



RNA-BASED BIOPESTICIDES FOR SUSTAINABLE CROP PROTECTION

Bindu Rajaguru*, Meenakshi Johri, Karan C. Choudhary and Shiksha V. Chauhan

Department of Biotechnology,

Pillai College of Arts, Commerce and Science (Empowered Autonomous), New Panvel, Navi Mumbai, India

*Corresponding author E-mail: bindurajaguru@gmail.com

ORCID iD: <https://orcid.org/0009-0001-1550-6624>

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Abstract:

RNA interference (RNAi) has emerged as a promising molecular technology for sustainable and environmentally compatible crop protection against diverse agricultural pests and pathogens. It relies on sequence-specific silencing of essential genes through double-stranded RNA (dsRNA) and small interfering RNA (siRNA), and has been explored against fungal pathogens, insect pests, nematodes, viruses and other disease-causing organisms. Approaches including spray-induced gene silencing (SIGS), host-induced gene silencing (HIGS), microbe-induced gene silencing (MIGS) and oral dsRNA delivery have expanded its applications. In *Botrytis cinerea*, BioClay improves dsRNA persistence and extends fungal protection compared with naked dsRNA. Similarly, chitosan-dsRNA nano complexes enhance RNA stability and induce effective gene silencing and mortality in *Piezodorus guildinii*. MIGS uses beneficial microorganisms to facilitate RNA-mediated protection against soil-borne fungal pathogens. However, rapid degradation of naked RNA, inefficient uptake, environmental exposure, formulation cost and variable target sensitivity remain limitations. Off-target effects, non-target exposure, environmental fate and resistance also require evaluation. Nanomaterials, clay-based carriers, chitosan formulations and biological delivery systems may overcome these barriers. Future research should optimize target-gene selection, dsRNA sequences, formulations and delivery systems, supported by large-scale field trials. Integration with integrated pest management (IPM) could reduce dependence on conventional chemical pesticides.

Keywords RNA Interference, Gene Silencing, Biopesticides, Crop Protection, RNA Delivery, Pest Management.

1. Introduction

Since its discovery, RNA interference (RNAi) has developed into a highly promising application for sustainable crop protection, due to its capability of triggering sequence-specific suppression of genes indispensable for the viability, growth or pathogenicity of agricultural pests and pathogens. The approaches of RNAi use the application

of double-stranded RNA (dsRNA) or small interfering RNA (siRNA) leading to the degradation and/or repression of complementary targets RNA transcripts. Among all of the different RNAi-approaches, spray-induced gene silencing (SIGS), was greatly highlighted for the protection of crops by exogenous application of RNA molecules to the host plant that will then elicit directly pest target genes without needing genetically transformation. SIGS efficacy is directly correlated to how successfully pathogen RNA is taken up, therefore enhancing RNA uptake for control efficiency is very important [1]. Other studies have also illustrated potential in using RNAi against viruses and various plant-pathogens interactions [2–4].

By improving the alternative strategy of RNA delivery into organisms, RNAi technology potential for crop protection is more likely to be applied. Engineered microbes-induced gene silencing (MIGS) is an application based on delivered small RNAs to soil borne pathogens with engineer microbes as the carriers and shows promising in fungal control [4]. Moreover, the usage of minicell-based delivery system is suggested as an alternative to protect the RNA molecule and help delivery functional RNA into pathogen, compensating the issues on using naked dsRNA [5]. The above mentioned delivery strategies based on utilizing organisms are valuable since the efficiency of both environment degradation and internal transport would heavily decrease the efficacy of RNAi. Effective delivery is essential and must meet the requirement of RNA stability, transportation and target organism uptake in the future practical of RNA biopesticides.

More formulation technologies, however, also improved the application limitation for conventional method. BioClay is a material developed using the layer stacking clay component to embed dsRNA and protect it from degradation with prolonged RNA activity in contrast to naked dsRNA in control of *Botrytis cinerea* [6]. BioClay could show the extended action in tomato leaves and fruits as well as chickpea under condition of experimental study. Nanocomplexes of dsRNA with chitosan and various nanomaterial-assisted systems are also used to protect RNA stability, transportation as well as promote RNAi efficacy [7,8]. Furthermore, cross-kingdom RNA trafficking provides a novel way to control organism interacting via transferring functional RNA among them [9,10]. This shows that both RNA delivery and cross-kingdom RNA transportation are the basic for next generation of RNAi technology.

While various achievements were obtained, there are still limitations hindering the practical application of RNAi technology on a large agricultural scale; they include: unstable RNA in the environment, low rate of endogenous RNA uptake, insufficient duration for persistent expression and target specificity (off-target effect), environment influences, costs and unpredictable effects. From the data from fungal pathogens, insects and other agricultural pest application on RNAi targeting the essential genes requires a sufficiently amount biological active RNA that reaching the pest [11-13]. These drawbacks could be overcome by stabilization formulations, Nanocarriers, biological delivery systems and accurate targeting strategies. Integrated approach of combined stabilized RNAi, precise delivery system and integrated pest management would present RNAi based biopesticides as a safe, selective and effectively technological innovation for the sustainable protection of crops.

2. Biological basis and molecular mechanisms of RNAi in crop protection

2.1 Double-stranded RNA formation and recognition

RNA interference is a sequence-specific RNA degradation and the start is formed by double-stranded (ds) or structured RNA. In plants, Dicer-like (DCL) enzymes convert dsRNA into small interfering RNA (siRNA) duplexes which contain single strands with complementary base pairing. The guide-strand is loaded onto Argonaute-

containing silencing complex that recognize target transcripts complementary in sequence to induce degradation or knockdown. This sequence-specific RNA interference machinery is a component of this crop protection mechanism [2].

2.2 Target-recognizing ability and gene knockdown

In plants after its formation, the guide strand leads the RNA-induced silencing complex towards target RNAs homologous to it. Sequence complementarity may allow gene silencing targeted to any function essential for the growth or development and pathogen or insect, such as virulence, metabolism and survival or reproduction, respectively. For example, hotspot-derived siRNAs are able to stimulate potent antiviral responses targeting African cassava mosaic virus through effective application to *Nicotiana benthamiana* and showed target specificity of appropriate RNA selection important for RNAi-mediated disease resistance.

2.3 Uptake of RNA and spray-induced gene silencing

First, the applied dsRNA used in Spray-Induced Gene Silencing need uptake by the target pathogen or organism [1]. It was reported that dsRNA was efficiently taken up by several pathogenic fungi namely *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoglyphus solani*, *Aspergillus niger*, *Verticillium dahliae* while few other species demonstrated little or no RNA uptake. Pathogenic species which absorb dsRNA well provided significant effect on disease resistance after spraying dsRNA.

2.4 Cellular delivery and nano-material delivery for RNAi

It is important for the effective utilization of RNAi for plant protection that RNA be delivered efficiently to target pathogen cells or plant cells [14]. Naked RNA is not very stable and crosses cell membrane poorly. RNA can be protected with nanodevices, and they are suitable for intracellular or intra-organism transport. Nanoparticles, especially clay nanoparticles may be a suitable delivery vector for siRNA into intact plant leaf cells.

2.5 Global pathway of RNAi mediated pesticide

The pathway: application of dsRNA/siRNA uptake DCL-mediated conversion small interfering RNA duplex synthesis choice of guide-strand loading onto AGO/RISC target- RNA targeting target recognition degradation or cleavage of target RNA suppression of crucial gene activity pest/pathogen growth/virulence to decrease protection of crops. This process should have suitable targets, RNA stability and ability to penetrate efficiently into the cells/organisms [14].

3. Target Specificity and Efficacy Across Agricultural Pests and Pathogens

3.1 Sequence-specific targeting selection

The RNAi technique provides highly specific silencing due to the design of dsRNA/siRNA targets to essential survival, growth and reproductive, or metabolism and pathogenic elements of specific organisms. For *S. Sclerotiorum* HIGS against the ABHYDROLASE-3 gene led to decrease the fungal transcript level, fungal bio-load and lesion expansion and the potential to use pathogen-specific RNAi for protection of crop, could thus be highlighted.

3.2 RNA uptake and silencing efficiency

The efficiency of the RNAi approach is strongly dependent on the ability of the target to internalize and process the RNA molecule. Qiao et al. Reported that dsRNA was effectively internalized by some of the fungal pathogens: *B. Cinerea*, *S. Sclerotiorum*, *R. Solani*, *A. Niger* and *V. Dahliae* but weak or zero take-up was observed in other species analysed. RNA take-up therefore plays critical part in determining effectiveness of SIGS

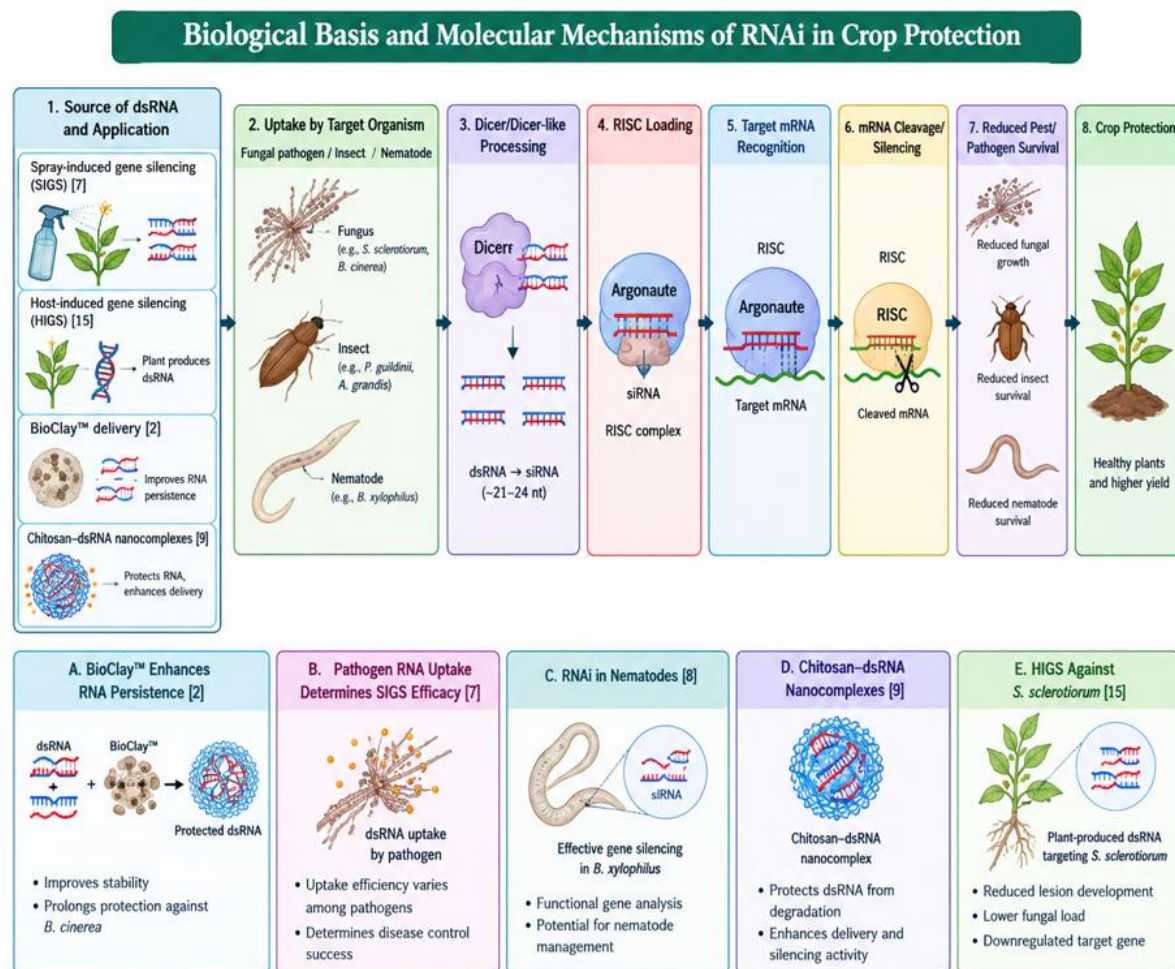


Figure 1: Mechanisms of RNAi in crop protection

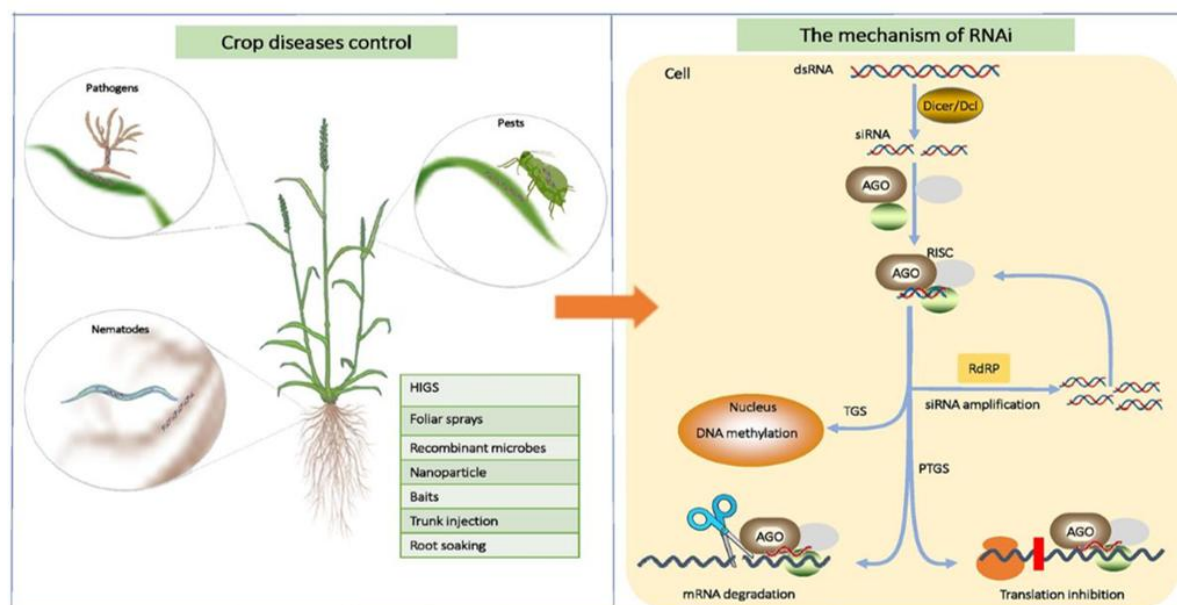


Figure 2: Biological Basis and Molecular Mechanisms of RNAi in Crop Protection

3.3 Selectivity and reduction of off-target effects

A significant potential benefit of RNAi based pesticides is the ability to specifically targets genes in a pest or pathogen organism, avoiding negative side-effect in beneficial organism or other non-pest organisms. In the HIGS research performed for *S. Sclerotiorum*, selective screening of the ABHYDROLASE-3 region allowed avoiding major affecting effects to closely related *B. Cinerca* which suggested great potential of organism specific RNAi technology [13].

3.4 Effects on insect pests

RNAi technique can suppress genes involved in indispensable survival aspects of an agricultural insect pest. In an example, 10 different target gene for a soybean pest insect *P. Guildinii* were searched and the significant impact upon administration to the aphid population with dsRNA against them have demonstrated mortality. Srp54k target genes were then delivered with chitosan-dsRNA nano-complex and showed about 66% corrected mortality and a significant reduced target-gene expression [7].

3.5 Nanomaterial Assisted delivery and Increased efficiency

Cellular permeability to uptake and processing of RNA is a critical aspect of RNAi. Clay nanoparticles efficiently deliver siRNA into living plant cells. Thus, it offers opportunities to enhance RNA delivery across biological barriers and can be overcome cellular permeability problems [14]. Chitosan based nano-complex protected dsRNA against degrade and enhanced the biological efficiency against agricultural insects.

3.6 Overall agricultural applications

All these studies indicate that RNAi may offer potential for precise control of fungi and insect pest pests, but application to viruses, nematodes and other agricultural pest control are also likely; while these factors determining potency seem include species specific choice of target genes, RNA uptake, stability, cellular permeability and uptake efficiency [14].

4. RNAi technology for gene function analysis and pesticide development

4.1 RNAi in functional gene analysis

RNA interference (RNAi) is an important reverse-genetic methodology to understand gene biological functions in agricultural pests and pathogens. When sequence-specific dsRNA is introduced, one target gene's expression could be reduced, then researchers could associate such gene knockdown with observable phenotypic impacts, like development, survival, reproduction, metabolism, or pathogenesis. For example, a dsRNA soaking methodology in pinewood nematode (*B. Xylophilus*). Enabling functional studies on the genes that are difficult to conduct traditional genetics.

4.2 Identification of essential genes as pesticides targets

Genes necessary to survival are ideal targets for RNAi-based pesticides. Researchers screened essential genes in wireworm (*Agriotes*) spp. And investigated gene suppression through ingestion dsRNA by targeting genes V-ATPase subunit A, V-ATPase subunit E, -actin and chitin synthase 1, respectively. Inhibition of V-ATPase A results in the strongest mortality rate (~50%), demonstrating how RNAi screens could identify promising pesticide targets [15].

4.3 RNAi-Based Development of Insecticidal Strategies

As shown in previous section, RNAi gene-function analyses could directly evolve into applied pest-management methods by targeting genes required for insect growth and survival. An investigation into stabilized dsRNA

targeting in cotton boll weevil (*A. grandis*) demonstrated an effort to improve resistance in cotton through RNAi technology. It emphasizes the importance in targeting the precise gene and also optimizing dsRNA stability and delivery system [16].

4.4 RNAi for control fungal pathogen

The usefulness of RNAi has been shown for identification and functional validation of pathogenesis genes in fungal pathogen. By using a host-induced silencing approach, the ABHYDROLASE-3 of *Sclerotinia sclerotiorum*, suppressing its expression leads to the reduced infection phenotypes, indicating that fungal pathogenesis functional genomics can identify RNA-targeting fungicide targets.

4.5 From gene function analysis to RNA-based pesticides

General schemes could be illustrated as: Identification of candidate genes, dsRNA/siRNA design, RNA delivery, Target-gene knockdown, Phenotypic validation, Identification of essential genes, Optimization of RNA formulation, Pest/ pathogen suppression, RNA-Based pesticide Development. RNAi successfully integrate functional genomics into precision pesticides management: A significant advantage of RNAi technique is to enable empirical validation of target candidates prior to their development into pesticides against pests. But optimization of RNA stability, delivery efficacy, and persistence of RNAs, cost efficiency and environmental safety still remain a challenge. [15].

5. Ecological Safety, Non-Target Risk Assessment, and Biosafety of RNAi-Based Crop Protection

5.1 Ecological safety of RNAi-based bio pesticides

RNAi crop protection offers a potentially safer alternative to broad-spectrum synthetic pesticides, given the possibility to design dsRNA specifically against defined genes of particular pests or pathogens and the ability of dsRNA to rapidly degrade into the environment. However, ecological safety is related to formulation, application rate, environmental persistence, entry pathways, and sensitivity of species in the application site. Environmental fate and exposure estimation should be assessed prior to field application of RNAi-based products.

5.2 Non target organism and off-target effects

Ecological safety depends largely on off-target effects on other organisms, such as on beneficial insects, pollinating insects, soil inhabitants, plants, aquatic organisms and other organisms. Sequence homology between dsRNA target sequences and transcriptome of non-target species is a primary determinant for potential off-target interactions. Highly related species may share higher sequence similarity, and similarity in the RNAi components might increase vulnerability to off-target effects. Sequence database screening coupled with experimental tests on specific on-target species is required for robust environmental risk assessment [18,19, 20].

5.3 Environmental fate and persistence of dsRNA

Environmental fate and persistence are important for RNAi safety. Factors like UV exposure, temperature, moisture, microbe and the soil conditions (pH, microbial content, soil texture etc.) as well as formulation can affecting the stability of RNAi bio pesticides. While a rapid degradation in the environment might limit prolonged environmental exposure, encapsulation and nanostructure-based delivery systems could increase the persistency of dsRNA, necessitating further environmental assessments. In the context of developing external RNA pesticides, the OECD recommends to evaluate the environmental fate and exposure and to assess the impact on non-target organisms [19,20].

5.4 Risk assessment and regulatory considerations

Hazard and exposure analyses are essential elements of a comprehensive risk assessment of RNAi-based crop-protection agents. Crucial parameters include the identity and biological function of the target gene, homology of dsRNA to genes of non-target organisms, the amount of dsRNA, environmental persistence, routes of exposure, potential for RNA uptake and the sensitivity to RNAi. Currently, the risk assessment frameworks should integrate the analysis of phylogenetic relationships, database searches and transcriptome data with data from real environmental exposures. This facilitates predictions on sequence-specific off-target effects and provides for evidence-based regulatory decisions [20].

5.5 Human health and biosafety

In addition to environmental aspects, the impact of RNAi-based pesticides on human health is also relevant, particularly for workers, consumers and people during application. The sequence-specific nature of RNAi technology affords opportunities for selective pest control but a thorough assessment of human health risk, including exposure route analyses for inhalation, dietary intake and dermal exposure, should be conducted at product development [19, 20].

5.6 Overview of biosafety

RNAi-pesticides offer a lot of opportunities and can reduce dependency on chemical conventional pesticides. However, environmental safety must not be taken for granted as biological products or based purely on specificity. Therefore, target specificity, environmental persistence, non-target exposure, off-target effects on organisms other than the pest, the formulation and development of resistance must be extensively scrutinized before large-scale field application. A combination of bioinformatic predictions and of field and Semi-field ecological assays are required for safe RNAi-based crop-protection products [20].

6. Therapeutic Applications of RNA Interference

6.1 RNAi as a therapeutic gene-silencing strategy

RNA interference (RNAi) using small interfering RNA (siRNA), have evolved into an essential therapeutic platform due to their ability to facilitate sequence-specific shutdown of disease-causing genes. SiRNAs designed to target specific gene transcripts down regulate their mRNA levels through the RNAi machinery to create potent, highly selective, and gene specific therapies. SiRNA therapeutics have moved from the laboratory into clinical application [1,2].

6.2 Therapeutic applications in genetic and metabolic diseases

One of the first successful applications of RNAi includes treating genetic and metabolic diseases-especially those caused by liver-expressed genes. SiRNAs can specifically silence expression of pathogenic protein and have the ability to maintain prolonged silencing following administration. The design of chemically stabilized siRNAs and improved delivery to liver tissues (eg. using lipid nanoparticles and GalNAc- conjugates) greatly contributed to success and prolonged treatment effect of these diseases [1,3].

6.3 RNAi against viral infections

RNAi also has the potential to control viral infections, through inhibiting viruses either targeting crucial viral RNAs, or host factors necessary for their replication, by designed against conservative sequence of different viral types which enables RNAi for treatment of infectious disease. Nevertheless the mutation, viral variation, as well as delivery to tissue where target is replicating is still one of the most essential obstacles of antiviral therapies using RNAi [4].

Table 1: Comparative Applications of RNAi in Crop Protection

Group	Target organism	Target gene	Species/Crop	Application method	Silencing outcome
Fungal pathogen	<i>Botrytis cinerea</i>	DCL1, DCL2	Tomato, lettuce, onion, grape, strawberry	Spray-induced gene silencing (SIGS)	Reduced fungal growth and lesion development
Fungal pathogen	<i>Sclerotinia sclerotiorum</i>	Virulence/essential genes	Brassica and other crops	SIGS	Reduced fungal growth and disease symptoms
Fungal pathogen	<i>Sclerotinia sclerotiorum</i>	ABHYDROLASE-3	<i>Arabidopsis thaliana</i>	Host-induced gene silencing (HIGS)	Reduced target-gene expression, fungal growth and disease severity
Fungal pathogen	<i>Aspergillus niger</i>	VPS51, DCTN1, SAC1, pgxB	Tomato, apple, grape	SIGS	Target-gene suppression and reduced disease development
Insect pest	<i>Piezodorus guildinii</i>	Srp54k and candidate genes	Soybean	dsRNA + chitosan nanocomplex	Reduced gene expression and increased insect mortality
Insect pest	<i>Anthonomus grandis</i>	Essential target genes	Cotton	Stabilized dsRNA	Improved cotton resistance and pest suppression
Nematode	<i>Bursaphelenchus xylophilus</i>	Candidate essential genes	Pine	dsRNA-mediated RNAi	Gene silencing and identification of potential control targets
Bacterial pathogen	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	cfa6, hrpL	<i>Arabidopsis thaliana</i>	Extracellular sRNA-mediated silencing	Reduced bacterial virulence and target-gene activity
Arthropod pest	Tick species	Functional/essential genes	Different host systems	RNAi-based gene silencing	Reduced gene expression; functional characterization of target genes

6.4 Applications in cancer and other diseases

RNAi also presents a variety of therapeutic opportunities for treating cancer, as well as cardiovascular and neurological disease, inflammation and others conditions, by reducing overactive expression of cancer genes, pathological proteins and signalling pathways that could hard to targeted with small molecule inhibitors. RNAis against almost any gene sequences that exist is a great promise, especially when facing previously incurable illness [5,7].

6.5 Delivery systems and clinical translation

Unfortunately, one of the biggest problems for therapy based on siRNA is delivering siRNA to cells. Native siRNA is not suitable for treatment application due to they are degraded quickly in circulation. Delivery based on viral vehicles is not widely employed for treating other illness than cancer [5,7]. Lipid nanoparticles, such as SLN, nanoparticles, liposome-based formulation as well as GalNAc and polymeric conjugated molecules have extensively explored and demonstrated improved siRNA stability and efficacy of tissue-specific delivery. The significant advances of improved delivery and formulation have been responsible for the transition of the experimental technology to marketed therapeutics. RNAi is a highly relevant strategy for a large number of unmet medical needs.

6.6 Future therapeutic potential

RNAi therapeutic is considered as one type of precision therapeutics which cover broad applications ranging from genetic disorders to viral disease and beyond. Delivery system, especially to extra-hepatic tissues, as well as brain and muscle cells. This field is still an active and exciting area of research aiming to broaden RNAi into the realm of other diseases beyond liver disorders. Ongoing work involves novel chemistries and formulation technologies, further development of effective delivery systems that can target extra-hepatic organs. [2,7].

7. Advanced formulation technologies and nanoparticle carriers

7.1 Nanomaterial-based RNA protection and delivery

One of the major obstacles for the field deployment of RNAi in agriculture remains the rapid and easy degradation of naked dsRNA in environmental conditions, thereby prompting advanced formulation technologies that protects RNA, ensures persistence, and facilitates penetration by target species. The use of nanomaterial carriers such as clay nanoparticles, layered double hydroxides (LDHs), chitosan and others nano complex can bind to dsRNA or siRNA, protect it and facilitate delivery. For instance, it has been demonstrated that the clay nanoparticles have the ability to delivery siRNA in complete plant leaf cells which could support an RNA-based control in plants [14].

7.2 BioClay formulation for prolonged RNAi activity

A formulation in which the dsRNA is associated with layered clay nano sheets called BioClay technology has protected dsRNA by the degradation process in vivo as the slow release of dsRNA on the surface of the plant surfaces can prolong RNAi protection from the pathogen *Botrytis cinerea* from that of naked dsRNA [6].

7.3 Chitosan-dsRNA nanocomplexes

Chitosan, a natural polymer with a positively charged surface that is readily interact with negatively charged RNA, forms stable nano complexes dsRNA with negatively charged cargo. Chitosan based formulation protects dsRNA from degradation and delivered the RNA molecule to the infected target cells for increased gene silence in vivo. The *Piezodorus guildinii* insect has been fed on chitosan-dsRNA nano complexes and substantial gene silencing of *P. Guildinii* gene showed significant protection for *P. Guildinii* [7].

7.4 Biological and nanoparticle-assisted delivery

Besides the synthetic nanoparticles. Biological platforms can also be explored to transport RNAs to the intended pathogen cells and achieve sustained release in the environments. For example, minicell-mediated delivery can be used to transport fungal RNAi molecules and microbe-induced gene silencing strategy can also deliver functional RNAs directly to the targeted pathogenic bacteria and soil borne fungal pathogens [5].

7.5 Improving field stability and application efficiency

In practice, the formulation can enhance RNAs stabilities in the environment, maintain longer persistence in the environment and improve the uptake and gene silencing efficiencies, as a result reducing the need to repeated application. However, maintaining RNA durability in nature can cause unintended effects on the environment, and is also require additional ecological and non-target risk evaluation, so this factor of long persistence should compromise the sustainability with the timely degrading capacity once the designed action is reached [14,19].

8. Mechanisms of Resistance and Sustainable Resistance Management

8.1 RNAi resistance

While target-specific and based on sequence, it can eventually lead to decreased sensitivity to dsRNA in target insects and pathogens (resistance). Resistance to RNAi can occur through multiple mechanisms. These include reduced RNA uptake into the target organism, degradation, transport/processing into RNA, a mutated target gene, or changed RNAi pathway components. Differences between pathogens' RNA uptake rates is most significant as greater uptake is associated with greater SIGS [1].

8.2 Target site, RNAi pathway resistance

The efficiency of an siRNA in silencing by hybridizing with target mRNA will be decreased if the target-site sequence mutated. Alterations in any protein components of dsRNA uptake, processing or Argonaute mediated silencing will also be manifested as resistance. It follows therefore that resistance management strategies must account for the specific target gene, and the biological machinery required to carry out uptake and silencing. Recent literature also places importance on altered susceptibility of the targets to dsRNA (target-site resistance) and enhanced biological factors that decrease efficacy [13].

8.3 Multiple target and stacked dsRNA techniques

Multiple target sequences are probably one of the first, most obvious ways to slow resistance development. Multiple dsRNA targets on separate pathways are required for the pest/pathogen to acquire resistance, unlike in target-site resistance. Regularly switching targets should also be an approach, reducing the rate of selection for single targets; these techniques would both be applicable within an RNAi pest-management regime.

8.4 Use RNAi together with conventional pest management techniques

RNAi can also be used within an integrated pest-management scheme rather than as stand-alone agents. Spraying dsRNA with conventional pesticides will not only combat any resistance present to either mode of action but decrease pressure to resist the other; literature is showing that the technology is beneficial against resistance factors (e.g. Insecticides detoxifying enzymes).

8.5 Better formulation in RNA delivery will slow resistance development

If the dsRNA cannot adequately get to its intended site, and a sublethal dose is received then selection for tolerance could occur. So to create the necessary levels of silencing RNA uptake must be increased while increasing resistance and stability, formulation is a way in which this can be increased. RNA Delivery could be optimized and

resistant develop postponed by using BioClay, chitosan based nanocomplexes and clay nanostructures to improve access and stability [1,20].

8.6 A sustainable strategy for managing resistance to RNAi

A well-designed RNAi resistance management program must combine appropriate target selection, multi-targeting of essential genes; formulation optimization; temporal or target switching and pest population resistance monitoring. RNAi should be used as one tool in an integrated pest management toolbox rather than a standalone alternative method, since many recent reviews of RNAi applications argue that dsRNA, being sequence-specific and compatible with resistance management programs provides a perfect means to create more sustainable replacements to current strategies.

9. Regulatory frameworks, scalability, and economic viability of RNAi-based crop protection

9.1 RNAi based pesticide regulations

To commercialize RNAi based crop protection products, regulatory frameworks capable to handle the distinct features of RNAi (sequence-specific nature, environment, non-target effects, exposure pathways, human health) must exist. Different countries have different regulatory approaches posing significant barriers for commercialization across countries. The OECD established guidelines to be followed for environmental and human health risk assessment of dsRNA that is applied on surfaces of crops and also guidelines to assess environment fate of the same. [19, 20]

9.2 Environmental and human health evaluation

Evaluating the identify and the function of target gene, sequence homology to non-target organisms and environmental exposure along with degradation, to finally assessing if human and non-target organisms are exposed is of critical importance. The OECD provides guidelines considering environment and non-target organisms as well as the factors of human exposure through appropriate routes (oral ingestion, skin application, inhalation) with sprayed dsRNA products [20].

9.3 Scalable dsRNA production

One of the limitations to a commercial scale RNAi based pesticide product development is a large-scale and economic production of high-quality dsRNA. Options available include; microbial fermentation, cell-free synthesis, in vitro transcription and plants as bioreactors, each having its advantages and disadvantages in term of yield, purity, processing steps and production costs. The reviews on recent commercialization identify high dsRNA production costs as an important hurdle [20].

9.4 Formulation and scalable application

For commercial scale RNAi based pesticides there is a need to develop formulation where dsRNA is stabilized throughout the supply chain, storage and field applications. DsRNA could be delivered protected through BioClay, chitosan-based nano complexes, and etc. For enhanced bioactivity and stability. Their production process needs to be cost effective and reproductive for an agro application and it is also important to consider efficacy in formulation design aside from factors such as cost, shelf life, interaction with other spraying substances, and application process. [6]

9.5 Economic viability and commercialization

Economic viability of RNAi-based pesticide relies on dsRNA costs during synthesis, formulation cost, application frequency, target crop value and compare well with conventional pesticides efficacy. Though initial production

costs are high, the use of RNAi pesticides, characterized by target specificity and negligible non-target effect, combined with an integrated pest management strategy, can ultimately provide significant cost-effectiveness as well as environmental benefits. Reducing the production costs and having improved comparative field efficacy data is the next step towards successful commercialization.

9.6 Futuristic regulation and commercialization perspective

Collaborative effort of researcher, biotech companies, farmers and regulatory agencies should foster research to commercialization process from laboratory settings of RNAi based pesticide development. harmonization on regulatory needs would minimize duplicated tests on product candidates and the time taken on testing would speed up introduction of RNAi based pesticide into market. Meanwhile, the production of inexpensive RNAi dsRNA, formulation strategies and automated production along with large scale application infrastructure are indispensable. In most of recent studies the authors report that crop protection with RNAi moves toward commercial stage, though limitations with regard to regulation, high production costs and field efficiency is recognized.

10. Challenges and future perspectives of RNAi-based crop protection

10.1 RNA stability and degradation in the field

One of the limitations for using RNAi-based protection in field crops is the poor stability of naked dsRNA/siRNA in the field. External application of RNA onto plants will lead to its rapid degradation when exposed to ultra-violet radiation, temperature, rainfall, nucleases and soil microbes thus reducing its bioactivity in field. Therefore, to enhance the RNA stability with delivery formulation such as BioClay, chitosan nanocomplexes and clay nanoparticles are an area of active research [6,14].

10.2 Delivery to the appropriate cells and cellular uptake

There remain critical limitations in the delivery of the RNA to the intended target pests or pathogen cells and uptake and processing by the RNAi machinery for effective gene silencing. Differential cellular uptake of RNA across pathogens could drastically affect the efficiency of SIGS [1]. This fact means that in future research ought to focus on optimized carriers including nanoparticles and biologics and formulations that could increase RNA uptake and still remain environmentally benign [5,20].

10.3 Specificity and impact on the non-target organisms

Since RNAi is a high sequence specificity technology, it has been observed that off-target effects can occur if homologous genes are present in beneficial organisms or a pest species due to binding of the dsRNA sequence. Prior to the commercial application, it will be essential to conduct comprehensive bioinformatics analyses on every target sequence and subsequent laboratory and field ecological tests to determine any off-target effects on non-target species. Regulatory guidelines require an assessment of the persistence, degradation rates and impact on non-target organisms.

10.4 Production and cost

High cost associated in the production of the dsRNA and application rate requirements, which dictates a substantial amount of RNA being required, can prohibit the use for low value crop products. A cost-effective strategy which can further yield improvements, purification efficiency, stability through the formulation as well as the ease of scalability in its production is required in the future. Microbial production, cell-free system and other low-cost platforms for RNA-based pesticides will prove beneficial [5].

10.5 Resistance development

This aspect can be observed with the repeated application of RNAi treatments and may involve down-regulation of the expression of the gene targeting. Alternatively, the plants may develop Resistance through other means, such as altered uptake and stability characteristics of dsRNA. Hence the strategy could involve multiple gene targeting and target rotation strategy along with integrated biological and conventional pest management techniques. The combinatorial treatment with IPM may further delay the resistance development [1,7].

10.6 Future prospects for sustainable Agriculture

Forward thinking strategies in RNAi-based pest management research should look toward rigorous field validations, standardized assessment of ecological risks and effective delivery technologies and costs-effective production. Cross kingdom RNA trafficking and cellular delivery via microbial delivery methods, nanotechnologies and better formulations can optimize the efficacy. Integration of RNAi-based product with IPM should bring selective environment-friendly crop protection against target insects, plant nematodes, plant pathogenic fungi and plants. [4,5].

11. Conclusion

RNA interference (RNAi) has established itself as a powerful, sequence-specific, and sustainable technology against crop-protecting pests, fungi, nematode, and virus. In this review, we demonstrated evidence that HIGS, SIGS, MIGS, and orally delivered dsRNA are effective against essential gene silencing and control efficacy on these insect pests or pathogenic microorganisms. Moreover, improvement in RNAi efficiency has been achieved through better protection of the dsRNA stability by using bio-based nanoparticles including bio- Clay, chitosan dsRNA nanocomplexes, clay nanoparticles and biological delivery agents.

Challenges such as environmentally labile, inefficient uptake, variable RNAi sensitivity, unintended gene targeting, non-target risk as well as poor dsRNA production, resistance and regulatory issue should be identified and circumvented if the RNAi for crop protection is to reach the field. To move RNAi from lab toward industrial application, researchers suggest that the efficacy tested in the laboratory should not be the sole benchmark and further validation in different field conditions, large-scale field studies and comparison of efficacy with traditional pesticides, rigorous environmental risk evaluation are needed for commercialization. Future perspectives that could boost the large-scale application of RNAi for crop protection in the field are likely involved development of efficient dsRNA manufacturing processes, new generation nanoparticles or biological delivery vehicles, smarter and better gene selection based on multiples targets as well as the integration of RNAi into integrated pest management programs. Increased cooperation among scientists in the lab and industry and governmental institutions and farmers are required to set up appropriate safety standards and regulation criteria. The emergence of RNAi based crop protection technology into the market by commercialization activities in recent years clearly showed that RNAi has gradually turned from lab studies into actual crop protectant products.

RNAi-based crop protection presents us with the opportunity to combat pervasive use of broad-spectrum chemical pesticides for effective, selective pest and disease control, and for protection of vulnerable and the environment compatible crop production system. Effective delivery technology as well as integrated development of risk assessment, product formulation and scalable production will provide RNAi based systems an essential role in the development of sustainable, resistant and climate friendly agricultural production systems worldwide.

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