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RNAi mediated gene silencing and it's application for crop improvement: A review

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Abstract
Gene silencing is a general term describing epigenetic processes of gene regulation. Gene silencing can occur during either transcription or translation and is often used in research. Gene silencing is considered a gene knockdown mechanism since the methods used to silence genes, such as RNAi, CRISPR, or siRNA, generally reduce the expression of a gene by at least 70% but do not completely eliminate it. It is a epigenetic process of gene regulation. It is used to describe the "switching off" of a gene by a mechanism other than genetic modification. The process to silence genes first begins with the entrance of a double stranded RNA (dsRNA) molecule into the cell, which triggers the RNAi pathway. The double stranded molecule is then cut into small double stranded fragments by an enzyme called Dicer. These small fragments, which include small interfering RNAs (siRNA) and microRNA (miRNA), are approximately 21-23 nucleotides in length. The fragments integrate into a multi subunit protein called the 'RNA induced silencing complex', which contains Argonaute proteins that are essential components of the RNAi pathway. RNAi is the key process of gene silencing. With RNAi, it would be possible to target multiple genes for silencing using a thoroughly-designed single transformation construct. Moreover, RNAi can also provide broad-spectrum resistance against pathogens with high degree of variability, like viruses.

Keywords: Gene silencing, RNA interference, miRNA, siRNA etc.

Introduction
Gene silencing is a general term used to describe the regulation of gene expression. It refers to "the ability of a cell to prevent the expression of a certain gene". Gene silencing can occur during either transcription or translation and is often used in research. Gene silencing is often considered the same as gene knockout. When genes are silenced, their expression is reduced. In contrast, when genes are knocked out, they are completely erased from the organism's genome and, thus, have no expression. Gene silencing is considered a gene knockdown mechanism since the methods used to silence genes, such as RNAi (RNA interference), CRISPR, or siRNA, generally reduce the expression of a gene by at least 70% but do not completely eliminate it. They provide a more complete view on the development of diseases since diseases are generally associated with genes that have a reduced expression. Silencing is a position effect. Genes are silenced at either the transcriptional or post-transcriptional level. Transcriptional gene silencing is the result of modifications of either the histones or DNA. e.g.- Silencing at the yeast telomere. Post-transcriptional gene silencing (PTGS) is the result of the mRNA of a particular gene being destroyed or blocked. A common mechanism of PTGS is RNAi. It refers to general processes of interruption or suppression of transcription or translation of the mRNA of the target gene by mechanisms other than genetic modification. Gene silencing is a general term describing epigenetic processes of gene regulation. It is used to describe the "switching off" of a gene by a mechanism other than genetic modification, i.e; a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell. The interruption or suppression of the expression of a gene at transcriptional or translational level is referred to as gene silencing. The silencing of a gene could be achieved by:

1. **Drugs:** These bind to target protein and cause protein inhibition
2. **RNase H-independent ODNs:** These oligo deoxynucleotides hybridize to target mRNA and cause inhibition of translation of target protein.
3. **RNase H–dependent ODNs:** These hybridize to target mRNA and mediate its degradation by RNase H.
4. **Ribozymes and DNA enzymes:** These catalyze cleavage of mRNA and hence cause its degradation.
5. **SiRNA and miRNA:** These hybridize to target mRNA by antisense strand and guide it into endoribonuclease enzyme complex, thereby causing its degradation or inhibition of translation.

History and Discovery

1. Rich Jorgensen *et al.* in an attempt to alter flower colours in petunias, introduced additional copies of a gene encoding *chalcone synthase*, key enzyme for flower pigmentation, into flowers of normally pink or violet colour. Unexpectedly the flowers produced were less pigmented, fully or partially white. It was observed that both the transgene and endogenous gene were down regulated in white flowers. This phenomenon was called co-suppression of gene expression.
2. Quelling was observed in fungus, *Neurospors crassa*, in an attempt to boost production of orange pigment produced by the gene *a L1* of the fungus. Attempts to enhance orange pigment in the fungus by introducing extra copies of carotenoid pigment genes failed when the orange pigment gene was suppressed in a third of the engineered mould. In some strains, the effect was passed on through multiple generations. This was later found to be similar to post – transcriptional silencing.
3. Plant virologists working on improving plant resistance to viral diseases observed a similar unexpected phenomenon. It was observed that plants carrying only short, non-coding regions of viral RNA sequences would show similar levels of protection as the plants expressing virus specific proteins. It was believed that viral RNA produced by transgenes could also inhibit viral replication. The reverse experiment in which short sequences of plant genes were introduced into viruses showed that targeted genes were suppressed in an infected plant. This phenomenon was labeled virus induced gene silencing (VIGS) and the set of such phenomenon was called as post transcriptional gene silencing.
4. Guo and Kempheus, attempted to use antisense RNA to shut down expression of the *par1* gene in *Caenorhabditis elegans* in order to assess its function. As expected injection of antisense RNA disrupted expression of *par-1* but injection of the sense strand control also did. The result remained a puzzle for three years and for this phenomenon they coined the term antisense mediated silencing.
5. Three years later Andrew Fire and Craig C. Mello (1998) [1] studied phenotypic effect of single stranded and double-stranded *unc-22* RNA into gonads of *C. elegans*. They observed that only the double stranded RNA consisting of both sense and antisense strand produced the typical twitcher in *C. elegans* while sense and antisense strands individually did not produced the twitcher. They concluded the results of their experiments as:

a) Silencing was triggered by injecting dsRNA but weakly or not at all by ssRNA
b) Silencing was specific for an mRNA homologous to dsRNA
c) The dsRNA had to correspond to mature mRNA sequence, neither intron nor promoter
d) Targeted mRNA was degraded.
e) dsRNA are amplified in cell, as very few are required to produce the effect.
f) Effect of dsRNA spread between tissues and even to progeny**.**

For their discovery of gene silencing by double stranded RNA they coined the term RNA interference (RNAi) and were subsequently awarded the Nobel prize in physiology or medicine (2006).

6. In 2001, Thomas Tuschl, discovered with his colleagues that RNAi could be prompted through the use of shorter pieces of RNA known as small interfering RNAs (siRNAs).
   Soon thereafter, they showed that duplexes of 21-nucleotide siRNAs mediated RNAi in cultured mammalian cells and demonstrated that siRNAs could be designed to silence specific genes without activating the interferon response. In other words, scientists could potentially silence any gene of interest in a highly predictable, reproducible, and accurate fashion (Ernie Hood, 2004).
7. Gregory Hannon and his colleagues identified, described, and named the "Dicer" enzyme, which chops dsRNA into siRNAs, as well as the RNA-induced silencing complex (RISC), which mediates the silencing process by degrading the homologous mRNA (Ernie Hood, 2004).

Materials for Gene Silencing: The Cellular components which are required for gene silencing are as follows: MicroRNAs (miRNAs), Small interference RNAs (siRNAs), Dicer, RISC, Histones, Chromatin and Heterochromatin and Transposons.

Methodology of Gene Silencing: It works at three levels,

1. **Transcriptional Gene silencing:** It includes the following mechanisms, *viz.*, Genomic Imprinting, Paramutation, Transposon silencing (or Histone Modifications), Transgene silencing, Position effect and DNA methylation.
2. **Post-transcriptional Gene silencing:** It works on the mechanisms like RNA interference, RNA silencing and Nonsense mediated decay.
3. **Meiotic Gene silencing:** It includes Transvection and meiotic silencing of unpaired DNA.

Mechanism of Gene Silencing: The mechanism of gene silencing is broadly divided into two major steps: initiation step and effector step In the initiation step the “trigger” dsRNA molecule, usually several hundred base pairs long, is cleaved to form 21- 23 bp double-stranded fragments known as short interfering RNAs or (siRNAs) or guide RNAs. siRNAs are produced when the enzyme Dicer, a member of the RNA se III family of dsRNS – specific rib nucleases, processively cleaves dsRNA in an ATP dependent processive manner.In the effector step the duplex siRNA are then unwound by the helicase activity associated with a distinct multiprotein complex known as RNA-induced silencing complex or RISC. An ATP dependent unwinding of siRNA duplex is required for activation of RISC. The siRNA strand that is complementary to the targeted mRNA is then used as primer by an RNA dependent RNA polymerase (RdRp) to convert the cognate mRNA into dsRNA itself. This dsRNA form of mRNA then becomes a substrate for Dicer cleavage activity, which leads to the destruction of the mRNA and formation of new siRNAs. Effectively this step amplifies the RNAi response and creates a self- perpetuating cycle of “degradative polymerase chain reaction” that will persist until no target mRNAs remain. This basic ‘core’ pathway defines the RNAi response as one of the most elegant and efficient biochemical mechanisms in nature. miRNAs, if they show perfect complementarity with the target mRNA cause RISC activation and target mRNA degradation similar to that by siRNA. If they do not show perfect complementarity with the

target mRNA which is usually the case, they cause RISC activation similar to that by siRNAs but the activated RISC binds to the target mRNA at its 3'-end and prevent its translation, thereby inhibiting gene expression.

Transcriptional Gene Silencing: The siRNAs work not only at the posttranscriptional stage but also leave their indelible marks on the genomes to repress the gene transcription activity or selectively remove portions of the genomes, especially of protozoans. Broadly speaking, the siRNAs bring about three different biochemical end products with the chromatin DNA: DNA methylation, as revealed mostly in plant systems; heterochromatin formation; and programmed elimination of DNA. DNA methylation had been reckoned a major source of transcriptional gene silencing (TGS), and mechanistically TGS had been viewed very distinctively from PTGS in the past. But recent developments have caused a blurring in the identity between these two pathways. The discoveries of such epigenetic changes have ignited a revolution not only in the field of gene regulation but also in gene maintenance and gene evolution.

RNA Interference (RNAi): RNA interference is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. This phenomenon is firstly discovered in transgenic plant *Petunia hybrida* **(**Andrew Fire and Craig Mello, 1998) [1] by enhancing anthocynin biosynthesis pathway. Unexpectedly, transgenic plants producing white or chimeric flowers were obtained instead of dark purple flowers due to the silencing of endogenous homologous gene and this phenomenon was termed as "co-suppression". RNAi occurrence is conserved among various organisms, also labeled as post-transcriptional gene silencing (PTGS) in plants, quelling in fungi (Romano and Macino 1992) [12] and RNA interference in animals (Fire *et al*, 1998) [1]. Fire *et al*., (1998) [1] elucidated the mechanism of RNAi in the nematode, *Caenorhabditis elegans*, and the term RNAi for the first time.

Mechanism of RNA Interference: It is triggered by double stranded RNA, which is cleaved by endoribonucleases, Drosha and Dicer to produce short RNA duplexes of 21-25 nt. in length. Drosha is responsible for the processing in the nucleus while Dicer further processes the precursors in the cytoplasm to yield short RNA duplexes called small interfering RNA (siRNA) or miRNAs (Bernstein *et al*, 2011) [3]. The siRNA is further processed such that one of the strands (the passenger strand) is destroyed, while the other strand (the guide strand or antisense strand) complexes with multiple proteins to form the RISC or RNA- induced silencing complex. It is the RISC that mediates sequence specific gene silencing by recognizing the m-RNA containing sequences complementarity with the guide strand of the siRNA (Tomari and Zamore 2005) [14]. There are three types of small RNA molecules responsible for the gene regulation *viz*., short interfering RNAs, MicroRNAs and short hairpin RNA.

Applications of RNAi mediated gene silencing for crop improvement

1. RNAi for disease and pathogen resistance**:** Gene silencing was first used to develop plant varieties resistant to viruses. Engineered antiviral strategies in plants mimic natural RNA silencing mechanisms. This was first demonstrated when scientists developed Potato virus Y-resistant plants expressing RNA transcripts of a viral proteinase gene (Obbard *et al*, 2009) [11]. Immunity has since been shown to other viruses such as the Cucumber and Tobacco Mosaic Virus, Tomato Spotted Wilt Virus, Bean Golden Mosaic Virus, Banana Bract Mosaic Virus, and Rice Tungro Bacilliform Virus among many others. In addition, plants can also be modified to produce dsRNAs that silence essential genes in insect pests and parasitic nematodes. This approach was used to develop root-knot nematode, corn rootworm and cotton bollworm resistant varieties.
2. RNAi for male sterility: RNAi has also been used to generate male sterility, which is valuable in the hybrid seed industry. Genes that are expressed solely in tissues involved in pollen production can be targeted through RNAi. For instance, scientists have developed male sterile tobacco lines by inhibiting the expression of TA29, a gene necessary for pollen development (Tehseen *et al*, 2010). RNAi was also used to disrupt the expression of Msh1 in tobacco and tomato resulting to rearrangements in the mitochondrial DNA associated with naturally occurring cytoplasmic male sterility (Nair, 1993) [10].
3. RNAi and plant functional genomics**:** A major challenge in the post-genomic era of plant biology is to determine the functions of all genes in the plant genome (Matthew, 2004) [8]. Compared to other techniques, RNAi offers specificity and efficacy in silencing members of a gene or multiple gene family. In addition, the expression of dsRNAs with inducible promoters can control the extent and timing of gene silencing, such that essential genes are only silenced at chosen growth stages or plant organs (Satto, 2005) [13].

Successful Examples of novel plant traits engineered through RNAi

Trait	Target Gene	Host	Application
Enhanced nutrient content	Lyc	Tomato	Increased concentration of lycopene (carotenoid antioxidant)
	DET1	Tomato	Higher flavonoid and b-carotene contents
	SBEII	Wheat, Sweet potato, Maize	Increased levels of amylose for glycemic management and digestive health
	FAD2	Canola, Peanut, Cotton	Increased oleic acid content
	SAD1	Cotton	Increased stearic acid content
	ZLKR/SDH	Maize	Lysine-fortified maize
Reduced alkaloid production	CaMXMT1	Coffee	Decaffeinated coffee
	COR	Opium poppy	Production of non-narcotic alkaloid, instead of morphine
	CYP82E4	Tobacco	Reduced levels of the carcinogen nornicotine in cured leaves
Heavy metal accumulation	ACR2	*Arabidopsis*	Arsenic hyperaccumulation for phytoremediation
Reduced polyphenol production	s-cadinene synthase gene	Cotton	Lower gossypol levels in cottonseeds, for safe consumption

Ethylene sensitivity	LeETR4	Tomato	Early ripening tomatoes
	ACC oxidase gene	Tomato	Longer shelf life because of slow ripening
Reduced allergenicity	Arah2	Peanut	Allergen-free peanuts
	Lolp1, Lolp2	Ryegrass	Hypo-allergenic ryegrass
Reduced production of lachrymatory factor synthase	lachrymatory factor synthase gene	Onion	"Tearless" onion

Conclusion

Biotic stress disproportionately affects farm productivity around the world with immense annual yield losses. For these reasons, control of microbial pathogens continues to be an agronomic and scientific challenge, and innovative and ground-breaking strategies are required to meet the requirement of a growing population. Recent work suggested that novel RNAi-based plant protection strategies may provide new opportunities for improving the world's food supplies and thus can have a huge impact on world's economy. A great number of basic research studies have enabled the rapid increase of knowledge in dsRNA-mediated silencing of target genes. Whereas the first investigations focused on the use of model organisms, it is now becoming possible to apply this knowledge towards modifying specific traits in agriculturally relevant crop plants. In addition to metabolic engineering and HIGS-mediated enhancement of disease resistance, RNAi strategies may be used to improve food safety by controlling the growth of phytopathogenic, mycotoxin-producing fungi. More research is required to optimize practical application strategies and to assess safety aspects. RNAi can also provide broad-spectrum resistance against pathogens with high degree of variability, like viruses (Andrew *et al*, 1998) [1]. Recent studies have hinted possible roles of RNAi-related processes in plant stress adaptation. Although much progress has been made on the field of RNAi over the past few years, the full potential of RNAi for crop improvement remains to be realized. The complexities of RNAi pathway, the molecular machineries, and how it relates to plant development are still to be elucidated.

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