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Phenomenal RNA Interference: From Mechanism to Application

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1. Introduction

The phenomenon of dsRNA-mediated interference (RNAi), was first demonstrated in nematodes in 1998 by *Professor Andrew Z. Fire at Stanford University, California, USA* and *Professor Craig C. Mello at University of Massachusetts Medical School in Worcester, USA*. It is thought to have evolved as a type of “genetic immune system” to protect organisms from the presence of foreign or unwanted genetic material. To be more specific, RNAi probably evolved as a mechanism to block the replication of viruses and/or to suppress the movements of transposons within the genome, because both of these potentially dangerous processes typically involve the formation of dsRNA intermediates. Cells can recognize dsRNAs as “undesirable” because such molecules are not produced by the cell’s normal genetic activities.

The RNAi was introduced to the public in mid-2001 and in just about few years it became one of the most widely used technologies in both academic and industrial research environments. In recognition of overwhelming importance of RNAi is an biological process and universally applicable tool, the leading *Journal Science* proclaimed it “The breakthrough of the year, 2002”. During the last decade, our knowledge repertoire of RNA-mediated functions has hugely increased, with the discovery of small non-coding RNAs which play a central part in a process called RNA silencing. Ironically, the very important phenomenon of co-suppression has recently been recognized as a manifestation of RNA interference (RNAi), an endogenous pathway for negative post-transcriptional regulation. RNAi has revolutionized the possibilities for creating custom “knock-downs” of gene activity. RNAi operates in both plants and animals, and uses double stranded (dsRNA) as a trigger that targets homologous mRNAs for degradation or inhibiting its transcription or

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translation (Almedia and Allshire 2005; Cotta-Ramusion et al., 2011) whereby susceptible genes can be silenced. Hence, RNA interference is the newest kid on the genetic block, allows the scientists to selectively turn off genes and finally promises to set the scientific world alight with its therapeutic potential and it has provided new platforms for developing eco-friendly molecular tools for crop improvement by suppressing the genes responsible for various stresses and improving novel traits in plants including disease resistance.

1.1 Landmarks in RNAi discovery

The discovery of RNAi was preceded first by observations of transcriptional inhibition by antisense RNA expressed in transgenic plants and more directly by reports of unexpected outcomes in experiments performed in 1990s. In an attempt to produce more intense purple coloured *Petunias*, researchers introduced additional copies of a transgene encoding chalcone synthase (a key enzyme for flower pigmentation). However, they were surprised at the result that instead of a darker flower, the *Petunias* were either variegated or completely white (Figure 1). They called this phenomenon co-suppression of gene expression (Napoli et al., 1990), since both the expression of the existing gene (the initial purple colour) and the introduced gene /transgene (to deepen the purple) were suppressed. It was subsequently reported by Christine, 2008 that suppression of gene activity could take place at the transcriptional level (transcriptional gene silencing, TGS) or at the posttranscriptional level (posttranscriptional gene silencing, PTGS).

Fig. 1. Upon injection of the transgene responsible for purple colouring in *Petunias*, the flowers became variegated or white rather than deeper purple as was expected.

Similar PTGS like effect “Quelling” (Cogoni and Macino, 1999) was also seen in fungi *Neurospora crassa*. Another RNAi related phenomenon, coat protein mediated protection (CPMP) in plants gave insight into the mechanism of PTGS. In 1995 Guo and Kempheus first studied RNA silencing in animals. They used antisense RNA technique to silence *par1* mRNA expression in *C. elegans* but found that *par1* mRNA itself repressed *par1* gene and concluded that both sense and antisense RNA could cause silencing. Their observation inspired the experiment of Fire, Mello and colleagues. Thus began the journey of the newly dubbed technology RNA Interference. The mystery of molecular mechanism responsible for gene silencing now known as RNA interference (RNAi) exploded in 1998. It was discovered that PTGS was triggered by double-stranded RNA (dsRNA), provided most unexpected explanation with many profound consequences.

Andrew Fire and Craig Mello published their break-through study on the mechanism of RNA interference in 1998, *Nature*. It was earlier known that antisense RNA, but remarkably also sense RNA could silence genes, but the results were inconsistent and the effects usually modest. However, due to the fact that both sense and antisense RNA could cause silencing Mello argued that the mechanism could not just be a pairing of antisense RNA to mRNA, and he coined the term RNA interference for the unknown mechanism. In their *Nature* paper, Fire and Mello tested the phenotypic effect of RNA injected into the worm *C. elegans*. They established that annealed sense/antisense RNA, but neither antisense nor sense RNA alone, caused the predicted phenotype. Furthermore, only injection of double-stranded RNA (dsRNA) led to an efficient loss of the target mRNA. Fire and Mello made the remark that RNAi could provide an explanation for a phenomenon studied in plants for several years: posttranscriptional gene silencing (PTGS). Finally, they ended their paper by speculating about the possibility that “dsRNA could be used by the organism for physiological gene silencing”. This discovery later won Fire and Mello the 2006 Nobel Prize in Physiology or Medicine. Thus it was clear that co-suppression in plants, quelling in fungi and RNAi in nematodes all shared a common mechanism. Further work showed that this effect was even more widespread, occurring in fruitflies and mammals too.

The biochemistry of RNAi was further elucidated in an *in vitro* system, built on *Drosophila* cultured cells, Elbashir et al., 2011 demonstrated that Double-stranded RNA is cut into short interfering RNA (siRNA) by the endonuclease Dicer (Lee et al., 2002). The antisense strand is loaded into the RISC (RNA-induced silencing complex) and links the complex to the endogenous mRNA by base-pairing (Martinez et al., 2002). The RISC complex cuts the mRNA strand, and the mRNA is subsequently degraded. In certain systems, in particular plants, worms and fungi, an RNA dependent RNA polymerase (RdRP) plays an important role in generating and/or amplifying siRNA. Thus, within few years a vast amount of information accumulated on the specific proteins and protein complexes involved in RNAi and molecular machinery involved in RNAi was subsequently revealed (Thakur, 2003).

2. Proteins involved in RNAi/ PTGS/Quelling

To understand the basis of RNA silencing both genetic and biochemical approaches have been undertaken. Genetic screens were carried out to search for mutants defective in quelling, RNAi or PTGS and a large number of genes whose products are somehow implicated in RNA silencing have been identified in *C. elegans*, *D. melanogaster*, *Homo sapiens*,

Dictyostelium discoideum, *N. crassa*, *Chlamydomonas reinhardtii* and *A. thaliana*. The identified genes encode various components some of which identified as initiators while others serve as effectors, amplifiers and transmitters of the gene silencing process. In the years to come, many other components as well as their interrelations will be revealed. Here, we outline what is known so far.

2.1 Dicer

The endonuclease enzyme called Dicer was first discovered in *Drosophila* by Bernstein et al., 2001. It belongs to the RNase III-family that shows specificity for dsRNAs and cleaves them with 3' overhangs of 2 to 3 nucleotides and 5'-phosphate and 3'-hydroxyl termini (Elbashier et al., 2001; Nicholson, 1999). Dicer is involved in the first step of RNA silencing -the production of siRNAs. Owing to its ability to digest dsRNA into uniformly sized small interfering RNAs (siRNA), this enzyme was named Dicer (DCR). Dicer is ATP-dependent and contains four distinct domains an N-terminal helicase domain, a PAZ domain, a 110-amino-acid domain conservative throughout evolution found in Piwi/Argonaute/Zwille proteins in *Drosophila*, *Arabidopsis* and involved in developmental control (Catalanotto et al., 2000; Tabara et al., 1999) dual RNase III domains and a double stranded RNA-binding domain, ruler helix. Evolutionarily conserved Dicer homologues from many different sources were also identified and tested in plants, fungi and mammals (Bernstein et al., 2001; Ketting et al., 2000). Furthermore, some recombinant Dicers have also been examined *in vitro*, and phylogenetic analysis of the known Dicer-like proteins indicates a common ancestry of these proteins (Golden et al., 2002).

2.2 RNA-induced silencing complex (RISC)

During studies on the biochemistry of RNAi several proteins engaged in RISC formation were characterised. After partial purification of crude extracts from *Drosophila* embryolysate and human HeLa cells through differential centrifugation and anion exchange chromatography, the nuclease cofractionated with a discrete ≈ 25 -nucleotide RNA species (siRNAs) are part of an effector nuclease which targets homologous RNAs for degradation (Hammond et al., 2000). This complex is referred to as the RNA-induced silencing complex (RISC). It is made up of a group of proteins which use the siRNA as a guide, presumably identifying the substrate through Watson and Crick base-pairing. The proteins in this complex are members of the Argonaute protein family, which are defined as having a PAZ and PIWI domains. The Argonaute PAZ domain most likely holds the 3' end of siRNA, providing the proper orientation for recognition and cleavage of mRNA. PIWI contains the active site for cleaving the mRNA, shown by the scissors in the schematic (Nykanen et al., 2001). The Argonaute family members have been linked both to the gene-silencing phenomenon and to the control of development in diverse species. The first link between Argonaute protein and RNAi was shown by isolation of *rde1* mutants of *C. elegans* in a screen for RNAi-deficient mutants. Argonaute family members have been shown to be involved in RNAi in *Neurospora crassa* (QDE3) as well as in *A. thaliana* AGO1 (Fagard et al., 2000).

2.3 RNA-dependent RNA polymerase

As a result of screening for genes involved in RNAi a family of proteins that exhibit the activity of RNA-dependent RNA polymerase (RdRP) was also identified (Birchler, 2009).

The identification of the quelling-defective gene *qde-1* in *Neurospora* was the first experimental evidence of the involvement of an RdRP in PTGS. The *C. elegans* nuclear genome also contains four members of this gene family: *ego-1*, *rrf-1*, *rrf-2* and *rrf-3*. Among all three of these *rrf-1* was found as the gene coding for RdRp involved in RNAi (Correa et al., 2010). Therefore, RdRP may also be responsible for the amplification and maintenance of the silencing signal by synthesis of secondary dsRNA trigger molecules, which in turn would be processed into secondary siRNAs. However, no RdRp has been identified by homology in the genomes of either flies or humans.

3. Mechanism of action

RNA interference is a classical mechanism of gene regulation found in eukaryotes as diverse as in yeast and mammals and, probably plays a central role in controlling gene expression, by inhibiting gene expression at the stage of translation or by hindering the transcription of specific genes. The RNAi pathway is initiated by the enzyme dicer, which trims long double stranded RNA, to form small interfering RNA (si RNA) or microRNA (miRNA). These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation. Using a recently developed *Drosophila in vitro* system, molecular mechanism underlying RNAi was examined. It was found that RNAi is ATP dependent yet uncoupled from mRNA translation. RNAi pathway can be divided into three major steps:

3.1 Initiator step: dsRNA cleavage

This is the first step in which, dsRNA is converted into 21-23bp small fragments by the enzyme Dicer. Dicer is the enzyme involved in the initiation of RNAi. It is a member of Rnase III family of dsRNA specific endonuclease that cleaves dsRNA in ATP dependent, processive manner to generate siRNA duplexes of length 21-23 bp with characteristic 2 nucleotide overhang at 3'-OH termini and 5' PO₄.

3.2 Effector step: Entry of si RNA into RISC

The siRNAs generated in the initiator step now join a multinuclease effector complex RISC that mediates unwinding of the siRNA duplex. RISC is a ribonucleoprotein complex and its two signature components are the single-stranded siRNA and Argonaute family protein. The active components of an RISC are endonucleases called argonaute proteins, which cleave the target mRNA strand complementary to their bound siRNA, therefore argonaute contributes "Slicer" activity to RISC. As the fragments produced by dicer are double-stranded, they could each in theory produce a functional siRNA. However, only one of the two strands, which is known as the guide strand, binds the argonaute protein and directs gene silencing. The other anti-guide strand or passenger strand is degraded during RISC activation. The process is actually ATP-independent and performed directly by the protein components of RISC. Although it was first believed that an ATP-dependent helicase separated these two strands, the process is actually ATP-independent and performed directly by the protein components of RISC (Senapedis et al., 2011).

3.3 Step 3: Sequence specific cleavage to targeted mRNA

The active RISC further promotes unwinding of siRNA through an ATP dependent process and the unwound antisense strand guides active RISC to the complementary mRNA. The targeted mRNA is cleaved by RISC at a single site that is defined with regard to where the 5' end of the antisense strand is bound to mRNA target sequence. The RISC cleaves the complementary mRNA in the middle, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA. This cleavage reaction is independent of ATP. The target RNA hydrolysis reaction requires Mg^{2+} ions. Cleavage is catalyzed by the PIWI Domain of a subclass of Argonaute proteins. This domain is a structural homolog of RNase H, a Mg^{2+} dependant endoribonuclease that cleaves the RNA strand of RNA- DNA hybrids. But each cleavage- competent RISC can break only one phosphodiester bond in its RNA target. The siRNA guide delivers RISC to the target region, the target is cleaved, and then siRNA departs intact with the RISC.

Thus the two important conditions to be fulfilled for the success of silencing by RNAi are established as: 5' phoshorylation of the antisense strand and the double helix of the antisense target mRNA duplex to be in the A form. The A-form helix is required for the stabilization of the heteroduplex formation between the siRNA antisense strand and its target mRNA.

Fig. 2. Mechanism of action of RNAi. Double stranded RNA is introduced into a cell is chopped by dicer to form siRNA, which binds to the RISC complex and is unwound. The antisense RNA complexed with RISC binds to its corresponding mRNA which is then cleaved by the enzyme slicer rendering it inactive (Christine, 2008).

4. Other forms of RNA interference

In addition to naturally occurring and manufactured siRNAs (Sigoillot and King, 2011), there have been recent publications of alternative forms of RNA, these are

4.1 Micro (mi)–RNAs

These are an abundant class of short (19–25 nt) single-stranded RNAs that are expressed in all higher eukaryotes. They are encoded in the host genome and are processed by RNase III nuclease Dicer from 70nt hairpin precursors). They can silence gene activity through destruction of homologous mRNA in plants or blocking its translation in plants and animals (Cullen, 2004; Novina and Sharp 2004). Recent work has identified their specific roles in the regulation of early haematopoiesis and lineage commitment). They are sometimes referred to as small temporal RNAs as a reflection of their importance in the regulation of developmental timing (Chen et al., 2004; Medema et al., 2004).

4.2 Piwi-interacting (pi) RNAs

These are single-stranded 25–31 nt RNAs which have recently been detected in mouse, rat, and human testes. They have been shown to associate with Piwi protein (a subclass of Argonaute proteins) and the human RecQ1 protein to form a Piwi-interacting RNA complex (piRC). These complexes are thought to regulate the genome within developing sperm cells (Carthew, 2006).

4.3 Short-Hairpin (sh) RNAs

Short hairpin RNA or shRNA or is a synthetically manufactured RNA molecule of 19–29 nucleotides that contains a sense strand, antisense strand, and a short loop sequence between the sense and antisense fragments. Due to the complementarity of the sense and antisense fragments in their sequence, such RNA molecules tend to form hairpin-shaped double-stranded RNA (dsRNA). shRNA is cloned into a vector, allowing for expression by a pol III type promoter. The expressed shRNA is then exported into the cytoplasm where it is processed by dicer into siRNA which then get incorporated into the siRNA induced silencing complex (RISC)(Medema, 2004).

4.4 Small modulatory (sm)RNAs

These are short, double-stranded RNAs which are found in the nucleus of neural stem cells of mice. They play a critical role in mediating neuronal differentiation through dsRNA/protein interaction (Kuwabara et al., 2004).

5. RNAi application

RNA interference occurs in plants, animals, and humans. It is of great importance for the regulation of gene expression, participates in defense against viral infections, and keeps jumping genes under control. Presently RNA interference has become attractive tool for various researchers and widely used in basic science as a method to study the function of genes and it may lead to novel therapies in the future.

Over the past decade “RNA interference” has emerged as a natural antiviral mechanism for protecting organisms from viruses. It blocks infection by RNA viruses especially in plants and lower animals. For instance, replication of plant viruses, many of which produce dsRNA replication intermediates, very effectively cause a type of RNA silencing called VIGS (Virus induced gene silencing). When viruses or transgenes are introduced into plants, they trigger a post transcriptional gene silencing response in which double stranded RNA molecules, which may be generated by replicative intermediates of viral RNAs or by aberrant transgene coded RNAs. The dsRNAs are then digested into 21-25 nt small interfering RNAs or siRNAs. The siRNAs subsequently assemble into a nuclease complex called RISC, guiding the complex to bind and destroy homologous transcripts. PTGS is believed to be an anti-viral response. Viral RNAs not only trigger PTGS, but they also serve as targets. Cleavages of viral RNA results in reduce virus titers in local and distant leaves and a plant recovery phenotype. In response numerous plant viruses have evolved proteins to suppress PTGS (Elbashir et al., 2001). The results by Silhavy and his colleagues suggest that tombavirus p19 protein suppresses local PTGS by binding to 21-25nt siRNAs. Therefore, siRNAs and not the longer dsRNAs, act as mobile silencing element since p19 can inhibit systemic silencing and p19 can only bind to siRNAs (Sui et al., 2002).

RNA interference besides being working as a genetic immune system against virus, it holds a promising key for maintaining the genome stability by suppressing the movement of mobile genetic elements such as transposons. Jumping genes, also known as transposons, are DNA sequences that can move around in the genome. They are present in all organisms and can cause deleterious effects in the genome if they end up in the wrong place. Many transposons operate by copying their DNA to RNA, which is then reverse-transcribed back to DNA and inserted at another site in the genome. Part of this RNA molecule is often double-stranded and can be targeted by RNA interference. . Thus in this way, foreign elements in the genome (viruses and transposons) can be kept silent.

Moreover, RNAi can be used greatly in both the mammal both “forward genetic” experiments (identifying the gene responsible for a given phenotype) and “reverse genetic” experiments (identifying the function of a known gene). From an application point of view, RNAi may also be useful as a therapy for diseases arising from aberrant gene expression. Typical reverse genetic experiments involve designing siRNAs (chemically or enzymatically synthesized) expressing constructs targeting a gene of interest. Following transient transfection of siRNAs, the phenotype of the cells is assessed using appropriate functional assays.

5.1 Functional genomics

The technology considerably bolsters functional genomics to aid in the identification of novel genes involved in disease processes. Genome sequencing projects have generated wealth of information regarding gene sequences but still clarity on functional role of all genes is missing. The use of small interfering RNA (siRNA) to knock down/ knockout expression of specific gene have opened up exciting possibilities in the study of functional genomics. The ability to easily and economically silence genes promises to elucidate numerous signaling, developmental, metabolic, and related disease pathways. Various

studies have been undertaken to elucidate the role of specific genes in basic cellular processes like DNA damage response and cell cycle control (Brummelkamp et al., 2002), general cell metabolism, signaling, the cytoskeleton and its rearrangement during mitosis, membrane trafficking, transcription and, DNA methylation.

Heterochromatin is composed of highly repetitive sequences interspersed with transposons and is non-coding. The condensation pattern is determined by both DNA and histone modification. Recently it has been found to produce RNAi which appear to be the key factor in epigenetic regulation of gene expression, chromosome behaviour and evolution. It may be the mechanism underlying genome imprinting whereby chromosomal condensation pattern is determined by parent-of-origin. Even the phenomenon of hybrid dysgenesis may be explained if siRNA pools that are largely maternal, do not match polymorphic repeats from the paternal chromosome it may result in mobilisation of transposons and consequent chromosomal disruption.

RNAi has been adapted with high-throughput screening formats in *C. elegans*, for which the recombination-based gene knockout technique was not established. Recently, a large-scale functional analysis of 19,427 predicted genes of *C. elegans*, was carried out with RNA interference. This study identified mutant phenotypes for 1,722 genes. RNAi technology has been similarly used in the identification of several genes in *D. melanogaster* involved in biochemical signaling cascade as well as embryonic development.

In plants, gene knockdown-related functional studies are being carried out efficiently with transgenes present in the form of hairpin (or RNAi) constructs. Plant endotoxins could be removed if the toxin biosynthesis genes are knocked out. SiRNA results in partial knockout, which is an advantage over complete knockout in that it helps in investigating the effect of various phenotypes. Thus the method holds a great potential to become the most commonly used technique for gene annotation in the near future.

5.2 Genetic improvement of crop plants

Prior to the discovery of RNAi, scientists applied various methods such as insertion of T-DNA elements, transposons, treatment with mutagens or irradiation. These approaches are very cumbersome and the above methods did not always work adequately. For instance, transposons and T-DNA elements were found to occasionally insert randomly in the genome resulting in highly variable gene expression. Furthermore, in many instances the particular phenotype or a trait could not be correlated with the function of a gene of interest. At the same time to improve crop plants transgenes are mainly introduced into the genomes of most model plant species using *Agrobacterium tumefaciens*, a common soil bacterium, and the mechanism of which relies on T-DNA (transfer DNA), that is carried on a resident plasmid. Single T-DNAs can integrate into the genome, but it is very common for multiple copies to integrate in variously permuted head-to-head, tail-to-tail and head-to tail arrays. As we all know till now the most effective genetic approach to pest control has been to make plants that produce a protein called Bt toxin, which causes insects to slow down, then stop eating crops, then die. More than 120,000 square miles of crops genetically engineered to produce Bt were grown last year. But Bt isn't effective against many pests, including corn rootworm, which can cause such extensive damage to corn plants' root systems that the plants blow over in the wind and researchers are concerned that insect pests are becoming

resistant to Bt. Here RNAi play a vital role. Now a day, researchers are trying to create plants that kill insects by disrupting their gene expression. The crops, which initiate a gene-silencing response called RNA interference, are a step beyond existing genetically modified crops that produce toxic proteins. Because the new crops target particular genes in particular insects, some researchers suggest that they will be safer and less likely to have unintended effects than other genetically modified plants. Moreover, the quality of crop plants can be improved by RNAi for example Kusaba M 2004 have made significant contribution by applying RNAi to improve rice plants. They were able to reduce the level of glutenin and produced a rice variety called *LGC-1* (low glutenin content 1). The rice mutant line LGC-1 (Low Glutenin Content-1) was the first commercially useful cultivar produced by RNAi. It is low-protein rice and is useful for patients with kidney disease whose protein intake is restricted. This dominant mutation produces hairpin RNA (hpRNA) from an inverted repeat for glutenin, the gene for the major storage protein glutenin, leading to lower glutenin content in the rice through RNAi. Rice down regulation can also be achieved through mutation-based reverse genetics and a gene targeting system (Terada et al., 2002; Shinozuka et al., 2003). However, RNAi has some advantages over these systems. One of these is its applicability to multigene families and polyploidy (Lawrence and Pikaard 2003), as it is not straightforward to knockout a multigene family by the accumulation of mutations for each member of the family by conventional breeding, particularly if members of the family are tightly linked. Another advantage of RNAi lies in the ability to regulate the degree of suppression. Agronomic traits are often quantitative, and a particular degree of suppression of target genes may be required. Control of the level of expression of dsRNA through the choice of promoters with various strengths is thought to be useful in regulating the degree of suppression. However, for wider application of transgene- based RNAi to the genetic improvement of crop plants further feasibility studies are needed.

Engineering of food plants that produce lower levels of natural plant toxins also possible through RNAi. Such techniques take advantage of the stable and heritable RNAi phenotype in plant stocks. For example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption. RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pest (Kumar et al., 2006). Similar efforts have been directed toward the reduction of the cyanogenic natural product linamarin in cassava plant (Siitunga and Sayre 2003). Although no plant products that use RNAi-based genetic engineering have yet passed the experimental stage, development efforts have successfully reduced the levels of allergens in tomato plants and decreased the precursors of likely carcinogens in tobacco plant (Gavilano et al., 2006). Other plant traits that have been engineered in the laboratory include the production of non-narcotic natural products by the opium poppy (Allen et al., 2004) resistance to common plant viruses (Zadeh and Foster 2004) and fortification of plants such as tomatoes with dietary antioxidants (Niggeweg et al., 2004). In plants, gene knockdown-related functional studies are being carried out efficiently when transgenes are present in the form of hairpin (or RNAi) constructs. Plant endotoxins could also be removed if the toxin biosynthesis genes are targeted with the RNAi constructs. Therefore, RNAi soon caught the world-wide attention and became a powerful and useful tool for molecular breeders to produce improved crop varieties.

5.3 RNA interference as a novel therapeutic agent

The ability to tap this native RNAi pathway has been recognized as one the most exciting biotechnology advances in the last decade. Given the gene-specific features of RNAi, it is conceivable that this method will play an important role in therapeutic applications and possibly of most commercial interest in the use of RNAi as a therapeutic agent. Indeed, RNAi has revolutionized biology research, including drug target discovery, by allowing for rapid identification and validation of gene function. There are three main time points at which a disease can be stopped. These are transcriptional, post-transcriptional, and post-translational intervention. Before the discovery of antisense RNA and RNAi, most of the drug targets have been proteins, and therefore, post-translational intervention. RNAi is a way to control the development of a disease earlier on in the process. Furthermore, the gene-specific features and potential of RNAi for knocking out a protein without harming a cell has established its most believable role into therapeutic applications. The inhibitory action of siRNAs has been documented for numerous diseases. Some of the examples are highlighted below:

5.3.1 Cancer protection

Gregory Hannon and colleagues have used RNAi to silence expression of p53 — the 'guardian of the genome', which protects against any tumour-associated DNA damage — by introducing several p53-targeting shRNAs into stem cells and looking at the effect in mice (Hemann et al., 2003). The shRNAs produced a wide range of clinical effects, ranging from benign to malignant tumours, the severity and type of which correlated with the extent to which the shRNA had silenced p53. As tumour suppressors such as p53 usually work as part of a complex and finely regulated network, the ability to dampen these networks to varying degrees in these libraries— which the authors term an epi-allelic series of hypomorphic mutations —will be of enormous value when it comes to investigating the early stages of disease. The success of these modified stem cells also gives hope that this could treat diseases in which stem cells can be modified *ex vivo* and then re-introduced into the affected individual.

Researchers at the charity Cancer Research UK and the Netherlands Cancer Institute have recently announced that they intend to generate a large library of human cells, each containing a silenced gene. They initially want to silence 300–8,000 cancer genes, and hope to eventually cover the entire human genome. Their aim is to uncover all the genes that become overexpressed in human cancers and to find out precisely what needs to be taken away from a cancerous cell in order to make it normal again (Hoffman et al., 2010).

5.3.2 HIV protection

HIV infection can be blocked by targeting either viral genes (for example, *gag*, *rev*, *tat* and *env*) or human genes (for example, *CD4*, the principal receptor for HIV) that are involved in the HIV life cycle. The strategy used was to silence the main structural protein in the virus, p24, and the human protein *CD4*, which the virus needs to enter the cells. This impairs the virus in infected cells and limits its spread into healthy cells (Paddison et al., 2002). Hence the production of virus is inhibited either by blocking new infections or blocking the production of new viral particles in infected cells (Lohman et

al., 1999). The concept of silencing genes in HIV is straightforward: Hit the virus where it counts by eliminating a protein it needs to reproduce or cause infection. siRNA molecules (shorter than 30 base pairs) are added to the cells, where the cell recognizes and degrades mRNA corresponding to the target sequence. As a result little or no protein is produced.

What makes RNAi so exciting to the researchers is its potential for knocking out a protein without harming a cell. By comparison chemotherapy kills tumors by destroying cancerous as well as healthy cells. RNAi strategy includes multiple targets to kill HIV. These could be the targets that block entry into the cells and disrupts the virus life cycle inside the cells. This technology will help researchers dissect the biology of HIV infection and design drugs based on the information. Researchers at City of Hope Cancer Centre in Duarte have developed a DNA-based delivery system in which human cells are generated that produce siRNA against REV protein, which is important in causing human disease (Yu et al., 2002).

5.3.3 Hepatitis protection

This has provided the first tangible evidence for RNAi as a therapy for diseases in live animals. Early RNAi studies noted that RNA silencing was prominent in the liver, which made this organ an attractive target for therapeutic approaches. Many immune-related liver diseases are characterized by apoptosis, which is mediated by a protein called Fas. So Judy Lieberman's group injected siRNA targeting Fas intravenously into two models of autoimmune hepatitis in mice. This decreased Fas mRNA and protein levels in hepatocytes and protected the cells against liver injury from apoptosis, even when siRNA was administered after the induction of injury. Extending these findings to other liver diseases looks hopeful, but the authors concluded that other strategies, such as viral vectors, might be required to target organs in which RNA silencing is less effective than in the liver.

6. Conclusion and future outlook of RNAi

The field of RNAi is moving at an impressive pace, generating exciting results and has established a novel archetype with far-reaching consequences in the field of transcription regulation. The RNA silencing has practical use because of the ability to reduce gene expression in a manner that is highly sequence specific as well as technologically facile, economical and having potential in finding out the function of genes at a faster speed and in agriculture specifically for nutritional improvement of plants and the management of mascotous plant diseases. In addition it has kindled hope for the treatment of several diseases, which have bothered mankind as untreatable by providing an innovative technology for development of therapeutics. However, the major obstacles hindering its immediate applications include selection of targeting sequences and in the delivery of siRNA. The key issues are 1) how to select silencing targets for a particular disease and 2) how to efficiently deliver siRNAs into specific cell types *in vivo*? Besides, RNAi technology can be considered an eco-friendly, biosafe ever green technology as it eliminates even certain risks associated with development of transgenic and it has already added new

dimensions in the various field of science. However, a better and comprehensive understanding of RNAi should allow future researchers to work effectively and efficiently in order to manage the phenomenon.

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This book provides us a thorough overview of Crop Plant with current advance in research. Divided into two section based on the chapters contents. Chapter 1 provides information about markers and next generation sequencing technology and its use. Chapter 2 is about how we can use Silicon for Drought tolerance. Chapter 3 is to deal with the major problem of rising CO₂ and O₃ causing environmental pollution. Chapter 4 covers the phenomena of RNAi and its use, application in crop science. Chapter 5 is a review for boron deficiency in soils and how to deal with it for better crops. Chapter 6-10 provide some information regarding recent works going on in crop science.

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