi	Kaldis *et al.*, 2018
4	Papaya	PRSV	60 dpi	Yin *et al.*, 2009

5. Method of Application of SIGS

The methods involved in the delivery of dsRNA sprays are;

1. Target gene identification
2. Synthetic dsRNA production system
3. DsRNA delivery systems.

Target gene identification

We have to select a vital gene to produce the dsRNA molecules. Because in the absence of the product of that gene the pathogen will not be able to survive. Conserved regions in the genome should also be selected since the spray has to be applied at different locations. From location to location there will be difference in some part of the genome. So we have to select such a region which is highly conserved in the region.

Eg: In the case of **Virus** we can select Coat protein and Movement protein in the absence of those virus won't be able to replicate and cause infect

In the case of **Fungi**, several enzymes involved in metabolic pathways like Ergosterol biosynthesis can be targeted.

6. Synthetic dsRNA production

Two type of production systems are available.

6.1 *In vitro* systems

Two *in vitro* systems are available for the production of dsRNA for application to plants to induce virus resistance. Initial studies by Tenllado and Diaz-Ruiz (2001) used an in vitro single stranded RNA (ssRNA) transcription system that required the annealing of products before topical application to plants. In this system, two DNA plasmids each encoding a sense and anti-sense complementary DNA (cDNA) sequence from the respective virus was transcribed into ssRNA by T7 DdRp and the respective sense and anti-sense ssRNA subsequently annealed. Appropriate for laboratory-based studies, the method generally yields RNA of excellent quality and purity, however, for large studies and field trials, is not economically feasible

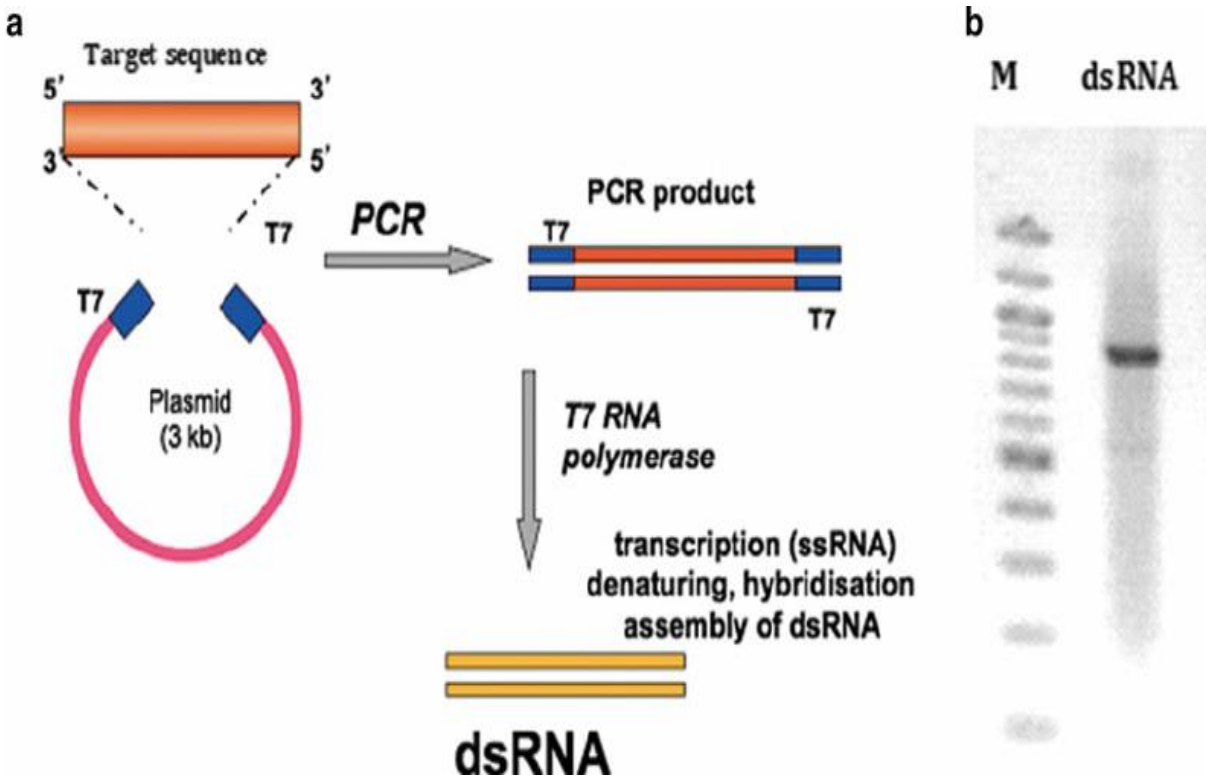


FIG.2: *In vivo* production system

A second in vitro system uses the Pseudomonas syringae dsRNA bacteriophage phi 6 (ϕ6) RNA dependent RNA polymerase (RDR) for generation of full-length dsRNA from ssRNA templates (Aalto et al. 2007). Analogous to the above ssRNA annealing protocol, this system makes use of only a single plasmid encoding either sense or antisense virus target sequence. The insert is transcribed by T7 DdRp to produce the ssRNA template strand for the RDR of ϕ6 that subsequently uses the transcribed ssRNA to generate the complementary strand. Although yields were not reported for this system, it was stated the quality and quantity of dsRNA was sufficient for small to medium scale laboratory based experiments (Aalto *et al*. 2007)

The above in vitro systems are appropriate for small laboratory based investigations however, to address the limits of production of the in vitro dsRNA production systems.

6.2 *In vivo* bacterial expression systems have been developed for production of large amounts of dsRNA. These systems are able to produce the required quantities for field application of dsRNA effector molecules. They encompass the use of various expression vectors within dsRNA-specific endonuclease deficient Escherichia coli strains or alternatively a P. syringae ϕ6 bacteriophage component based production system.

The majority of studies to date concerning the topical application of dsRNA have used E. coli strain HT115 (DE3) to produce large amounts of dsRNA for silencing studies. Initially used to silence endogenous genes in *Caenorhabditis elegans* (Timmons *et al.* 2001), the E. coli strain HT115 harbours the pro-phage λDE3 encoding the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 polymerase gene for dsRNA transcription. Transposon mutagenesis of the *rnc* gene encoding the RNAse III enzyme abrogates *rnc* function (Takiff *et al*., 1989) defining *E.coli* HT115 (DE3) as RNAse III defective and able to constitutively express the T7 DdRp when induced. This approach has been shown to be efficient and scaleable for the production of large amounts of dsRNA (Gan *et al*., 2010; Tenllado *et al*., 2003)

7. Carriers for dsRNA delivery

Under laboratory conditions we can utilise carriers like buffers and surfacatants. Because the environment is highly controlled and the activity of RNases will be limited or we can use RNase inhibitors.

But in order to apply it into field conditions, we have to sort to nanoparticle mediated delivery. There are 3 types of nanoparticles;

1. Organic
2. Inorganic
3. Polymer based

7.1 **Organic based Nanoparticles :** Organic compounds are used as carriers.

7.1.1 Chitosan based nanomaterials – Chitosan is a natural polysaccharide which is made of β-1-4 linked *N*-acetyl-D-glucosamine and D-glucosamine.It is usually produced by the deacetylation of chitin from crustacean shells. Due to its cationic nature, natural abundance, low toxicity and biodegradability it can be used as carrier for dsRNA delivery.

7.1.2 Lipid based nanomaterials -

1. Solid lipid nanoparticles - SLNs are considered to be one of the most effective lipid-based colloidal carriers. SLNs are in the submicron size range of 50–1000 nm and are composed of physiologically compatible lipids recognized as safe, which are in solid state at room temperature. They consist of a solid lipid core surrounded by a layer of surfactants in an aqueous dispersion, with multiple potential combinations of lipids and surfactants. The interest on SLNs has led to the development of different types of production methods (i.e., high-pressure homogenization) successfully implemented in pharmaceutical industry. Cationic lipids are used to prepare SLNs

due to their positive surface charge that interacts electrostatically with the negative charge of the nucleic acids

2. Cationic Emulsions - Emulsions are dispersions of one immiscible liquid in another stabilized by a third component, the emulsifying agent therefore, they present in their composition three components: oil, water, and surfactants. When cationic surfactants are used, these dispersed systems make them suitable for gene delivery. The presence of cationic surfactants causes the formation of positively charged droplets that promote strong electrostatic interactions between emulsion and the anionic nucleic acid phosphate groups. Cationic emulsions composed of cationic lipids and core oil have been shown to be useful for dsRNA delivery.
3. Liposomes - Liposomes are colloidal lipid- and surfactant-based delivery systems, composed of a phospholipid bilayer surrounding an aqueous compartment. They may present as spherical vesicles and can range in size from 20 nm to a few microns. Cationic lipid-based liposomes are able to complex with negatively charged nucleic acid via electrostatic interactions, resulting in complexes that offer biocompatibility, low toxicity, and the possibility of large-scale production required for *in vivo* applications. The lipid to RNA ratio and overall lipid concentration used in forming these complexes are very important for efficient ds RNA delivery and vary with applications. Liposomes can fuse with the plasma membrane for uptake; once inside the cell, the liposomes are processed via the endocytic pathway and the genetic material is then released from the endosome/carrier into the cytoplasm.

7.2 Inorganic nanoparticles as carrier : Inferable from nano-measurement size to volume proportion and its strength, inorganic (metal) nanoparticles are broadly utilized as promising quality bearers in dsRNA applications. Inorganic nanomaterials have remarkable electrical and optical properties, biocompatibility and in addition low cytotoxicity. The different inorganic nanoparticles used for the delivery are;

1. Gold nanoparticles - Among the different metals, suitability of gold as nanoparticles is highest due to its inactive nature, ease of synthesis, high functionalization, ability, higher absorption coefficient, simplicity of location and potential ability of targeted capacity

2. Graphene nanoparticle - Graphene is also an attractive nano-materials because of its optical, thermal and electrical properties

3. Silica nanoparticles – It can be easily synthesized with a controlled size, shape, and structure, making them highly advantageous delivery vehicles. They are commonly produced in a spherical

shape with pore-like holes; for example, porous hollow silica nanoparticles (PHSNs) or mesoporous silica nanoparticles (MSNs). PHSN and MSN commonly load the pesticide into the inner core to protect the active molecules and, therefore, provide a sustained release. The shell structure of PHSNs protect the active molecules inside the nanoparticles against degradation by UV light. The literature suggests that silicon has already been used to enhance plant tolerance against various abiotic and biotic stresses and, therefore, silica nanoparticles seem to be the natural choice for the development of agri-products for pathogens.

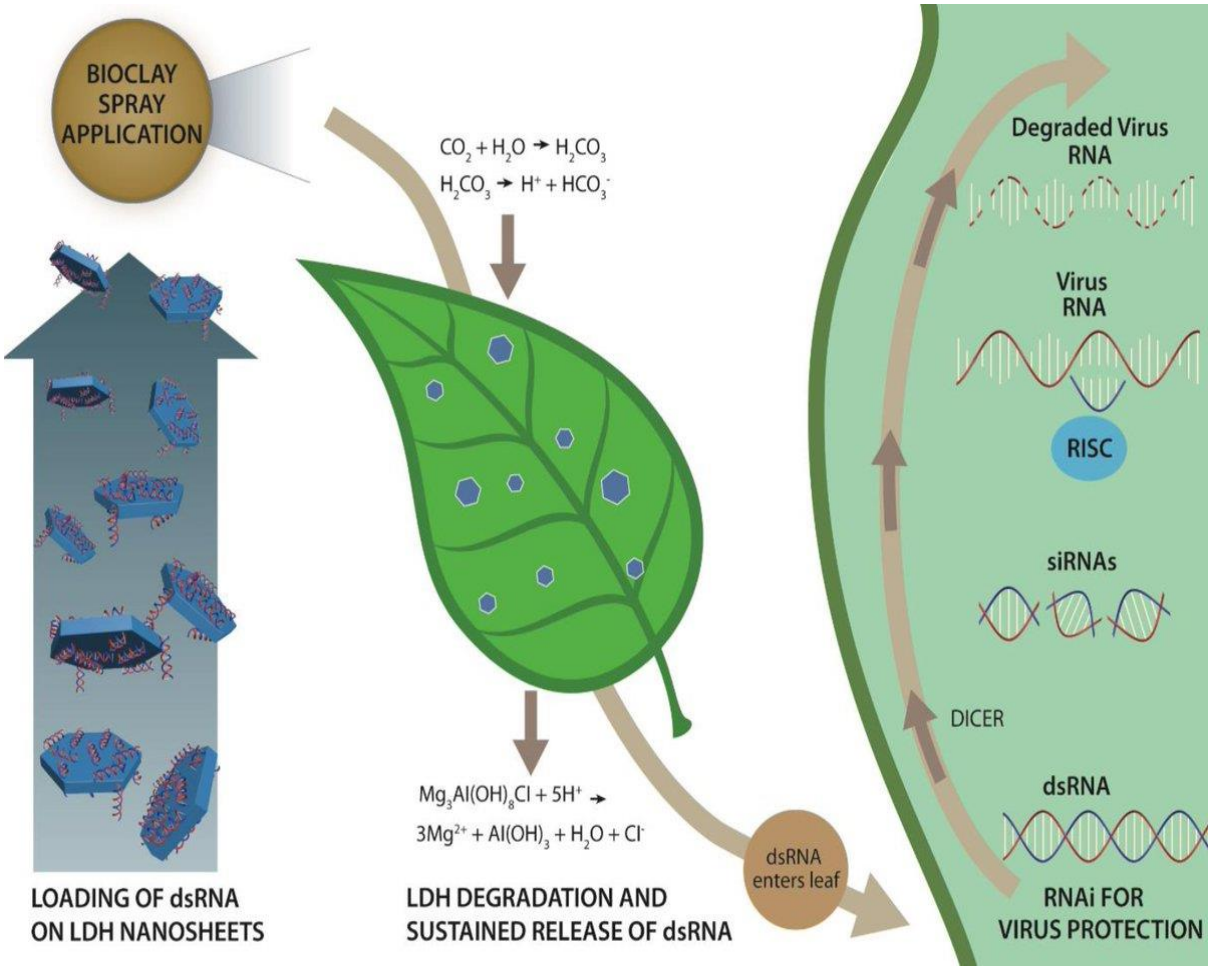
7.3 Polymer mediated delivery of dsRNA : Both natural and synthetic polymers show variation in their structure, molecular weight together with the chemical properties of the repeat units(s), affect their physico-chemical properties. Due to the large diversity in polymers, polymer based nanomaterials have drawn interest as potential delivery vectors. Polymer mediated delivery provides unique advantage because of their stability in biological fluids. It includes;

1. Poly Ethylene Imine (PEI): It is most extensively studied polymer for use in gene delivery. PEI is utilized for delivery of nucleic acid to the cells because of its buffering limit. It is available as linear PEI or branched PEI. Linear PEI contains all secondary amines while branched PEI contains primary, secondary and tertiary amines. Furthermore, secondary and tertiary amines of the PEI impart buffering capacity.
2. Dendrimers: These are mono disperse, nano sized, highly branched macromolecules which are chemically synthesized in a controlled manner to obtain varied surface functionalities. These are important class of polymers used in delivery of oligonucleotides. They have high drug loading capacity either by encapsulation or conjugation
3. PLGA based nanoparticles**:** Poly (lactic-*co*-glycolic acid) PLGAs are a family of water-insoluble polymers that have been widely used in biomedical applications. These can be readily formed into nanoparticles which entrap biologically active molecules.

8.1 Case study 1: Clay nanosheets for the topical delivery of RNAi for sustained protection against viruses**.**

They have bound dsRNA to layered double hydroxide clay nanosheets having an average particle size of 80 to 300 nm (BioClay). The dsRNA bound to BioClay was significantly protected from nucleases, while the dsRNA/BioClay complex did not wash off, even after rigorous rinsing. On the leaf surface, atmospheric CO2 and moisture resulted in a gradual breakdown of BioClay into a biocompatible residue, releasing the dsRNA in the plant cell either by passive diffusion or active transport. A 330 bp dsRNA targeting the CMV CP and bound to BioClay could be detected even 30 days post application (dpa) and upon single application in tobacco resulted in CMV protection for at least 20 days (Mitter *et al.*, 2017). Similar results were recently obtained, when spraying of 461 bp dsRNA bound to BioClay and targeting the bean common mosaic virus (BCMV) CP protected N.benthamiana and cowpea (Vigna unguiculata) plants against BCMV infection (Worrall *et al*., 2019).

FIG 3: Mode of action of bioclay



Efficacy of BioClay

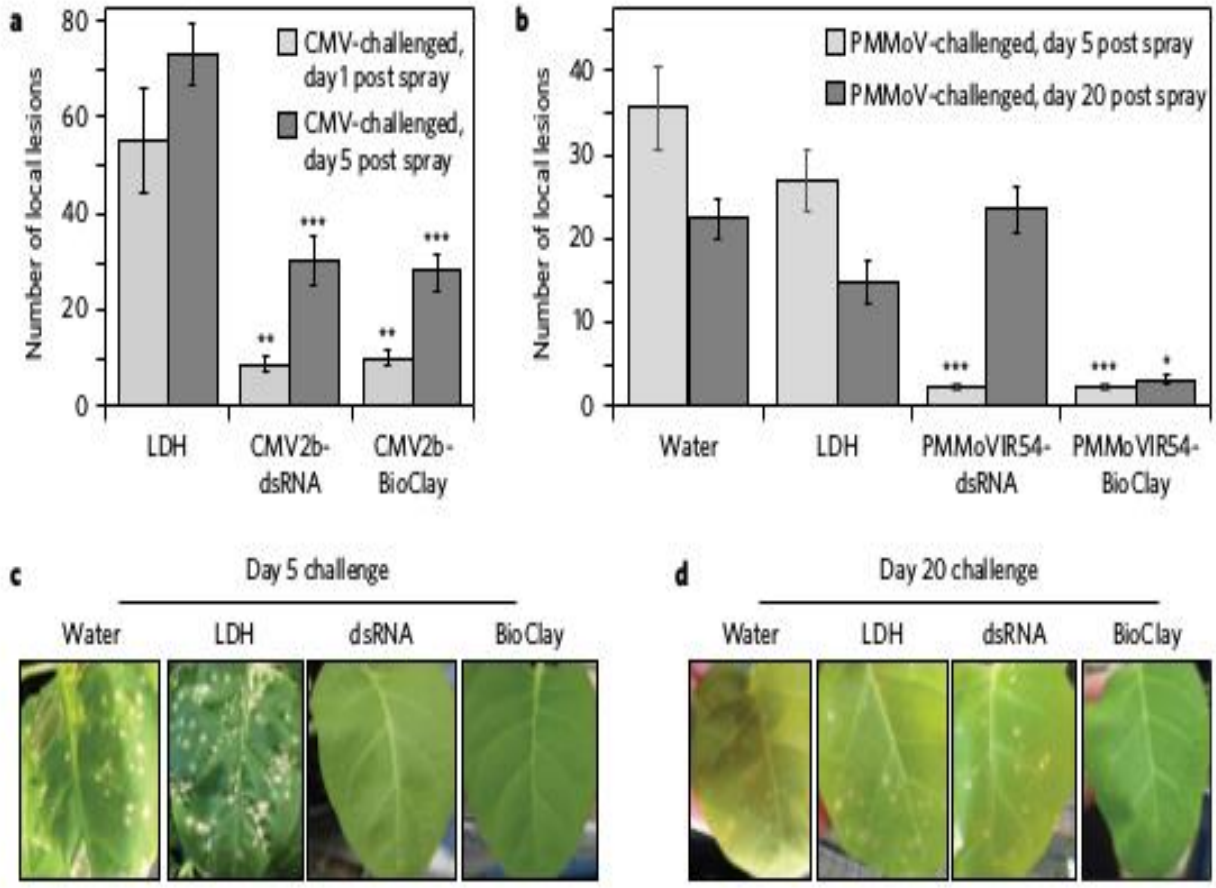


FIG 4: Efficiency of Bioclay

8.2 Case study 2

Using the agronomically important barley – *Fusarium graminearum* pathosystem, we alternatively demonstrate that a spray application of a long noncoding dsRNA (791 nt *CYP3*-dsRNA), which targets the three fungal cytochrome P450 *lanosterol C-14α-demethylases*, required for biosynthesis of fungal ergosterol, inhibits fungal growth in the directly sprayed (local) as well as the non-sprayed (distal) parts of detached leaves. Unexpectedly, efficient spray-induced control of fungal infections in the distal tissue involved passage of *CYP3*-dsRNA via the plant vascular system and processing into small interfering (si)RNAs by fungal DICER-LIKE 1 (*FgDCL-1*) after uptake by the pathogen. We discuss important consequences of this new finding on future RNA-based disease control strategies.

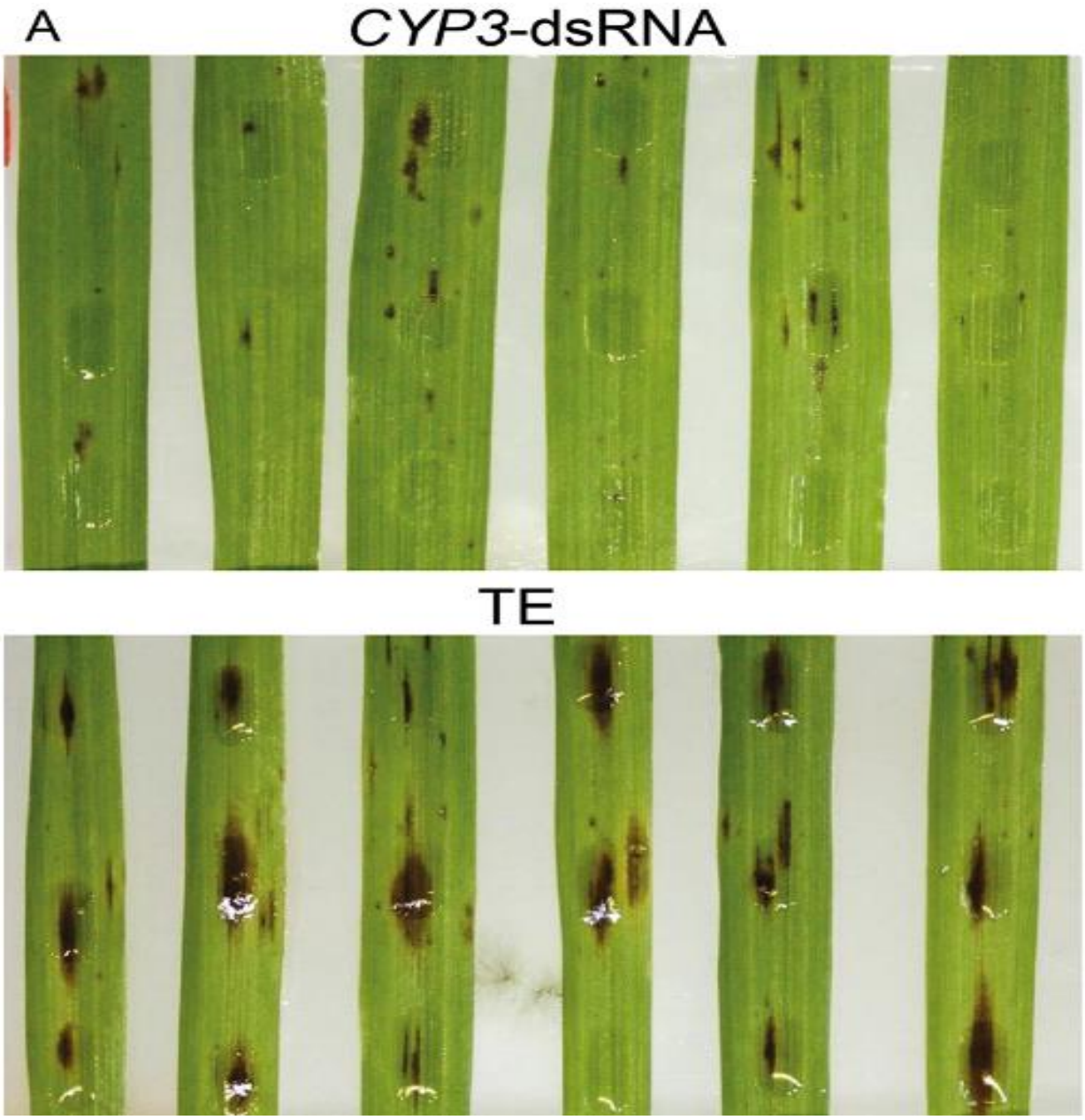


FIG 5: Comparison of lesion size in dsRNA treatment v/s control

Inference.: There is significant reduction in the lesion size of fungus in areas which was sprayed with dsRNA compared to unsprayed areas.

9. Advantages of this approach over conventional approaches

1. Non transgenic approach – There for public concerns will not be there in the delivery of this dsRNA molecules.
2. High specificity – Here we are developing dsRNA molecule specific to each pathogen specifically. Therefore, there is less chance for off target effects.
3. Effective against viral diseases – Right now we are not having an ultimate answer for the virus diseases. So this approach will provide a solution for this.
4. No residue problem – Since the dsRNA molecule is not thet much stable, the concerns regarding the residues on the products is not a serious issue.
5. Chances of developing resistance is low – Each time the dsRNA molecules cut at different locations by the DICER. Therefore, the chances of pathogen getting in contact with the same molecule is low and also the selection pressure is low. So chance of developing resistance is low.

10. Conclusion

Exogenous application of RNA molecules (SIGS) with the potential to trigger RNAi is a very powerful tool in modern crop protection platform considering the political and public pressure for sustainable solutions to current agricultural problems. Compared to conventional disease management strategies, exogenous RNAi promises significantly lower off-target effects, since its activity can be narrowed down to a window of few nucleotide complementarity with its target. With the rapidly accumulating sequence datasets, non-conserved regions can be selected as targets to exclude as much as possible off-target effects. To the same end, applying unique siRNAs, rather than long dsRNAs which are processed in a diverse population of siRNAs, would also greatly decrease off-targets. Arguably, the lack of genome data for any existing organisms near the site of exogenous RNA application undermines the proper ecological risk of RNAi technology in the agroecosystem. Nevertheless, the chance to silence non-intended targets is very low and far beyond any other available crop protection strategy has yet to offer. It cannot be excluded that certain pathogens may display gene mutations rendering them resistant to RNAi sprays. In cases of monogenic resistance, alternating ~200 bp regions of the target sequence could tackle this issue, while for polygenic resistance cases, using chimeric RNAs or a mixture of different RNAs could offer a solution, underpinning the overall flexibility of the RNA-based approaches. In the near future, it is reasonable to assume that optimization of RNA production, delivery and stability methods may pave the way for the exogenous application of diverse RNA molecules not necessarily triggering RNAi. Indicatively, such molecules could include: capped and polyadenylated mRNAs when accumulation of a protein is desired; miRNA decoys/sponges for sequestering of a given miRNA and thus upregulating its endogenous target; and even clustered regularly interspaced short palindromic repeats (CRISPR) components (Cas9 mRNA, sgRNAs) for the induction of editing events in a GMO-free manner.

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11. DISCUSSION

1. Is dsRNA sprays effective against bacterial diseases?

   Since the RNA intereference machinery is absent in the bacterial cells (because it is a prokaryotic), dsRNA sprays will not be effective against the bactereial diseases. In the case of virus, there is uncoating of the genetic material inside the plant cell. Therefore, the viral mRNA is accessible to the plant RNA interference machinery. In the case of fungal diseases, there is RNA intereference machinery within the fungal system. Even though the dsRNA cannot be used against the bacterial pathogen, anti sense RNA based methods can be used as a strategy.

2. Whether the method is cost effective?

   The cost of production of per gram dsRNA is about 2 USD. It was an estimate the about 4 gram dsRNA is required for one hectare. The exact cost of the formulation is not known. Still, when we are considering the cost of dsRNA production, the method is cost effective.

3. What is PAZ domain?

   It is the domain present in the proteins; Piwi and Argonaute. The domain is said to be having importance in protein-protein interaction.

4. What is the recommendation dose?

   It was estimated that about 4g dsRNA is required per hectare. But this may vary depend on the crop and pathogen.

5. Whether transgenic approach or non transgenic approach is advantageous?

   Transgenic approach is good and durable because once it is developed, the protection is assured. But in a country like India where transgenics are not supported, it is not an option. Therefore we have to adopt other methods. Here comes the importance of this non transgenic approach.

6. How the stability of dsRNA molecule is enhanced in the field level?

   In the field level, the stablility of the dsRNA against adverse conditions can be enhanced by the use of suitable carriers like nanoparticles. This nanoparticles will help to protect the dsRNA molecules from uv light and nucleases.

7. How the dsRNA from dsRNA- nanoparticle complex is released inside the system?

   Inside the plant system the change in the pH will help separate dsRNA from the nanoparticles and there by provide the sustained release of the dsRNA molecules.

8. Is the spray effective against wilt diseases or the diseases affecting roots?

   Right now there is no reports of dsRNA sprays against the root diseases. But there was a study conducted to check the stability of dsRNA on soil. So in the future there may have dsRNA sprays against root diseases.

9. Whether there is systemic action for the dsRNA sprays?

   Yes. The RNA intereference mechanism is showing some systemic action. Some amount of protection is observed in the unsprayed and newly emerged leaves also.

10. Whether short or long dsRNA is efficient in inducing the RNA interference mechanism?

    Long dsRNA are having more efficiency than small dsRNA because, when the long dsRNA is cut large number of siRNAs are formed. Therfore, the efficiency will be more.

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Department of Plant Pathology

Pl. Path 591: Master's Seminar

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Admission No : 2018-11-016

Major Advisor : Dr. Anita Cherian K.

Venue : Seminar Hall

Date : 13-12-2019

Time : 11.30 am

RNA interference in Plant disease management: A non-transgenic approach

Abstract

Current practices to protect plants and improve crop productivity rely on chemical methods, which are hazardous to the environment and exert deleterious effects on human health. As an alternative, RNA interference (RNAi) technology has emerged as one of the most promising strategies for sustainable disease management by enhancing resistance in plants (Singh, 2005). But, this technology involves the development of transgenic plants that enable the production of pathogen specific double stranded RNA (dsRNA) molecules through host induced gene silencing (HIGS). Insufficient information about the consequences of genetically modified organisms raised public concern which led to legislative limitations on the commercialization of transgenic plants in many countries (Bawa and Anilakumar, 2013). Recently, a non-transgenic approach with RNAi has been developed that involves direct exogenous delivery of dsRNA molecules to crop plants *via* topical application *i.e.* Spray induced gene silencing (SIGS).

The RNA interference machinery comprises of DICER, short interfering RNAs (siRNAs), RNA induced silencing complex (RISC), argonaute protein and RNA dependent RNA polymerase. dsRNAs are the interfering agents in RNAi technology. The steps involved in this mechanism are digestion, RISC activation, unwinding, binding and degradation. Once dsRNAs are introduced into the plant cell, these are processed by the endonuclease, DICER into siRNAs of 21-24 nucleotides length (Liu *et al*., 2009). These siRNAs, after incorporation into the RISC have the potential to degrade the pathogen specific messenger RNAs.

Currently, various methods have been standardized to produce dsRNA molecules by *in vitro* and *in vivo* production systems. This involves enzymatic processes which exploit the catalytic activity of polymerases obtained from bacteriophages (Timmons, 2006). The *in vitro* production system

utilizes hybridization of sense and anti-sense single strands of RNA that are synthesized by T7 DNA dependent RNA polymerase or by combining with RNA transcription catalyzed by the RNA dependent RNA polymerase of bacteriophage *phi*6. *In vivo* production systems involve use of bacterial expression systems with T7 polymerase gene complexes.

Plant cells have tough cellulose-rich cell wall which forms a physical barrier for biomolecule delivery. Hence, the delivery of dsRNA molecules into the plant cell is facilitated by conjugating with nanoparticles as carrier compounds. Organic, inorganic and polymer based nanoparticles are being utilized for this purpose. BioClay, a product of dsRNA with high stability and prolonged protection against plant viruses has been developed by Mitter *et al*. (2017). It has dsRNAs bound to layered double hydroxide clay nanosheets with a particle size of 80 to 300 nm.

The global growth of population, reduction of cultivable land, climate change, concerns about the safety of genetically engineered plants, *etc.* led to the development of novel, non-transgenic and environment friendly approaches to manage plant diseases. Hence, non-transgenic exogenous application of dsRNA molecules with the potential to trigger RNA interference is one of the powerful tools in modern crop protection platform and a sustainable solution to the current agricultural biotic challenges.

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