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RNA Interference and its Application in Crop Improvement

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Abstract

The discovery of RNA interference (RNAi) in mid-ninety's added a new dimension in the regulation of gene expression by different types of RNA. It soon caught the worldwide attention and a number of reviews have been published to describe the RNAi phenomenon both in plants and animals. The technology became a powerful tool to understand the functions of individual genes and also proved useful for molecular breeders to produce improved crop varieties. This review article summarizes the historical background of RNAi, describes the role of different classes of RNA molecules, particularly of ds (double stranded) RNAs, hp (hairpin) RNAs, siRNAs and mi (micro) RNAs and two important polymerase III enzymes, namely, Dicer and RISC (RNA-induced Silencing Complex) that help RNAi's carry out its function through a unique process. The article gives a few plant examples in which this technology has been successfully used to produce improved crop varieties and to analyze gene function. Finally, pointing out that this proven technology is freely available to research scientists, the article advocates that more and more laboratories both in developed and developing countries use this powerful tool to understand the functions of plant genes and improve crop production.

Introduction

Rightfully christened 2002's "Technology of the year" by Science (Couzin 2002), and Fortune Magazine's "Billion Dollar Breakthrough" (Stipp 2003) in 2003, RNA interference (RNAi), as the name implies, suppresses gene expression by degrading specific messenger RNAs (mRNA). Introduction of a piece of double stranded RNA (dsRNA) into the cytosol initiates the phenomenon of RNAi, in turn activating a pathway culminating in the degradation of the targeted gene

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transcript (Agrawal et al. 2003; Kuznetsov 2003; Arenz and Schepers 2003). In addition to RNA degradation upon activation of the RNAi pathway, there are also cases where the promoter region of the gene is silenced through methylation (Mette et al. 2000; Wassenget et al. 1994). Initially, the progress of research in this field was slow. In the early 1990's, scientists reported for the first time that the expression of a gene of interest could be suppressed by means of RNA-mediated antisense silencing. This discovery stimulated a great deal of interest in the area of deciphering gene functions at various laboratories around the world. This early research was mainly confined to plants and fungus until Fire et al. (1998) reported RNA interference in *C. elegans.* The study of RNAi has become increasingly more rewarding and continually expanding following the completion of several recent genome sequencing projects such as human and *Arabidopsis* projects.

More recently, research in this field has been directed to several other areas including micro RNA's (Bartel 2004; Pasquinelli 2002), promoter methylation (Matzke et al. 2004; Jones et al. 1998), and hairpin RNA (Smith et al. 2000; Wesley et al. 2001). Simultaneously, results obtained in these areas have found practical applications in crop improvements such as in the production of potato virus Y (PVY) resistant potatoes (Smith et al. 2000). These results and applications illustrate RNAi's potential to bioengineer horticultural and cash crops. The ability for RNAi in addressing the specific needs of plant breeders has increased due to the ease with which this tool can be used towards the realization of this objective. A number of papers including reviews (Agrawal et al. 2003; Kuznetsov 2003; Arenz Schepers 2003 and Kusaba 2004) have been published recently in this field. The intention of this review article is to present information scattered through the literature to help molecular breeders use this powerful tool to produce transgenic crops with more success than before. Other important areas of RNAi will also be discussed with special reference to its applicability for production of improved varieties of cereal-, fruit-, cash- and vegetable crops.

RNAi: The History and Overview

Prior to the discovery of RNAi, scientists applied various methods such as insertion of T-DNA elements, and transposons, treatment with mutagens or irradiation and antisense RNA suppression to generate loss-of-function mutations. These approaches allowed scientists to study the functions of a gene or gene family of interest in an organism. Apart from being time-consuming, the above methods did not always work satisfactorily. 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. It is in this backdrop that the RNAi phenomenon was discovered.

Eventually leading to the discovery of RNAi, antisense RNA suppression was an early form of RNA silencing employed mainly by plant scientists. This process involved the introduction of the antisense strand of RNA into the cell that corresponded to the target mRNA, the transcript intended to silence (Brantl 2002; Knee and Murphy 1997). After entry into the cell, the introduced antisense RNA and the native target mRNA would bind via complementary base pairing preventing the translation of mRNA. This is due to the inability of ribosomes to bind to dsRNA (Arenz and Schepers 2003; Brantl 2002). This process, however, did not always result in a loss of function of a targeted gene. This led concerned scientists to continue the search for other methods of gene silencing. Fire et al. (1998) took the antisense silencing approach a step further, in *C. elegan*s, with simultaneous introduction of both the sense and antisense strands of the targeted mRNA resulting in a ten-fold higher potency at silencing the gene than treatment with the antisense or sense strand alone. By injecting the two strands (sense and antisense) simultaneously the scientists were, in fact, creating the double stranded RNA required for starting the RNA interference pathway. This foundational experiment prompted many more scientists into looking at the complex process of RNAi in more detail. They studied several forms of RNA as well as two highly conserved enzymes, (a) Dicer in animals and Dicer-like elements in plants; (b) RISC (RNA-induced Silencing Complex) that are involved in RNAi. Their studies shed enough light for the understanding of the RNAi phenomenon, including unraveling the mystery of individual gene function. Such an understanding prompted molecular breeders to use the RNAi technology as a powerful tool in the production of crop plants with specific traits, particularly in situations where existing methods failed.

Different classes of RNAs

A brief description of different classes of RNAs seems appropriate before the topic of RNAi is discussed in detail. The most abundant type of RNA inside a cell is ribosomal RNA (rRNA) followed by transfer RNAs (tRNAs) and messenger RNAs (mRNAs). In addition, there are hairpin RNAs (hpRNAs), double stranded RNAs (dsRNAs), small interfering RNAs (siRNAs) and micro RNAs (miRNAs). Only the last four classes of RNA, which take active roles in RNAi, will be described here in some detail.

The first of the classes of RNA taking an active role in RNAi is dsRNA, which is formed by complementary base pairing of two single-stranded fragments of RNA (Agrawal et al. 2003). Found naturally in the cell, long dsRNAs generally derive from such events as transposition of transposable elements (Schramke and Allshire 2004) or virus induction (Rovere et al. 2002; Marathe et al. 2000). With the production of dsRNA, the RNAi process is initiated (Fig. 1).

As previously stated, the long dsRNA is cut into smaller fragments known as siRNA (Fig. 1 B). The term, small interfering was coined due to their use as a targeting sequence, by RISC, aimed at mRNA for degradation. First isolated by Hamilton and Baulcombe (1999), the siRNAs are composed of 21-25 base pairs (bp) with a 3′ two-nucleotide (nt) overhang. In addition to the 3′ nt overhang, they also have 5′ phosphate and 3′ hydroxyl groups. Lipardi et al. (2001) found that the 3′ hydroxyl group is required in order to direct RNAi *in vitr*o. While Dicer may incorporate siRNAs into RISC following their synthesis, they do not require this event to occur *in viv*o. Instead, siRNAs constructed *in vitro* can be introduced to silence the intended gene transiently.

In addition to dsRNA and siRNA, there still remain two more participating classes that deserve mentioning. The hpRNA is simply another form of dsRNA derived from a long piece of single stranded RNA containing inverted repeats and a hairpin lop connecting them (Wesley et al. 2001). The miRNA, closely resembles siRNA (Bartel 2004; Nelson et al. 2003). Both are short (generally in the range of 25 bp) and are used as targeting sequences aimed at degrading a specific mRNA. The miRNAs are endogenous and appear to represent a novel way of regulating gene expression during development. Both of these classes of RNA will be discussed in more detail later.

Dicer and RISC

The dsRNA and siRNAs alone cannot degrade mRNA, but require the assistance of two enzymes namely, Dicer and RISC. Dicer, which was first discovered by Bernstein et al. (2001) in *Drosophil*a, is a complex enzyme belonging to the RNase III family. A closer look at the enzyme reveals that it has four different domains each with a very specific task. They are: a) an N-terminal helicase, b) dual RNase III motifs, c) C-terminal dsRNA binding domain, d) PAZ (Piwi/Argonaute/Zwille) domain (Agrawal et al. 2003; Kuznetsov 2003; Arenz and Schepers 2003). The PAZ domain is believed to physically interact with the corresponding PAZ domain of the RISC complex. However, further work is necessary to verify this hypothesis. The dual RNase III motifs perform the actual cutting of the dsRNA, hence the characteristic 5′ phosphate

and 3′ hydroxyl residues on the resulting siRNAs. Experiments involving human Dicer enzymes showed that the cutting mechanism of the enzyme is ATP-independent (Kuznetsov 2003), however, there may be involvement of some ATPase activity during the releasing of siRNA from the enzyme. The helicase domain is also believed to take part in the process.

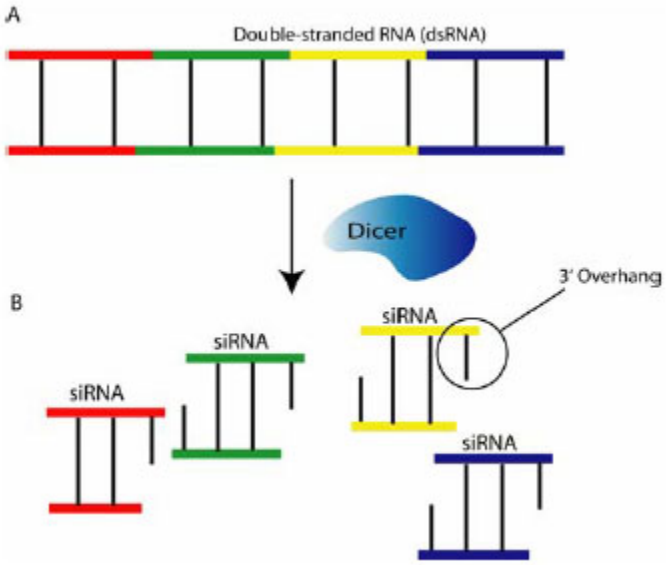

Fig. 1. (A) A visual representation of a fragment of long dsRNA. Colored fragments represent siRNAs prior to cutting by Dicer. (B) A visual representation of siRNAs after Dicer has excised them from dsRNA. Colored fragments represent their position in the dsRNA.

RISC is the component of the RNAi machinery that uses siRNAs to track down and degrade the complementary mRNAs. First discovered in *Drosophil*a, by Hammond et al. (2000), RISC consists of both protein and RNA. The protein component of the complex has ribonuclease activity with the ability to cut RNA. In addition to the ribonuclease activity RISC also contains a PAZ domain. Additional RISC components include two RNA binding proteins, Vasa intronic gene and dFMR proteins (Agrawal et al. 2003; Kuznetsov 2003; Arenz and Schepers 2003). There are still other components of RISC yet to be identified. For example, it remains unclear as to how the siRNAs become incorporated into RISC, as well as how the siRNA operates within the complex. The general consensus among scientists in the field is that there is protein-protein interaction between Dicer and RISC through the PAZ domain, enabling small single stranded 19-29 nucleotides long RNA fragments to enter the RNAi pathway. This phenomenon poses two questions: (1) Does Dicer first pick up the

small single stranded fragment of RNA or does RISC recruit it into the complex itself and (2) How does the siRNA work within the complex? Researchers found only the answer to the second question. They obtained partial evidence to suggest that RISC degrades the sense strand and only uses the antisense strand of the siRNA. RISC utilizes the siRNA and searches for the complementary base sequence of the targeted mRNA. The degradation process is initiated once successful locating and cutting of the complementary mRNA occurs, by the siRNA-RISC complex, thus exposing the freshly cut mRNA to exonucleases (Fig. 2).

RNAi : How Does It Work ?

The RNAi process engages the participation of several pathways. Two of the premier pathways involved in the process are RNA introduction (Waterhouse and Helliwell 2002) and mRNA degradation (Fig. 2). In addition to the above, an unclear amplification pathway exists.

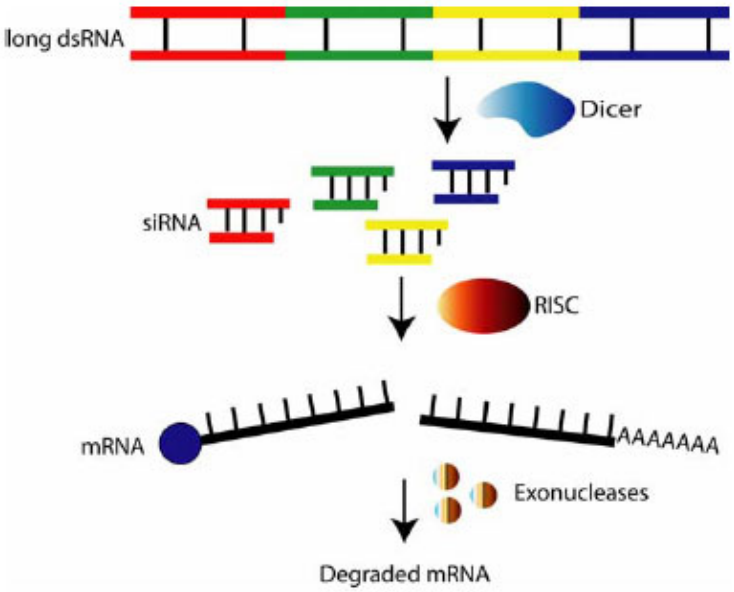

Fig. 2. Dicer cuts Long dsRNA in cut into smaller fragments, called siRNA. The siRNAs are then incorporated into RISC. The siRNA-RISC complex then targets a sequence, complementary to the siRNA, in a piece of mRNA. The mRNA is cut by RISC exposing it to cellular endonucleases that eventually degrade the mRNA.

Insertion of double-standed or small-interfering RNA into a cell can be accomplished in several different ways, such as by bombardment, *Agrobacterium*-, viral mediated dsRNA transfer or by infiltration (Sijen and

Kooter 2000). Most of these methods utilize an RNAi vector to produce stable or transient dsRNA *in vivo*. In other words the plant in study must be transformed with a vector that produces dsRNA, using one of the above techniques. Unnamalai et al. (2004), on the other hand, have recently devised a way of introducing dsRNA without transforming the plant with an RNAi vector. They used cationic oligopeptides for delivering dsRNA into plant cells. Specifically, the scientists introduced dsRNA into tobacco cells using POA, which is a cationic oligopeptide 12-mer. Their results showed efficient silencing of the target genes.

The path to mRNA degradation begins when a piece of dsRNA is introduced into the cytosol resulting in the recruitment of Dicer. This recruitment initiates chopping of the long dsRNA into a number of smaller double-stranded fragments (Fig. 1). These smaller pieces, generally in the range of 21-25 base pairs with 3' two-nucleotide overhangs attached to 5′ and 3′ ends, are siRNAs as mentioned earlier. Soon after their formation, the siRNAs are incorporated into RISC via an undetermined pathway, initiating the process of mRNA degradation (Agrawal et al. 2003; Kuznetsov 2003; Arenz and Schepers 2003). Researchers currently believe that a physical interaction occurs between Dicer and RISC through a common PAZ domain. Thereafter, RISC utilizes the siRNA as a targeting sequence seeking the complementary mRNA. Successful docking of the RISC-siRNA complex at the targeted mRNA site initiates the degradation process. The mRNA degradation is completed by the action of cellular exonucleases.

A third, not yet fully understood, pathway seems to enhance effectiveness of RNAi, through the amplification of siRNAs. Current consensus on the issue is that the siRNAs undergo amplification by an RdRP (RNA-dependant RNA polymerase). The site of siRNA amplification is yet to be determined. Lipardi et al. (2001) reported that siRNA might be involved in the synthesis of long dsRNA. Further studies into RdRP-mediated amplification revealed the presence of secondary siRNAs (Sijen et al. 2001). Secondary RNAs, not detectable in the introduced dsRNAs are derived from siRNAs that complement the targeted mRNA. These secondary RNAs actively participate in the degradation of the complementary mRNA.

Hairpin RNA and Micro RNA

RNAi also utilizes two other unique forms of RNA: hpRNA (Fig. 3) and miRNA. Hairpin RNA (hpRNA) is formed as a result of the folding back of two closely positioned complementary sequences from a single-stranded RNA molecule (Wesley et al. 2001, Smith et al. 2000). This folding brings the two complementary sequences together where they will hybridize. hpRNA can be

accomplished through sequences of inverted repeats where a normal sequence reads in the 5′-3′ direction followed by the same sequence read in the 3′-5′ direction. t-RNAs are examples of naturally occurring hairpin RNAs originating from genes encoding respective t-RNAs. A slight problem with hpRNA, however, is its lack of stability.

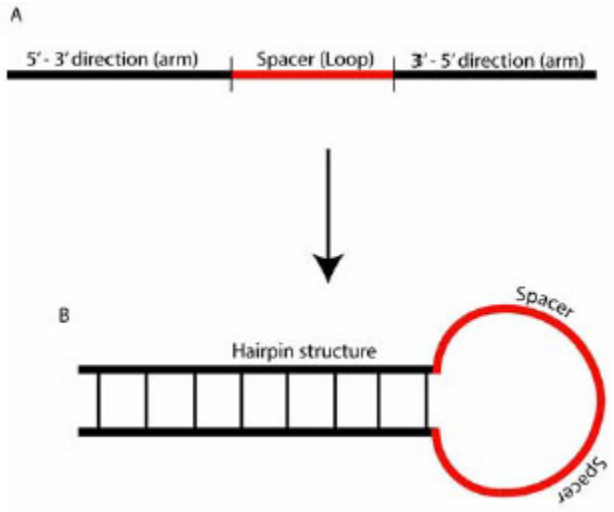

Fig. 3. (A) A diagram of hpRNA prior to folding into the characteristic hairpin structure. It has two sequences in inverted repeat orientation with a spacer in between. The two sequences compose the arms and the spacer composes the loop. (B) The unfolded sequence in (A) folded into a hairpin structure.

Stabilization of hpRNA can be accomplished through the introduction of a spacer sequence located between the two inverted repeat sequences (Wesley et al. 2001, Smith et al. 2000). The spacer sequence can be composed of any sequence, not complementary to the inverted repeats, and creates the loop structure of the hairpin. Recent studies illustrate that a much higher rate of silencing may be achieved, if the constructed spacer contains an established active intron sequence (Wesley et al. 2001). The use of an intron sequence as a spacer has been termed intron-hairpin RNA (ihpRNA). The reason for this added stability and silencing may be due to the alignment of the two arms (inverted sequences) of the hpRNA during its docking at the splicing machinery. In other words, the splicing machinery creates a clean and perfectly aligned dsRNA sequence that exits the nucleus and initiates RNAi in the cytosol.

The miRNAs (Fig. 4), while not universally required for RNAi, are strikingly similar to siRNAs in many respects: (a) they originate from double stranded structure (b) they are more or less of the same size consisting of approximately 20-30 base pairs (c) both are processed by Dicer or Dicer-like enzyme (DCL) (Hutvagner et al. 2001), (d) RISC uses both as targeting sequences and (e) they play a vital role in the RNAi process by directing PTGS (Bartel 2004; Nelson et al. 2003). They differ from each other in their origin. While miRNA is derived from genomic DNA, siRNA is generated as a result of chopping long dsRNA into smaller segments. Active miRNA, produced endogenously from native DNA, has two phases: primary-miRNA (pri-miRNA) and pre-miRNA. Occurring first, pri-miRNA is transcribed from a chromosomal sequence, which is then processed by Dicer into pre-miRNA. Both pri- and pre-miRNA are characterized by a hairpin structure (Bartel 2004; Nelson et al. 2003; Tijsterman and Plasterk 2004).

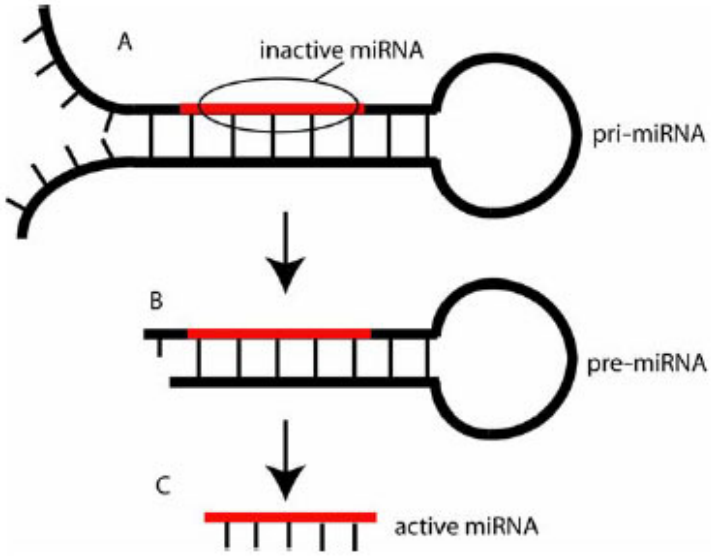

Fig. 4. (A) A diagram of unprocessed pri-miRNA. The red fragment represents the active miRNA that will be excised. (B) Processed pri-miRNA, which is now called pre-miRNA. Again the red fragment represents the active miRNA. (C) Active miRNA that has been excised from pre-miRNA by Dicer.

Processing of miRNAs occurs both at the nuclear, as well as cytoplasmic levels. There is, however, a slight difference in the process between plants and metazoans. For instance, in plants the processing of the pre-miRNA happens inside the nucleus, while in metazoans the processing pre-miRNA occurs in the cytosol (Bartel 2004). Once a miRNA gene is transcribed, the transcript will

form a roughly 40-60 bp long hairpin structure, with two arms of approximately the same length. One of these arms produces the active miRNA via Dicer (Nelson et al. 2003).

The discovery of miRNAs led scientists to reflect back at some previously established genes closely resembling miRNAs. Two of these genes, let-7 (Reinhart et al. 2000) and lin-4 (Lee et al. 1993), regulating timing of different developmental stages in *C. elegans* were known for some time (Pasquinelli 2002). Since the expression of these two genes was time-related, i.e., associated with different development stages, they were termed small temporal RNAs (stRNAs) (Reinhart et al. 2000; Hutvagner and Zamore 2002). After the discovery of micro-RNAs, it was realized that these two genes, both in their structure and function, share characteristic features with over 100 recently discovered micro-RNAs reported in *Drosophila,* humans and worms (Pasquinelli 2002; Hutvagner and Zamore 2002). Other than size, one of the common features that characterize the two classes of RNA is the presence of a loop flanked by two slightly unequal arms, one containing the base sequence of the micro-RNA. This striking similarity led the researchers to conclude that the two genes, namely, let-7 and lin-4 represent micro-RNAs.

Several miRNAs in plants act as negative regulators on systems such as determination of meristem cell identity and organ polarity. This is best illustrated in the differentiation of an *Arabidopsis* dorsiventral leaf. Three mi-RNAs, namely, miR162, miR165/166 are involved in this process (Carrington and Ambros 2003). When miR162 is formed from its precursor, pre-miR162, by DCL, it inactivates DCL1 (Dicer-like1) mRNA. In other words, as DCL generates active miR162, the active miR162 blocks DCL production creating a negative feedback loop (Xie et al. 2003). Conversely, in the absence of miRNA162, DCL acts on miR165/166 precursor generating the active miRNAs. These active miRNAs in turn block translation of two transcription factor genes, PHV/PHB. When no miR165/166 is present in the cytosol of the leaf primordial cells, the upper surface (adaxial) is developed. On the other hand, their presence turns off the two transcription factor genes, leading to the development of the lower (abaxial) surface of the leaf. miR165/166 is normally found at positions distant from the meristem. The study of mutant *phv* and *phb* genes, as have been reported by Carrington and Ambros (2003), support the conclusion that *Arabidopsis* leaf development is regulated by a negative feedback loop. In wildtype leaf primordial cells, signals are normally transmitted to the abaxial leaf surface, while in mutants signals are redirected to the adaxial leaf surface

converting the abaxial- into the adaxial surface. All the characteristic features of the adaxial surface are present on the abaxial side of the mutant leaves.

In addition to RNAi-like degradation of gene transcripts miRNAs are also active in translational repression. Here, silencing of the gene transcript does not occur immediately following transcription, but rather during translation. Once the ribosome has attached to the mRNA and translation has begun, the miRNAs interfere with the process. It has yet to be determined how the miRNAs achieve this translational repression. It has been proposed that translational repression occurs sometime after initiation either by slowing and stalling the actively translating ribosome or by specifically degrading the newly translated polypeptide (Bartel 2004; Olsen and Ambros 1999). It may be mentioned here that miRNA is a seemingly natural phenomenon, but may prove yet, to be another powerful tool for gene suppression.

Small RNAs and Promoter Methylation

Gene silencing also occurs through what is termed, Transcriptional Gene Silencing (TGS). First discovered by Wassenegger et al. (1994) in tobacco, this form of gene silencing, termed RNA-directed DNA Methylation (RdDM) takes place when the promoter region of a gene is methylated. Either dsRNA or siRNA directs methylation of cytosine residues in both the promoter region and the open reading frame (ORF) (Agrawal et al. 2003, Jones et al. 1998). Interestingly, methylation in the ORF region alone is not sufficient to initiate TGS. On the other hand, methylation of the promoter region alone is sufficient for producing stable and heritable silencing of a transgene. Scientists, however, do not agree on whether TGS is related to Post Transcriptional Gene Silencing (PTGS), even though there is evidence to suggest that there is a direct correlation between the amount of DNA methylation and the amount of siRNA within a cell (Ingelbrecht et al. 1994). Another interesting finding in this connection is the demonstration by Mette et al. (2000) that dsRNA can induce promoter methylation resulting in the cleavage of dsRNA into smaller fragments similar in size to siRNAs. Further research into RdDM has illustrated that DNA methylation may be helpful in preventing the incidence of potato spindle tuber viroid (Wassenegger et al. 1994), plant potyviruses (Jones et al. 1998), cereal yellow dwarf virus (Wang et al. 2001), and others.

DNA Directed RNAi in Plants

As discussed earlier, some applications of RNAi in plants have relied on non-*Agrobacterium*- mediated methods of introduction of dsRNA into the cells. For example, particle bombardment was used to show that RNAi worked at the

single cell level in cereals (Schweizer et al. 2000), while Klink & Wolniak (2000) and Stout et al. (2003) demonstrated that there is a direct uptake of dsRNA by fern spores during imbibition. DNA-directed RNAi makes use of dsRNA-expressing vectors introduced into plants via *Agrobacterium*-mediated transformation (Fig. 5). This approach has been shown to be effective in reducing the amount of a specific gene product. One of the first studies testing DNA-directed RNAi in plants was done to compare the ability of sense, antisense, or dsRNA at generating RNA-mediated virus resistance via PTGS in tobacco and silencing of an endogenous GUS reporter gene in rice (Waterhouse et al. 1998). In both cases it was shown that duplex RNA was more effective than either sense or antisense RNA at silencing the target gene.

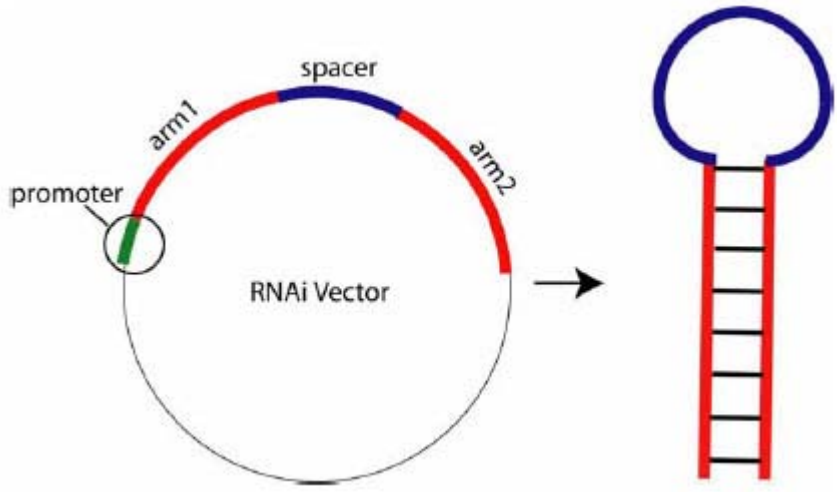

Fig. 5. Diagram representing DNA directed RNAi. An RNAi vector contains a promoter region (green), two arms (red), and a spacer (blue). As RNA polymerase transcribes the RNAi vector, it will produce hairpin structures as shown on the right.

Another study that provided evidence that DNA-directed RNAi was a viable approach in plants targeted four different genes involved in different phases of flower development in *Arabidopsis* (Chuang and Meyerowitz 2000). The phenotypes produced via RNAi for each of these genes matched the previously characterized phenotypes for already existing loss of function mutants in each case. *In situ* RNA hybridization and Western blot analysis confirmed that there was a reduction of the specific target mRNA caused by RNAi. The progeny from the RNAi mutants also showed the same mutant phenotypes, indicating that RNAi transgenic plants resulted in heritable phenotypes. Interestingly, the RNAi

transgenic plants produced a series of phenotypes ranging in their severity. The transformation vector used in these experiments was designed to produce dsRNA for the target gene with expression of the duplex RNA under the control of a strong constitutive viral promoter.

As previously discussed, a gene vector encoding an intron-spliced RNA with a hairpin structure (ihpRNA) was found to improve efficiency in silencing of the target gene in plants (Smith et al. 2000). This innovative study has revealed that this approach results in PTGS with nearly 100% efficiency when targeting viruses or endogenous genes. Using this procedure, researchers at the CSIRO, in Australia, have developed several powerful RNAi vectors (Cf. the next section for details). Currently, it is unclear how the presence of the functional intron increases silencing efficiency; however researchers believe that this technique will work with a wide range of genes in plants. More recently, a chemical-regulated inducible ihpRNA vector (Guo et al. 2003) was used to achieve DNA-directed RNAi in plants. This additional feature allows silencing of genes for basic cell function and/or development in situations where silencing a gene can be lethal. The chemical-inducible system used is the CLX (Cre-Lox) system, which has the advantage over other inducible systems in that it is under stringent control in addition to a very high rate of recombination after induction. This study showed that the inducible system resulted in silencing of a GFP transgene and an endogenous desaturase gene in *Nicotiana* and *Arabidopsis* with high efficiency.

One important practical question concerning the use of RNAi by plant biologists is their inability to decide what part of a target gene should be used for production of dsRNA for most effective silencing. There is some information available regarding this question from a study of silencing of GUS in transgenic tobacco (Hutvagner et al. 2000). In this study the small RNA fraction in the GUS-silenced line was analyzed. The analysis revealed the presence of small RNAs corresponding to the two-thirds of the GUS coding region at the 3′ end but none from the 5′ coding region and 3′ UTR region of the GUS mRNA. In 2002, Dharmacon was the first company to develop an algorithm for the rational design for highly potent siRNA gene silencing tool. The initial data for this discovery was published in Cell and Nature Biotechnology (Khvorova et al. 2003; Reynolds et al. 2004). Now there are several companies that have developed computer algorithms for analysis of a gene sequence based on a number of parameters and predict the most effective siRNA sequences for silencing that particular gene. There are several free software programs (applications) available on the internet for selecting the most effective region to be used for RNAi.

There are several interactive resources available on the internet free to use by any researchers. One would benefit by learning more about each software application and use the right tool to design silencing RNA fragment for a particular gene of interest. The web sites containing such RNAi related software applications are listed here.

Silencing RNA Target Finder from Ambion
http://www.ambion.com/techlib/misc/siRNA_finder.html

RNAi Design Tool from Integrated DNA Technologies
http://biotools.idtdna.com/rnai/

Gene Specific siRNA Selector
http://hydra1.wistar.upenn.edu/Projects/siRNA/siRNAindex.htm

RNAi Designer from Invitrogen
https://rnaidesigner.invitrogen.com/sirna/

siRNA Selection Program, Whitehead Institute for Biomedical Research.
http://jura.wi.mit.edu/siRNAext/register.php

Summary of siRNA Design Rules and Tools
http://www.protocol-online.org/prot/Molecular_Biology/RNA/RNA_Interference/siRNA_Design_Rules/

General Resources on RNAi in Ambion
http://www.ambion.com/techlib/resources/RNAi/

The previously mentioned studies used endogenous genes with known functions or alleles with known mutant phenotypes to easily assay the ability of the RNAi vectors to effectively silence the target genes. However, when using RNAi to study a gene of interest with an unknown function there are controls needed to insure that phenotypes produced by RNAi are indeed due to loss-of-function for the gene being studied. There is a report of nonspecific changes in gene expression induced by siRNAs (Persengiev et al. 2004) but it should be of no concern to plant scientists as it applies to mammalian cells. The siRNAs are very specific and the potential for non-specific effects is marginal. For experiments using siRNAs, it is important to titrate the siRNAs to determine the lowest possible effective concentration. In addition, two more controls that are recommended are the use of scrambled control siRNAs (random sequences of the same length and nucleotide length), and single or double amino acid substitutions in the control siRNA. These and other guidelines and others for the use of RNAi have been made by participants of the Horizon meeting on RNA and the editor of Nature Cell Biology and can be found in Nature Cell Biology 5: 489-490 with additional information available at the Horizon symposium website at http://www.nature.com/horizon/rna/index.html.

Application of RNAi for crop improvement

Directed by T. J. Higgins (Current publications by CSIRO), scientists at CSIRO, in Australia, have played a pioneering role in demonstrating that RNAi technology may be used for such applications as gene silencing thereby generating improved crop varieties in terms of disease-, insect resistance, enhancing nutritional qualities, and much more. To facilitate gene silencing through RNAi they also developed several versions of the pHannibal and pKannibal plasmid vectors from the original constructs. The above scientists have shown that by replacing the loop in hpRNA with an intron, the efficiency of gene silencing can be enhanced from about 50% to nearly 100%. The vector sequences for pHannibal and pKannibal are available in Genbank as well as a published paper by Wesley et al. (2001).

Scientists all over the world working with RNAi will benefit from their findings and the vectors (pHannibal, pKannibal) which are available free of charge for academic research. *BayerCrop* Science has acquired an exclusive worldwide license to develop, market, and sell selected crop plant varieties in which the RNAi technology has been successfully applied by the CSIRO scientists. Using this technique this group has developed varieties of barley that are resistant to BYDV (barley yellow dwarf virus) (Wang *et a*l. 2000). Their results showed that the barely plants developed through RNAi technology are resistant to viral infection while the control plants became infected with the yellow dwarf virus.

Kusaba and his team (Kusuba et al. 2003) have recently 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 low glutenin content was a relief to the kidney patients unable to digest glutenin. The trait was stable and was transmitted for a number of generations. They showed that the procedure may apply to both monogenic and polygenic agronomic characters. They advocated the use of either a weak promoter to regulate the level of expression of dsRNA or the use of sequences with various homologies to the target gene. Since the use of a weak promoter reduces the frequency of suppression rather than weakens it, they favor the latter method. To reduce the level of suppression, they recommend the use of both closely- or distantly related species that bear various degrees of homologies to the target gene. Close homology between the target gene and the host plant would enable each resulting siRNA to cleave the target mRNA.

Prospects of utilizing RNAi techniques

In Ethiopia, Bangladesh and India, the people in the lower socioeconomic class use a leafy vegetable known as *Lathyrus sativu*s. It is a leguminous crop and contains a neurotoxin called β-oxalylaminoalanine-L-alanine (BOAA) (Spencer et al. 1986). People consuming this vegetable suffer from a paralytic disease called, lathyrism. The disease paralyses people both temporarily and permanently, however the effects can be somewhat reduced if the plant is boiled prior to consumption. Paralysis in the limbs is a known symptom of BOAA, yet people still consume this vegetable in times of famine. This species is remarkably suited to grow in marginal and inhospitable land without irrigation, fertilizer, and pesticides. It flourishes also times of devastating flood and drought, when no other food crop survives. This is an instance where RNAi technology can be used to silence the gene(s) responsible for production of BOAA. There may be one difficulty; in that the BOAA genes may be linked to genes, which confer immunity to this unique crop or impart drought and flood tolerance. Bringing down the levels of BOAA to a safe concentration, rather than totally silencing the concerned genes, may overcome this obstacle.

Another instance where RNAi may be fruitfully applied is in the production of banana varieties resistant to the Banana Bract Mosaic Virus (BBrMV), currently devastating the banana population in Southeast Asia and India (Rodoni et al. 1999). In certain years, the entire banana crop in certain areas is lost due to the attack by the above virus. The BBrMV infects banana plants destroying the fruit producing bract region, rendering them useless to farmers. The virus is spread by small plant eating insects called aphids, as well as through infected plant materials. The problem is further compounded when further banana crops are raised in the infected field because the infection spreads from the previous diseased crop. However, by carefully designing an RNAi vector aimed at silencing the Coat Protein (CP) region of the virus, scientists may be able to develop a banana variety that is resistant to BBrMV and yet safe to eat. The CP region of the different strains of virus is highly conserved and as such silencing of this gene in other varieties of banana will not pose a problem. Another novel approach here would be to utilize an inducible promoter system in order that dsRNA is produced only upon infection and not constitutively.

A possible application of RNAi involves the down regulation of a key enzyme in the biosynthetic pathway of lignin in the two economically important *Corchorus* species, namely, *C. capsularis* and *C. olitoriu*s. The enzyme 4-coumarate:CoA ligase (4-Cl) is one of the key enzymes in the early stages of lignin biosynthesis. This makes it a promising target for regulating the quantity of lignin, produced in the jute plant. The present quantity of lignin in the

commercial varieties of jute increases the cost of pulp production for manufacture of high quality paper. Hence, reduction in the lignin content will be welcome to the paper industry. With the availability of the sequence of the 4-Cl gene, it would be possible to create a transgenic jute variety expressing the RNAi construct to down regulate the quantity of 4-Cl mRNA thereby reducing the lignin production. With this approach, it would also be possible to vary the quantity of lignin synthesis by the help of different promoters and altering the length of interfering RNA.

Thus RNAi technology may prove to be a powerful molecular tool by generating jute varieties with low lignin content, allowing for easier, environmentally friendly and cost effective processing of fiber for the production of various economically important commodities such as high quality paper and cloth.

Conclusion

Current agricultural technology needs more and more molecular tools to reduce current crop loss and feed extra mouths, which according to a recent estimate by the FAO (Food and Agriculture Organization) will increase by two billion over the next 30 years. The RNAi technology, described in this article, describes one such powerful innovation. If judiciously used, this technology may go a long way to narrow the gap through production of disease-, insect- and virus resistant, nutritionally rich and toxic-free crops. The cost effectiveness is always a big question, whenever a new technology is developed. In fact, it becomes a stumbing block for a resource-poor developing country to adopt a new technolgy, if it is to buy the patent from a multinational company at an prohibitive price. Fortunately, the situation in case of RNAi technology is different. Hopefully, the technology that has been developed by the scientists from developed countries will be available to any lab including those in the developing countries, where work utilizing RNAi technology is either in progress or going to be launched shortly. The technology is well developed and can be applied directly to evolve a crop resistant to stresses caused by virus, bacteria, fungi, insects or natural disasters.

One of the major purposes of the present review article is to help policy makers in food-deficient countries to understand how scientific breakthroughs such as RNAi technology may be helpful in tackling this gigantic problem of feeding an additional 2 billion people over the next 30 years from an increasingly fragile natural resource base. However, any new technology involving the gene manipulation may be opposed by anti-GM groups severely limiting its effectiveness or wider use. Since this technology offers a great potential in

understanding gene functions and utilize them to improve crop quality and production, it is a matter of time before we see the products of this RNAi research in the farmers' fields around the world.

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