NEW ERA AGRICULTURE MAGAZINE

Gene Silencing and it's Implications in Crop Improvement

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Introduction:

Gene silencing is describing as epigenetic processes of gene regulation. Gene silencing is a technique used to turn down or switch off the activity of genes by a mechanism other than genetic modification. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell.

Gene silencing (GS) is defined as a molecular process involved in the down regulation of specific genes, the mechanisms of Gene silencing that suppress gene activity in plants has extended that control of gene expression. Currently, there are several routes of GS identified in plants, such as: transcriptional gene silencing and post transcriptional (PTGS or RNAi) gene silencing (Fire et al. 1998), microRNA silencing and virus induced gene silencing. All these pathways play an important role at the cellular level, affecting gene regulation and protection against viruses and transposons. The post-transcriptional gene silencing involves breakdown of the mRNA itself by various techniques like Ribozymes, antisense RNA, DNAzymes and RNA interference (RNAi). Among all these techniques RNA interference has emerged as most potent tool to effect targeted gene silencing and is being used to determine the function of genes which are expressed in a constitutive or cell-fate dependent manner.

Short History of Gene Silencing:

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.

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2. Quelling was observed in fungus, *Neurospors crassa*, in an attempt to boost production of orange pigment produced by the gene aL1 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. 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.
4. Three years later Andrew Fire and Craig C.Mello studied phenotypic effect of single- stranded and double- stranded **unc-22** RNA into gonads of *C .elegans* (1998). 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. For their discovery of gene silencing by double stranded RNA they coined the term RNAinterference (RNAi) and were subsequently awarded the Nobel Prize in physiology or medicine (2006).
5. 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.
6. 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

Levels of Gene Silencing:

Post transcriptional gene silencing (PTGS):-

It is known commonly as RNA interference (RNAi). It causes silencing by destruction of the mRNA of the gene to which

the siRNA shows perfect complementarity (Zhang et al. 2016). Mechanisms involved in PTGS are as follows.

a) RNA interference (RNAi)
b) Virus induced gene silencing (VIGS)
c) c.Host induced gene silencing (HIGS)
d) d.DNA interference (DNAi)

Transcriptional gene silencing (TGS) It Causes gene silencing by:

a) DNA methylation
b) Histone modification
c) Genomic imprinting
d) Paramutation
e) Position Effect
f) Transposone Silencing
g) RNA directed DNA Methylation (RdDM)

Before PTGS:- Antisense RNA:-

Prior to the discovery of RNAi, scientists applied various methods such as insertion of TDNA 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. The antisense technology is based on blocking the information flow from DNA via RNA to protein by the introduction of an RNA strand complementary to (part of) the sequence of the target mRNA (Lee et al. 2003). This so-called antisense RNA is thought to base pair to its target mRNA thereby forming double-stranded RNA.

Disadvantages:

- Some genes could not be silenced at all.
- The degree of silencing varied considerably between cells and tissues within an experiment.
- Silenced cells tended to revert and lose silencing after prolonged growth.

Post transcriptional gene silencing (PTGS)

RNA interference (RNAi) :-

The term RNA interference (RNAi) was coined after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA. RNAi is a cellular mechanism that degrades unwanted RNAs in the cytoplasm but not the nucleus. RNA silencing is a sequence specific RNA degradation process that is triggered by the formation of double stranded RNA that can be introduced by virus or transgene.

RNA interference (RNAi) is a molecular mechanism in which fragments of double stranded nucleic acid (dsRNA) interfere with the expression of a particular gene that shares a homologous sequence with the dsRNA. DsRNA triggers the specific

degradation of homologous RNAs only within the region of identity with the dsRNA. The dsRNA can be either, MicroRNA (miRNA) or Small interference RNA (siRNA).

Salient features of RNAi

- Double stranded RNA rather than single–stranded antisense RNA is the interferring agent
- High degree of specific gene silencing with less effort.
- Highly potent and effective, only a few double stranded RNA molecules per cell are required for effective interference.
- Silencing can be introduced in different developmental stages
- Systemic silencing
- Avoids problems with abnormalities caused by a knocked out gene in early stages which could mask desired observations.
- Silencing effects passed through generations

Major component of RNAi

- Small RNA
    - siRNA
    - miRNA
- Dicer
- RISC

Small interfering RNAs (siRNAs):-

These are double stranded RNA from exogenous sources like viruses, transgenes or transposons, i.e; they originate from double stranded RNAs. These result from the Dicer enzyme cutting up a larger fragment of perfectly complimentary double stranded RNA. They have Symmetric 2nt 3'overhangs, 5' phosphate groups and are 19- 24 nucleotides in length, slightly shorter than miRNAs. These fragments then bind to the nuclease complex RISC and serve as a guide to recognition of complementary base pairing on target mRNA, ultimately bringing its degradation

MicroRNA (miRNA):-

These are double stranded RNA molecules which originate endogenously from ***MIR*** genes, i.e; these originate from single stranded RNA. Micro-RNAs (miRNAs) constitute a novel, phylogenetically extensive family of small RNAs (~22 nucleotides) with potential roles in gene regulation. miRNAs are produced by Dicer from the precursors of ~70 nucleotides (pre-miRNAs). Some miRNA genes have been found in close conjunction, suggesting that they are expressed as single transcriptional units. miRNAs which are 21–24 base duplexes that are usually incompletely based paired and form partial duplexes within the 3′-untranslated region (UTR) of targeted transcripts via an association with RISC. Their functional roles vary depending on the organism, but in mammals the primary mechanism of miRNA action is to inhibit mRNA translation.

These clustered miRNAs are expressed polycistronically and are processed through at least two sequential steps.

- Generation of the ~70 nucleotide precursor micro RNAs from the longer transcripts (termed pri-miRNAs
- Processing of pre-miRNAs into mature miRNAs

MicroRNA biogenesis:-

These are fragments originally from large DNA sequence,that has miRNA sequence and reverse complement present. Mature miRNAs are derived from two major processing events, driven by sequential cleavage by the RNAse –III enzymes Drosha and Dicer. miRNAs are transcribed by RNA polymerase II, producing primary microRNAs (pri-miRNAs). These are several kilobases long, polyadenylated and capped, similar to the production of mRNAs from protein encoding genes. These pri-miRNAs are then subjected to processing by the microprocessor complex, composed of Drosha and its associated binding partner, Pasha (also known as DGCR8),Which results in the excision of a 65-75 nucleotide stem-loop structure called as precursor microRNA (pre-miRNA). These pre-miRNAs are then recognized and transported from the nucleus to the cytoplasm via the Ran-GTP dependent nuclear transmembrane protein, Exportin 5, where they are subjected to a second cleavage step by Dicer. Processing by Dicer results in the production of a small double- stranded miRNA duplex containing 2-nucleotide- long 3'overhangs. These double-stranded products are quickly unwound by helicase and a single mature strand is produced which is asymmetrically incorporated into RNA-induced silencing complex (RISC) where they can act by translational repression

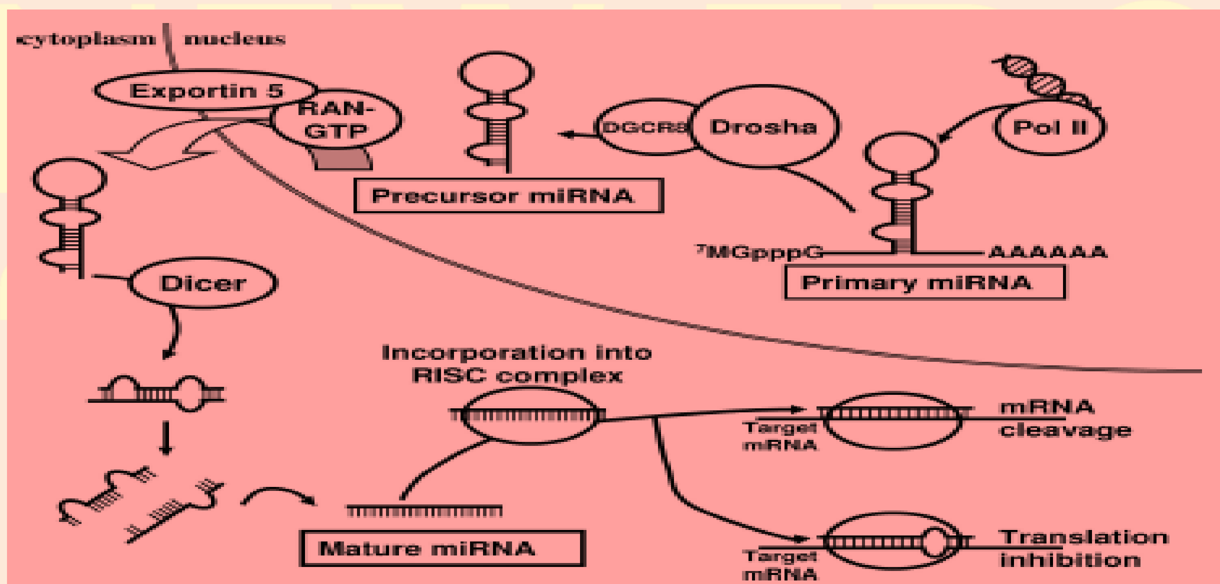


Fig. 1:- miRNA synthesis

by a cleavage incompetent RISC or mRNA degradation by a cleavage-competent, slicer-containing RISC.

- PAZ domain (110-130 amino-acid domain present in protein like Argo, Piwi.);it is thought to be important for protein-protein interaction

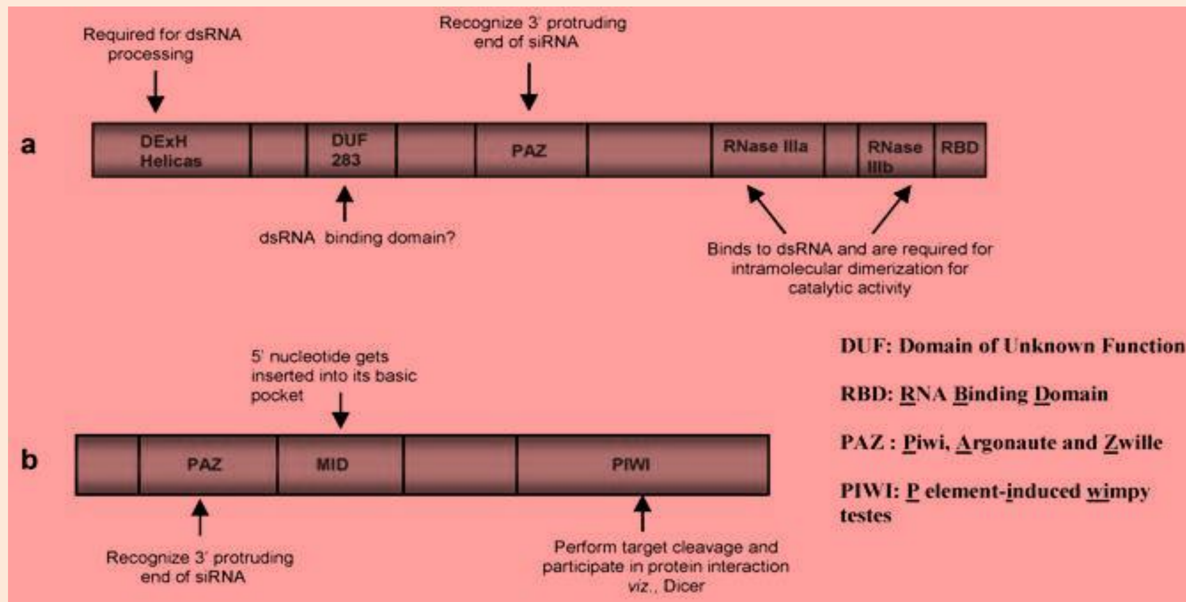


Fig. 2: - DICER's domain

Dicer:- Dicer is an endo-ribonuclease in the RNase III family. It cleaves double-stranded RNA (dsRNA) into short double-stranded RNA fragments called small interfering RNA(siRNA) about 20-25 nucleotides long, usually with a two-base overhang on the 3' end. Dicer catalyzes the first step in the RNA interference pathway and initiates formation of the RNA- induced silencing complex (RISC),

DICER's domain:- Dicer have four distinct domains**:**

- Amino-terminal helicase domain
- Dual RNAse III motifs in the carboxyl terminal segment
- dsRNA binding domain

RNA-Induced Silencing Complex (RISC):- it is a large multi-protein complex (~500-kDa) specifically a ribonucleoprotein, which incorporates one strand of a ssRNA fragment, such as miRNA, and double- stranded siRNA. The active components of an RISC are endonucleases called argonaute proteins which cleave the target mRNA strand. Unwinding of double- stranded siRNA by ATP independent helicase.

Mechanism of RNAi:- Mechanism of RNA interferences as understood is that it comes into play when a double stranded RNA is introduced either naturally or artificially in a cell. An endo-ribonuclease enzyme cleaves the long dsRNA into small pieces of RNA. The

small pieces could be mi RNA or si RNA depending upon the origin of long dsRNA i.e. endogenous or exogenous respectively. A double stranded RNA may be generated by either RNA dependent RNA polymerase or bidirectional transcription of transposable elements or physically introduced (Das et al. 2011).

The mechanism of RNA interference can be divided into 2 stages:

1. Initiation
2. Effector

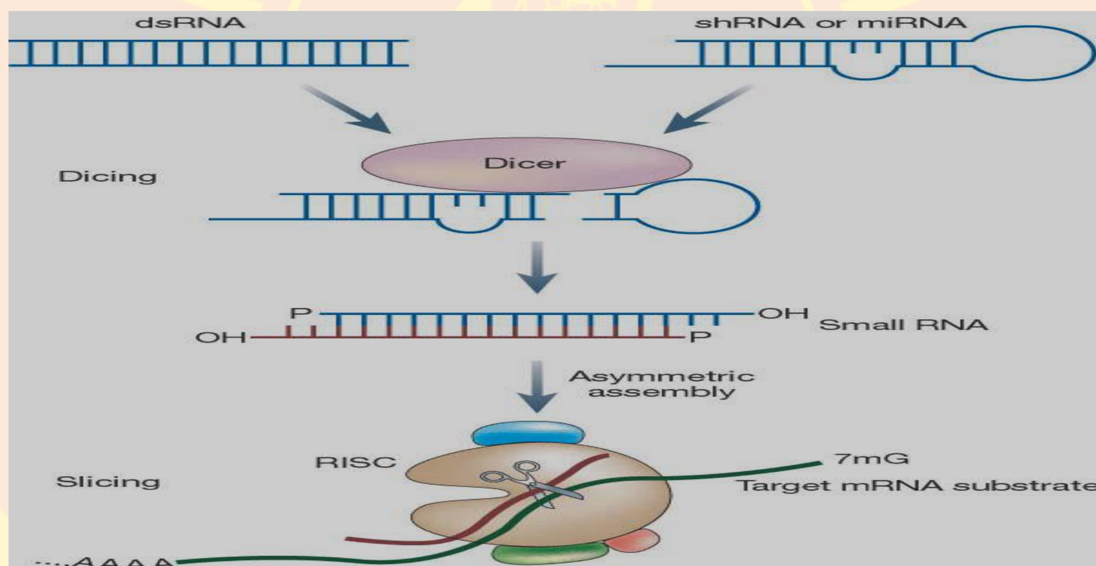


Fig. 3:- Mechanism of RNAi

Initiation step:

- dsRNA molecule is cleaved to form 21-23 bp double stranded fragments called siRNA

Effector step:

- siRNA is unbound by helicase activity associated with a multiprotein complex known as RNA-induced silencing complex(RISC).
- The antisense RNA complexed with RISC binds to its corresponding mRNA which is cleaved by the enzyme Slicer rendering it inactive.

Virus induced gene silencing (VIGS):- VIGS is one of the plant defense mechanisms against invading viruses. Both RNA and DNA viruses are recognized by the plant defense machinery and siRNA-mediated PTGS is provoked that eventually silences the viral sequences. VIGS utilizes the RNAi pathway in plants to induce transient gene knock-down. This process begins with the introduction of modifi ed virus that also contains fragment of endogenous gene sequence. Once expressed in vivo, dsRNAs are generated as the virus replicates and spread through the plant. Silencing persists

until proliferation of viral RNAs is overcome by the silencing response (Pandey et al. 2015).

The first step of VIGS method involves selection of the gene fragment to be used for silencing the target gene. The gene target should be carefully chosen such that they do not produce off targets. This is followed by cloning gene fragments into the viral vector by conventional restriction digestion based or Gateway cloning method. The viral vector harboring the gene of interest is then inoculated into plants by agrodrench/rub inoculation (depending on the type of viral vector and plant). After 2–3 weeks, the inoculated plants are analyzed for down regulation of genes by various means like RT-qPCR and measurement of the viral titer (Unver et al. 2009).

Host-induced gene silencing (HIGS):- HIGS involves silencing the vital and highly conserved genes of pathogens by the host plants. HIGS is based on the finding that RNA molecules are able to move from plants into fungal cells and effectively silence their target genes. Recently, host plant derived siRNAs have been successfully used to silence fungal and other pathogen-specific genes. The first step involves identification of pathogen gene to be silenced. The first option is to choose the native gene that does not share any homology with the plant gene. The second option is to design synthetic siRNAs corresponding to the selected gene using siRNA design tools like siMax and then test them for their silencing effectiveness in pathogen. For HIGs, the genes are cloned in either ihpRNA vectors or VIGS

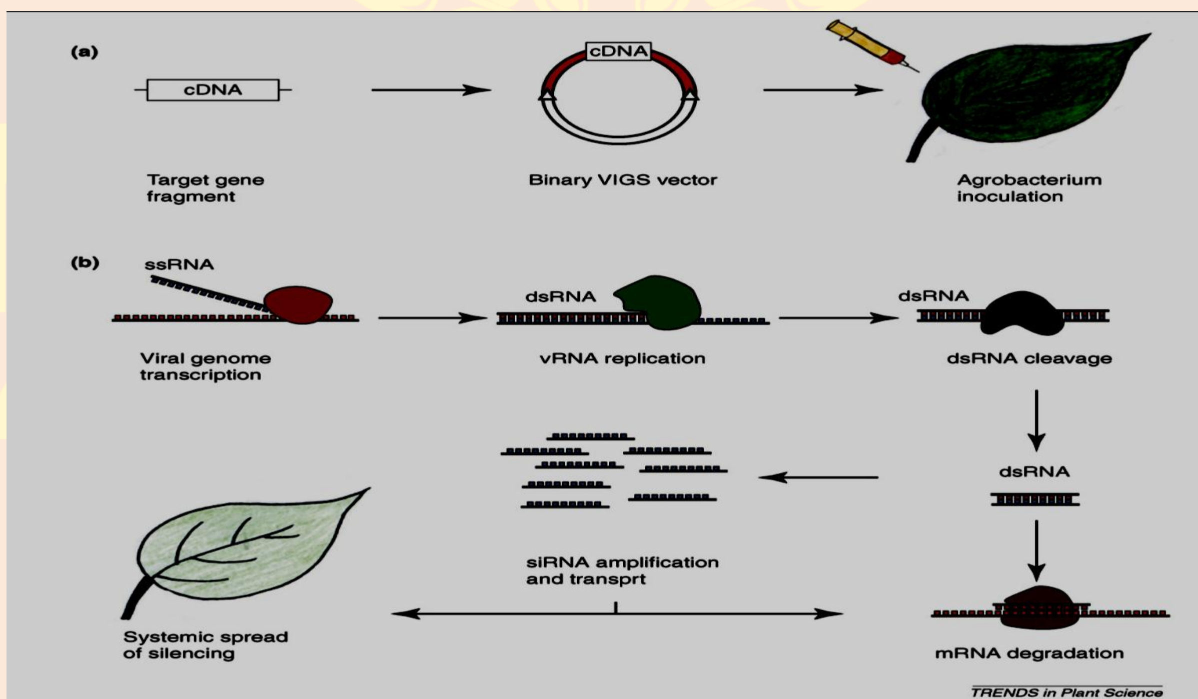


Fig. 4: - Method and molecular mechanism of VIGS

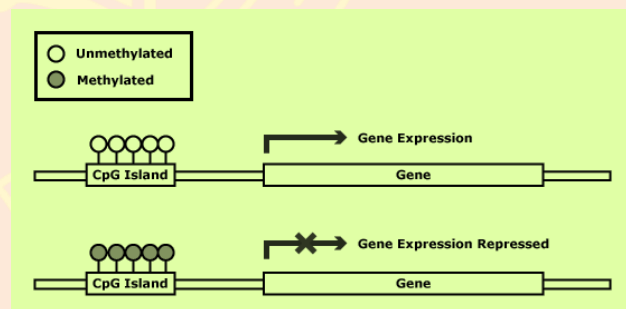
vectors and then transformed into plants by a suitable method. The transformants can then be analyzed by plant phenotype, different molecular biological methods like PCR and pathogen growth assay.

DNA interference (DNAi):- Apart from RNA molecules, promoter-less dsDNA molecules can induce sequence-specif c gene silencing in plants. This DNA-mediated gene silencing is termed as DNA interference or DNAi. The protocol involves amplification of the DNA by PCR followed by cloning in suitable cloning vector. The digested DNA fragments are then introduced into plants by particle bombardment. The transformed plants are then analyzed for silencing of the target genes. This method has been developed and used by Tsuboi et al. wherein they showed that the delivery of dsDNA fragment into fern (*Adiantum capillus - veneris*) gametophytic cells induces a sequence-specific gene silencing. The PCR-amplified DNA fragments can be used for the induction of gene silencing by DNAi. Like RNAi, DNAi-induced gene silencing was also shown to be systemic. Unlike RNAi, where the heritability of gene silencing is believed to be limited to a promoter region of a target gene, DNAi by gene fragments derived from coding regions was also found to be inherited to the next generation.

Transcriptional gene silencing (TGS)

DNA methylation: - DNA methylation is a process by which methyl groups are added to DNA. Methylation modifies the function of the DNA, typically acting to suppress gene transcription.

DNA methylation is essential for normal development and is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, suppression of repetitive elements, and carcinogenesis. Two of DNA's four nucleotides, cytosine and adenine, can be methylated. Adenine methylation is restricted to prokaryotes.



DNA methylation can stably alter the expression of genes in cells as cells divide and differentiate from embryonic stem cells into specific tissues (Shah et al. 2012). The resulting change is normally permanent and unidirectional, preventing a cell from reverting to a stem cell or converting into a different cell type. However, DNA methylation can be removed either passively,

by dilution as cells divide, or by a faster, active, process. The latter process occurs via hydroxylation of the methyl groups that are to be removed, rather than by complete removal of methyl groups. DNA methylation is typically removed during zygote formation and re-established through successive cell divisions during development. DNA methylation at the 5 position of cytosine has the specific effect of reducing gene expression and has been found in every vertebrate examined.

Histone modification/ chromatin remodeling:- It can be the state of protein modification, rather than the presence of the protein per se, that is responsible for an epigenetic effect. Histones (H1, H3, H4, H2A, and H2B) are also subject to several different covalent modifications.

- Methylation,
- Acetylation,
- Phosphorylation,
- Ubiquitination,
- Sumoylation

- Histones modification such as Methylation and Acetylation have important role in regulation of gene expression.

Histone methylation:- Histone methylation is a process by which methyl groups are transferred to amino acids of histone proteins that make up nucleosomes, which the DNA double helix wraps around to form chromosomes. Methylation of histones can either increase or decrease transcription of genes, depending on which amino acids in the histones are methylated, and how many methyl groups are attached. Methylation events that weaken chemical attractions between histone tails and DNA increase transcription, because they enable the DNA to uncoil from nucleosomes so that transcription factor proteins and RNA polymerase can access the DNA. This process is critical for the regulation of gene expression that allows different cells to express different genes.

- Histone methylation is associated with either transcriptional activation, inactivation, or silent genomic regions.

Methylation at H3 lysine 4 characterizes active genes – it facilitates transcription in part by protecting active coding regions from deacetylation and by promoting recruitment of transcriptional complexes

Methylation of H3 lysine 9 characterizes inactive genes – it leads to recruitment of HP1(heterochromatin protein) which induce heterochromatin formation and silencing.

Histone Acetylation:- Histone acetylation occurs by the enzymatic addition of an acetyl group (COCH3) from acetyl coenzyme A. The process of histone acetylation is tightly involved in the regulation of many cellular processes including chromatin dynamics and transcription, gene silencing, cell

cycle progression, apoptosis, differentiation, DNA replication, DNA repair, nuclear import, and neuronal repression. The modifying enzymes involved in histone acetylation are called histoneacetyltransferases (HATs) and they play a critical role in controlling histone H3 and H4 acetylation. Histone H3 acetylation may be increased by inhibition of histone deacetylases (HDACs) and decreased by HAT inhibition. Histone deacetylaces (HDACs) catalyze the hydrolytic removal of acetyl groups from histone lysine residues. An imbalance in the equilibrium of histone acetylation has been associated with tumorigenesis and cancer progression. The lysine residues at the N-terminal of histone tails are subjected to either acetylation by histone acetyltransferase enzymes (HATs), or deacetylation by histone deacetylases (HDACs). These lysine residues have a positive charge that can bind tightly to the negatively charged DNA to condense nucleosomes and form a closed chromatin structure, which is inaccessible to transcription factors. Acetylation removes positive charges and reduces the affinity between histones and DNA, thereby opening the condensed chromatin structure to allow transcriptional machines easier access to promoter regions. Histone hyperacetylation is thus considered a more reliable epigenetic regulator of transcriptional activation.

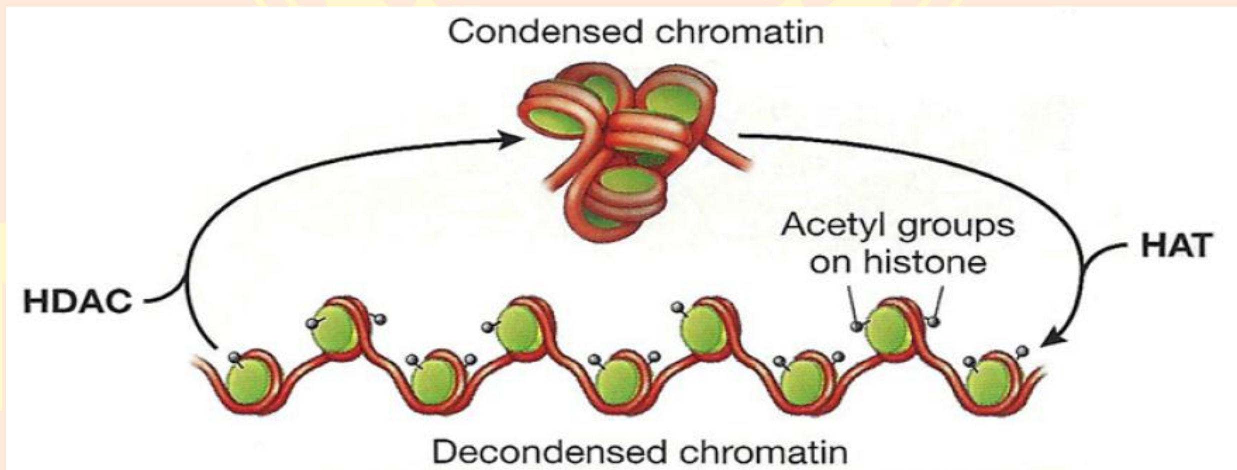


Fig. 5:- Histone Acetylation and chromatin remodeling

Genomic imprinting:- Genomic imprinting refers to differential expression of maternally versus paternally inherited alleles Or defiend as genetic phenomenon by which certain genes are expressed in a parent-of-origin-specific manner. It is independent of the classical Mendelian inheritance and found in insects, mammals and flowering plants.

Paramutation:- Paramutation is an interaction between two alleles at a single locus, whereby

one allele induces a heritable change in the other allele. The change may be in the pattern of DNA methylation, for example. The allele inducing the change is said to be paramutagenic, whilst the allele that has been epigenetically altered is termed paramutant (or paramutated). This change is meiotically inheritable, and therefore paramutation violates Mendel's first law. A paramutant allele may have altered levels of gene expression, which may continue in offspring which inherit that allele, even though the paramutagenic allele may no longer be present. Through proper breeding, paramutation can result in sibling plants that have the same genetic sequence, but with drastically different phenotypes. Paramutation was first discovered and studied in maize (Zea mays) by R.A. Brink in the 1950s. Brink noticed that specific weakly expressed alleles of the red1 (r1) locus in maize, which encodes a transcription factor that confers red pigment to corn kernels, can heritably change specific strongly expressed alleles to a weaker expression state. The weaker expression state adopted by the changed allele is heritable and can, in turn, change the expression state of other active alleles in a process termed secondary paramutation. Brink showed that the influence of the paramutagenic allele could persist for many generations. Allelic interactions similar to paramutation have since been reported in other organisms, including tomatoes, peas, and mice.

Position effect:- Position effect is the effect on the expression of a gene when its location in a chromosome is changed, often by translocation. This has been well described in Drosophila with respect to eye colour and is known as position effect variegation (PEV).The phenotype is well characterised by unstable expression of a gene that results in the

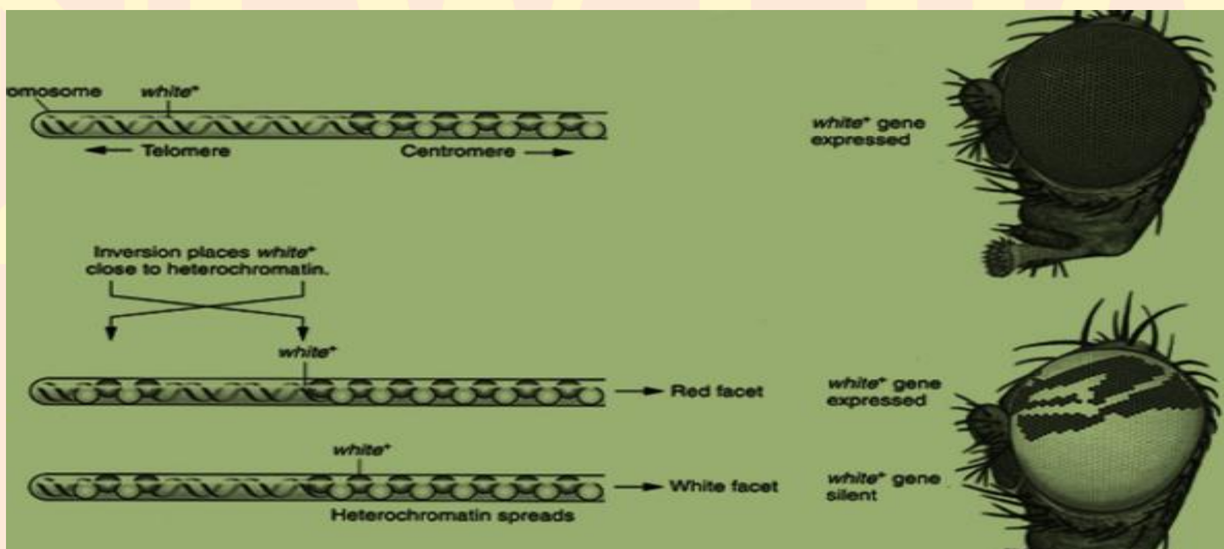


Fig. 6: - Position effect variegation

red eye coloration. In the mutant flies the eyes typically have a mottled appearance of white and red sectors. These phenotypes are often due to a chromosomal translocation such that the colour gene is now close to a region of heterochromatin. The heterochromatin can spread stochastically and switch off the colour gene resulting in the white eye sectors. Position effect is also used to describe the variation of expression exhibited by identical transgenes that insert into different regions of a genome.

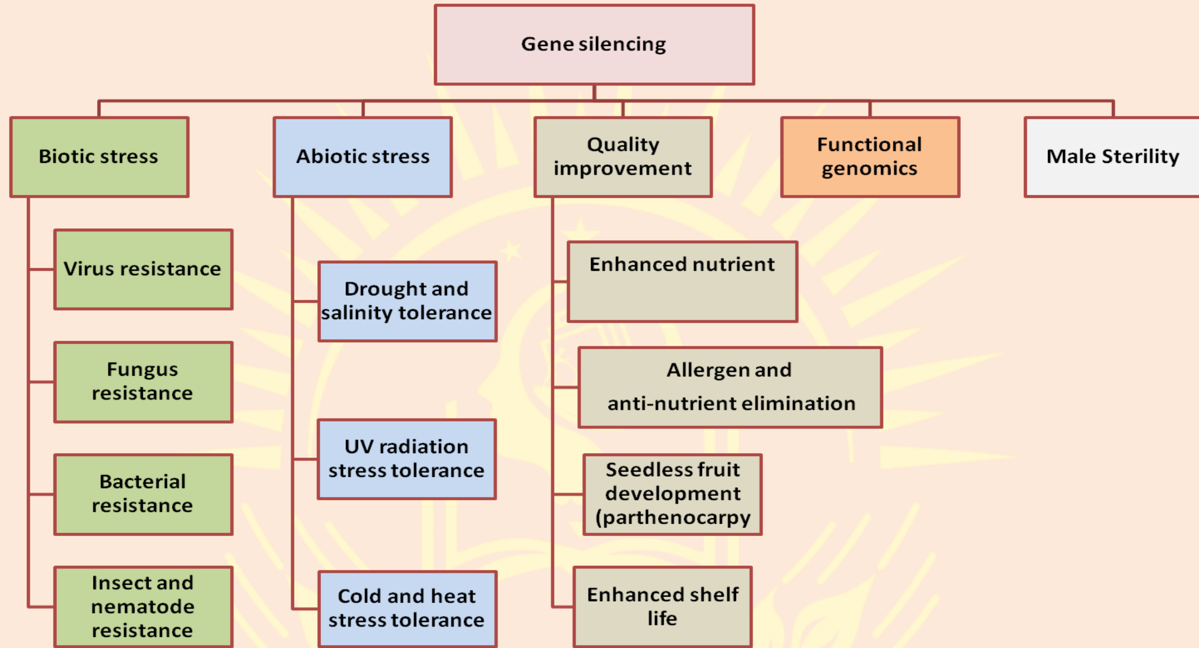
Transposon silencing:- It is a form of transcriptional gene silencing targeting transposons. Transposon silencing is achieved through DNA methylation, histone modification and small RNA-based pathways.

RNA directed DNA Methylation (RdDM):- A role for RNA in guiding *de novo* cytosine methylation of homologous DNA sequences was first discovered in viriod-infected plants and subsequently also in nonpathogenic plant systems. When the dsRNA degradation mediated PTGS occurs in plants, the genomic DNA regions homologous to dsRNA are often found methylated at almost all the sensitive cytosine residues. This process is generally referred to as RNA-dependent-DNA methylation and the corresponding part of the genome, especially the promoter region might remain transcriptionally silent. The initiator of RNA-dependent DNA methylation / TGS could be either the transgene-derived dsRNA or the consequent siRNA. Depending on the sequence information of the dsRNA, RNA-dependent DNA methylation was found to occur at the open reading frame and/or the promoter region of the genome. If methylation occurred only at the open reading frame, TGS did not result. However, RNA-dependent DNA methylation at the promoter sequences induced TGS, which, unlike PTGS, was stable and heritable.RNA-dependent DNA methylation within the host genes has also been found to occur preponderantly during virus-induced gene silencing, a type of RNAi that is generally initiated by plant virus vectors carrying portions of host genes It is now known that the movement of transposons was controlled by transcriptional suppression (TGS) and that methylation also played a role in this suppression, depending on the nature of the transposon. The level of target DNA methylation is directly related to the amount of siRNA present in the cell, and thus the apparent differences between these observations can be resolved. In other words, the availability of siRNA may determine the level of RNA-directed DNA methylation. In the events of RNA-dependent DNA methylation, the chromodomain containing DNA methylases acts either alone or in combination with other proteins, such as piwi-containing proteins, to form complexes with

the siRNAs and cause sequence-specific RNA-dependent DNA methylation, finally resulting in TGS.

Application in gene silencing in crop improvement

produce dsRNAs that silence essential genes in insect pests and parasitic nematodes. This approach was used to develop rootknot nematode, corn rootworm and cotton bollworm resistant varieties.



Biotic Stress 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. 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 acilliform Virus among many others. In addition, plants can also be modified to

Abiotic Stress Tolerance:- Water deficit or drought is one the major environmental stresses limiting crop productivity. RNAi has been applied successfully to develop drought tolerant crops. RNAi mediated down regulation of farnesyl transferase in canola using the ***AtHPR1*** promoter showed more resistance to seed abortion during flowering induced by water deficiency without affecting yield during drought stress (Kamthan et al. 2015). Similarly gene silencing approach has been utilized for salinity tolerance heat and cold tolerance etc.

Table 1: Showing improved traits against biotic stress using target genes in crops			
Traits improvement	**Targeted gene**	**Plant**	**Reference**
Virus resistance			
***Bean Golden Mosaic Virus* (BGMV)**	***AC1***	**Bean**	**Bonfim et al. (2007)**
***Barley Yellow Dwarf Virus* (BYDV)**	***BYDV-PAV***	**Barley**	**Wang et al. (2000)**
***Rice Dwarf Virus* (RDV)**	***PNS12***	**Rice**	**Shimizu et al. (2009)**
***Turnip Mosaic Virus* (TuMV)**	***HC-Pro***	**Tobacco**	**Niu et al. (2006)**
Insect resistance			
Helicoverpa armigera	***CYPAE14***	**Cotton**	**Mao et al. (2007)**
Corn rootworm	***V-ATPase A***	**Maize**	**Baum et al. (2007)**
Nematode resistance			
Meloidogyne incognita	**Splicing factor and integrase**	**Tobacco**	**Yadav et al. (2006)**
Meloidogyne incognita	***16D10***	***Arabidopsis***	**Huang et al. (2006)**
Bacterial resistance			
***Xanthomonas citri* subsp. citri (Xcc)**	***PDS* and *CalS1***	**Lemon**	**Enrique et al. (2011)**
Agrobacterium tumefaciens	***iaaM* and *ipt***	***Arabidopsis***	**Escobar et al. (2001), Dunoyer et al. (2006)**
Fungal resistance			
Magnaporthe grisea Xanthomonas oryzae	***OsSSI2***	**Rice**	**Jiang et al. (2009)**
Magnaporthe grisea	***OsFAD7* and *OsFAD8***	**Rice**	**Yara et al. (2007)**
Phytophthora infestans	***SYR1***	**Potato**	**Eschen-Lippold et al. (2012)**
***Blumeria graminis* f. sp. tritici**	***MLO***	**Wheat**	**Riechen (2007)**

Table 2: Showing improved traits against abiotic stress using target genes in crops			
Trait improved	Target	Plant	Reference
Drought tolerance	NFYA5	*Arabidopsis*	Li et al. 2008
	GmNFYA3	Soybean and *Arabidopsis*	Ni et al. 2013
	OsDSG1	Rice	Park et al. 2010
Cold tolerance	PCF5/PCF8	Rice	Yang et al. 2013
Heat stress tolerance	CSD1 CSD2 CCS	Arabidopsis	Guan et al. 2013

Quality improvement:- gene silencing has been used to modify plant metabolic pathways to enhance nutrient content and reduced toxin production. The technique takes advantage of the heritable and stable RNAi phenotypes in plants

Table 3: 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
	ZLKR/SD H	Maize	Lysine-fortified maize
Reduced alkaloid production	CaMXMT1	Coffee	Decaffeinated coffee
	COR	Opium poppy	Production of nonnarcotic alkaloid, instead of morphine
	CYP82E4	Tobacco	Reduced levels of the carcinogen nornicotine in cured leaves
Reduced Polyphenol production	a-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

RNAi and Plant Functional Genomics:- A major challenge in the postgenomic era of plant biology is to determine the functions of all genes in the plant genome. 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. There are several ways of activating the RNAi pathway in plants. The various RNAi techniques have advantages and disadvantages with respect to how persistent their effects are and the range of plants to which they can be applied. These include the use of hairpin RNA expressing vectors, particle bombardment, Agrobacterium mediated transformation and virus induced gene silencing (VIGS).

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. 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.

Advantage of gene silencing:

- Down regulation of gene expression
- Easier than use of antisense oligo-nucleotides.
- siRNA more effective and sensitive at lower concentration.
- High Specificity
- blocking expression of unwanted genes and undesirable substances.
- Inducing viral resistance
- Powerful tool for analysing unknown genes in sequenced genomes.
- Useful approach in future gene therapy.
- Oligonucleotides can be manufactured quickly, some within one week; the sequence of the mRNA is all that is needed (Borkar et al. 2013)

Disadvantages:

- **Delivery system**:- getting those exquisitely specific siRNAs to the appropriate sites in the appropriate amounts to ensure appropriate uptake and the intended silencing remains a considerable challenge
- **Off target effect:-** when siRNA can affect unintended genes in the organism which may be vital

Conclusions and future perspectives:- Gene silencing based on PTGS and TGS methods has been successfully used for functional characterization of abiotic and biotic stress responsive genes, improvement of nutritional traits, forage quality and bio-fuel production. Among the different methods used for TGS and PTGS, RNAi and VIGS are popular. RNAi-mediated gene silencing is one of the methods that are commonly used in the laboratories for plant species that can be easily transformed. VIGS is commonly used in some plant species for quick and transient gene silencing since it does not require stable plant transformation. In addition to reverse genetics,

VIGS can also be used for forward genetics. The recent advances in bioinformatics have led to the development of useful web tools that can accurately and efficiently predict the dsRNA targets thus overcoming the main drawback of PTGS.

Further efforts should be focused towards improving gene silencing methods by addressing the problem of off-target silencing and the virus-associated symptoms. This can be achieved by developing better bioinformatics tools for accurate prediction of off- target gene silencing, further improvement of MIGS, a miRNA mediated silencing, MIR-VIGS for more precise silencing of target genes, and identification of new viral vectors which have high infectivity but reduced adverse affects on the plants. In addition, there is a need to develop easy and efficient plant transformation protocols which can be applied to a wide variety of plants.

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