CHAPTER – 22

Antisense RNA Technology

Neha Srivastava, Shama Parveen and Sudheer Kumar Pathak

INTRODUCTION:

Ascribing the association between structure and function of gene and modulating its appearance to evident the desired phenotype have been major challenges for scientists. In order to elucidate the phenotypes associated with a given gene, various gene-targeting techniques have been tried with diverse success. Such hurdles in gene identification and manipulation can be overcome by Gene silencing. Gene silencing can be executed at transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) levels. The TGS is caused by DNA methylation, heterochromatin formation and Programmed DNA elimination whereas the PTGS techniques rely upon the breakdown of mRNA by various technologies, including antisense RNA, ribozymes, DNAzymes, micro RNAs, and RNA interference (RNAi). Among all these techniques, antisense RNA technology is the most efficient tool for targeted gene silencing. Antisense technology is a method that can dispute protein production which is used for the inhibition of gene expression.

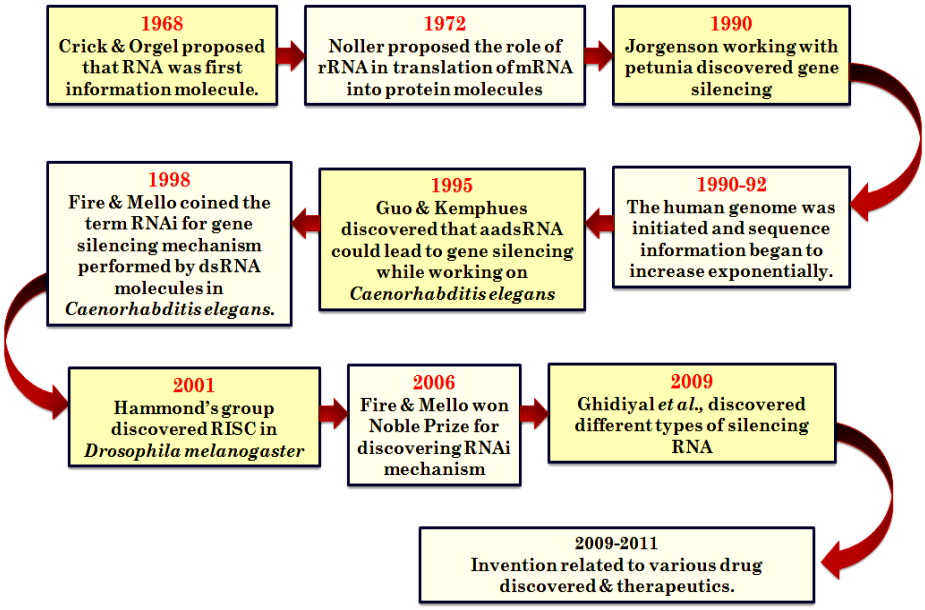
The inhibition of gene expression is depend upon the conventional central dogma of genetics in which DNA stores genetic information and proteins execute the biological functions, while RNA serves as a bridge in the transmission of genetic information. However, in the human genome less than two percent of the total DNA are translated into proteins whereas more than 90% of genes are transcribed into non coding RNAs (ncRNAs) (**Qi and Du, 2013**). Antisense RNAs represent a specific type of ncRNA used for regulating genetic activity at multiple levels in a cell, such as at DNA, RNA and chromosome structures, transcription, translation, RNA and protein stabilities (**Rusk, 2015**). Antisense RNAs are unique DNA transcripts with small, non coding and diffusible molecules containing 19–23 nucleotides that are complementary to mRNA. With the development of antisense RNAs, their application will gradually replace traditional technology for gene-specific silencing. In this review, we summarize the current understanding of antisense RNAs, particularly of the formation and their mechanism regulating the expression of their target genes.

Time line of Anti sense RNA Technology

THE BASICS OF ANTISENSE TECHNOLOGY:

Antisense technology is the process in which the antisense strand hydrogen bonds with the targeted sense strand. A sense strand is a 5' to 3' mRNA molecule or DNA molecule. The complementary strands or mirror strand to the sense is called an antisense. When an antisense strand binds with mRNA sense strand, cell recognize the double helix as foreign to the cell and proceed to degrade the faculty mRNA molecule which prevents the production of undesired protein. m RNA is the nucleic acid molecule that carries genetic information from the DNA to the other cellular machinery involved in the protein production.

The basic idea is that if an oligonucleotides which is a short RNA or DNA molecule complementary to a mRNA produced by a gene that can be introduced into a cell, it will specifically bind to its target m RNA through the exquisite specificity of complementary based pairing the same mechanism which guarantees the fidelity of DNA replication and of RNA transcription from the gene. This binding forms an RNA dimmer in the cytoplasm and halts protein synthesis. This occurs because the mRNA no longer has access to the ribosome and cytoplasm by ribonucleotides. Therefore, the introduction of short chains of DNA complementary to mRNA will lead to a specific diminution, or blockage, of protein synthesis by a particular gene. In effect, the gene will be turned off. The current hypotheses include "Blocking RNA splicing, accelerating degradation of the RNA molecule and preventing introns from being spliced out of the mRNA, impeding the exportation of mRNA into the cytoplasm, hindering translation and the triplex formation in DNA". (**Gupta *et al.*, 2011)**



Source: ***RNAi Web* (*http://www.rnaiweb.com/RNAi/RNAi_Timeline*)**

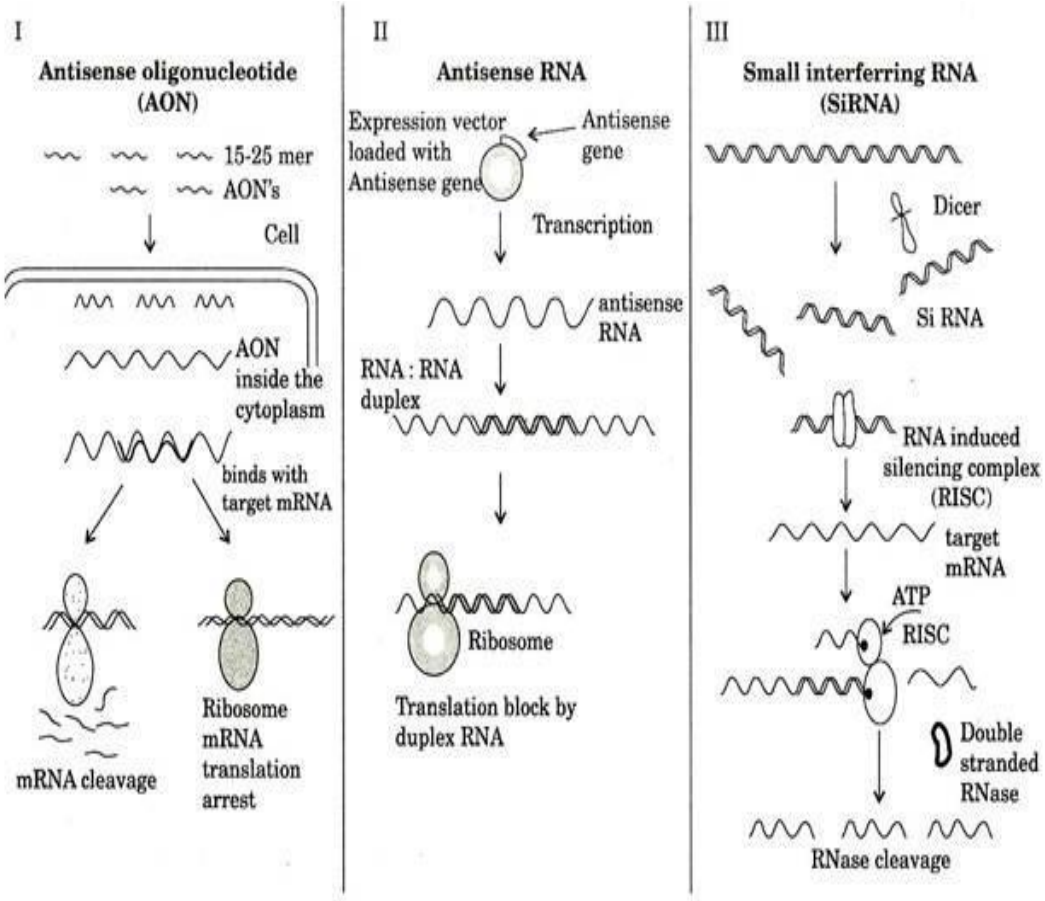


Fig. 1- Mode of action of antisense

PROTEIN FORMATION:

Protein molecules are the expression of a gene which has being produced in the body. However, to get a protein the cell must undergo two complex processes transcription and translation. Transcription is the process in which an RNA copy is made of the DNA. In order to get the copy, many enzymes such as polymerase, helicase, exonuclease, ligase and single stranded binding protein work together to unwind the double helix and match the base pairs of RNA (adenine, guanine, cytosine and uracil) to the DNA, once the copy is made, the RNA molecule, which is now in the heterogeneous nuclear RNA (hnRNA) made, is still not ready to go express the gene by making a protein. The hnRNA must be spliced to remove non coding sequence, and protected from the cellular environment with a 5'cap and poly A tail. Finally, the hnRNA is transported out of the nuclear membrane and into the cytoplasm where it achieves the status of mRNA. In the cytoplasm, the mRNA molecule hooks up with the ribosomes where the protein production can start. Every three nucleotides in the mRNA molecule codes for a

specific amino acid and are appropriately called a codon. The codon pair with an anticodon of t-RNA that has attached to an amino acid. In this manner a polypeptide chain is formed. It will eventually twist and contort itself into a unique configuration which aids in the function of the protein.

Occasionally, a bad mRNA molecule is synthesized so that the resulting protein cannot function properly. Abnormalities of protein cause many diseases that afflict humans. Therefore, it seems logical to conclude that if the expression of these malfunctioned proteins could be stopped, the sources of disease would be obliterated and the disease will be treated. This idea is the basis for the antisense technology.

MODE OF ACTION OF ANTISENSE TECHNOLOGY:

Several possible mechanisms are involved during antisense inhibition of specific gene. Following are some of the most promising mode of actions have been suggested (Fig. 1)-

(i) Antisense oligonucleotides (AON)
(ii) Antisense RNA
(iii) Small interference RNA (Si RNA)

ACTION IN THE NUCLEUS PREMISE:

One of the earliest methods in antisense inhibition is carried out by blocking transcription. This happens to be within nuclear premise. It was shown that transcribed antisense RNA can have influential role in interfering RNA processing stage. Successful interferences are inflicted by blocking splicing of the mRNA. Thus, unspliced RNA or unprocessed RNA itself is not in a position to move out from nuclear premise and enter for translation process.

Formation of RNA-RNA duplex due to binding of sense and antisense transcript is quite unstable and are susceptible to be attacked by nuclease. Degradation of duplex RNA is then inevitable. In addition, duplex state of RNA hinder the processing or easy transport of sense mRNA across the nuclear membrane.

ACTION IN CYTOPLASMIC PREMISE:

RNA-RNA duplex may sometime escape from nuclear premise without being killed by nucleases. Inside the cytoplasm inhibition may also occur at translation stage when antisense transcript would compete with the ribosomes to bind specifically at 5′ end of the sense RNA (mRNA).

Generally, ribosomes are assembled on single-stranded mRNA and initiates translation. But binding of sense and antisense RNA (RNA duplex) completely prevents ribosome assembly on RNA duplex molecule in duplex status or assembled ribosomes cannot move along with duplex RNA. Therefore, completely arrest the translation process.

Binding of antisense RNA to target mRNA at specific sequence play a role in inhibiting translation process. While constructing artificial antisense gene on several occasions, a small fragment containing Shine—Dalgarno sequence for ribosome binding and the coding region will be inserted into expression vector into opposite orientation.

The resulting gene yields antisense RNA that is complementary to the ribosome binding site region of the mRNA. A shorter, antisense RNA lacking most of the sequence

complementary to the 5′ leader region of the mRNA was found to be less effective. These results clearly indicated to be importance of 5′ untranslated complementarily or the length of the complementary region in antisense RNA activity.

Further support for such evidence comes from the finding that cells engineered to produce antisense RNA complementary to the 5′ region of the tetracycline mRNA than controlled cultures. The extent of antisense RNA length and amino terminal complementarity appear to be correlated with high inhibition levels. Similarly, in the case of CAT antisense RNA, the RNAs complementary to the 5′ end of the target mRNA was more effective.

TYPES OF ANTISENSE METHODS

The three main types of antisense methods can inhibit the expression of a targeted gene. These antisense methods are - Antisense oligonucleotides

-Antisense RNA

- Small interfering RNA (Si RNA)/ RNAi

Both the methods are basically designed to blockade or degradation of a specific target mRNA as a consequence of binding of nucleic acids complementary to sequence of the mRNA.

METHOD 1. ANTISENSE OLIGONUCLEOTIDES (AON):

Transient inhibition of a specific gene expression can be achieved by using antisense oligonucleotides, which are short (14-18 bases) DNA molecules complementary to the 5′ leader sequence or 3′ end of mRNA. In cases where oligonucleotide is complementary to the coding sequence, the 5′-fragment can be translated to generate a truncated polypeptide.

The binding of antisense deoxy oligonucleotide and target mRNA leads to formation of a DNA-RNA duplex which is unstable and is recognized by RNase- H which selectively degrades the RNA strand in a DNA-RNA duplex, thus inhibiting translation. In case of blockage of RNase-H activity, oligonucleotide directed to cap sites can only inhibit translation as it inhibits binding of 40S subunit of ribosome.

Another class of oligonucleotides exists which are called the code blockers or triplex forming oligonucleotides, these bind in the major groove of DNA target sequence, thus inhibiting transcription. They can either inhibit the binding of transcription factors by binding upstream to the coding region or can inhibit movement of RNA polymerase. Triplex formation requires the target site to have a homopurine or homopyrimidine sequences and third strand binds by hoogsten base pairing, triplex strategy though is quite effective, especially in case of actively transcribing genes, yet it has its disadvantages like the requirement of a homopurine/homopyrimidine target sequence and further the problem of triplex instability under physiological conditions.

The effectiveness of oligonucleotides depends on several factors like position of target site against which they are directed, length of the oligonucleotide and further the presence or absence of secondary structure at the binding site. Stability of oligonucleotide is very

essential for effective inhibition, and, thus, in case of unmodified oligonucleotides, poor uptake and nuclease degradation were the limiting factors.

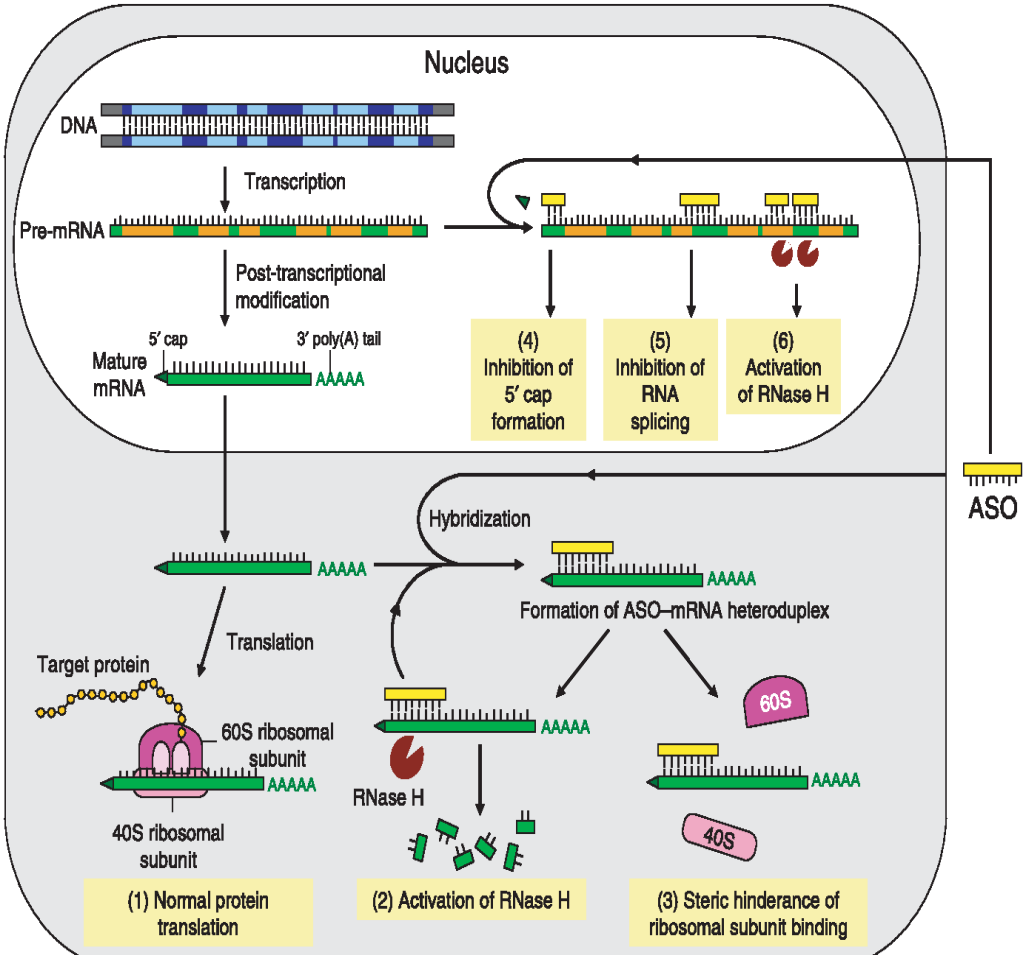


Fig: 2- Antisense oligonucleotides mechanism

Therefore, various types of modifications have been developed which render the oligonucleotide resistance to nucleases. The modifications are either of the phosphate backbone (e.g. methylphospho- nates, phosphorothioates, and phosphoroselonates) or the oligomer ends may be modified (K-oligo decarboxynucleotide).

Antisense oligonucleotide once placed inside the cell, its next destiny is to reach the site of mRNA and able to bind to its mRNA target. The target mRNA generally shows inaccessible to binding of AON due to its intramolecular base pairs formed between complementary sequences in single stranded mRNA. Thus, antisense oligonucletide methods of gene silencing still shows limited success and require many refined strategies.

METHOD 2. ANTISENSE RNA:

Another gene silencing approach is the antisense RNA technique which discussed above in detail. The technique involves the introduction of expression vector contains antisense gene into host cells, which inhibits expression of the gene from which it is derived.

It is presumed that expression of antisense gene results in RNA duplex forms between antisense RNA and transcript complementary mRNA preventing translation, thereby preventing expression of specific target gene.

FACTORS INFLUENCING ANTISENSE RNA REGULATION OF GENE EXPRESSION:

While making antisense constructs for specifically regulating the expression of a particular gene, certain factors have to be taken into account. The presence of antisense transcript much in excess of target mRNA is a prerequisite for effective inhibition, therefore the choice of promoter is important. Cauliflower mosaic virus (CaMV) 35S RNA promoter is a constitutive one and is the most widely used one.

Other commonly used promoters include nopaline synthase promoter, chlorophyll a/b-binding protein gene promoter, and CHS gene promoter. Identical promoters and terminators may be employed for both sense and antisense constructs but usually an excess of antisense transcripts is required, thus antisense gene is cloned along with constitutive promoters (**Robert *et al.* 1989**).

Since the formation of duplex of sense and antisense transcripts is the critical step for inhibition, the degree of homology and homology in certain specific regions is important. Though some heterogeneity is tolerated, e.g. starch synthase antisense gene from cassava could suppress the starch synthase in potato antisense apple ACC oxidase has been reported to inhibit ethylene production in tomato yet low inhibition is seen in cases where degree of homology is low.

Transgenic tobacco plants carrying antisense gene for tomato ACC oxidase showed variable inhibition in different parts and physiological states; this may be due to varying degree of homology or fluctuations in different tissues (**Einset, 1996**).

APPLICATIONS OF ANTISENSE RNA TECHNOLOGY:

Antisense strategies have been applied to plant systems as well as animal systems not only for production of novel mutants but also for studying the steps involved in particular metabolic pathways, identifying gene function, plant development, crop improvement and other novel uses.

Antisense RNA provides an opening in the study of regulation of viral genes, as an antisense inhibition can be taken to be a leaky mutation which would be useful in studying genes, mutations in which are lethal and their partial inhibition also leads to a significant change in phenotype.

Such partial inhibition was used to create a tobacco mutant deficient in NADH-hydroxypyruvate reductase to study the role of photo-respiration in stress protection. Besides unravelling the vital gene functions, antisense RNA inhibition has been used to observe various steps in metabolic pathways. Modified the activity of carbonic anhydrase

which had no significant impact on CO_2 assimilation but it brought forward the effect of decline in carbonic anhydrase activity on stomatal conductance and susceptibility to water stress.

Antisense mutants of tobacco with drastic decrease in Rubisco content resulted in low photosynthetic rate; however, the leaf development was normal and independent of Rubisco content though leaf development was delayed. The biochemical target of various herbicides is acetolactate synthase, and this was confirmed by raising transgenic potato plants expressing antisense acetolactate synthase which were inviable without amino acid supplementation, thus an in vivo model for herbicide action was put forward (**Hofgen *et al.* 1995**).

Similarly, the effect of ethylene on shoot morphogenesis was studied via the production of transgenic mustard plants expressing antisense 1-aminocyclopropane -1-carboxylic acid (ACC) oxidase gene, and such plants showed marked increase in regeneration potential and corresponding decrease in ethylene production. Cotton fibre protein genes have also been characterized using antisense RNA inhibition of a particular gene. Antisense inhibition has been utilized to work out the role of lipoxygenase (LOX) in lentil protoplast by the introduction of antisense LOX gene (**Maccarone *et al.* 2000**).

The antisense RNA technology has formed the basis for elucidating the flavonoid biosynthetic pathway, and, as a matter of fact, CHS gene was the first endogenous gene targeted by antisense RNA in plants. Antisense CHS petunia plants produced flowers with pale corolla pigmentation but the steady state levels of mRNA of other flavonoid-specific genes were not Other than elucidating the steps of metabolic pathways, antisense RNA inhibition has found its use in identification of gene function as in case of a ripening gene (pTOM5), which was found to be a part of carotenoid pathway (**Bird *et al.* 1991**).

Transgenic Flaveria bidentis plants with antisense Rubisco gene were used to study the relationship between CO_2 assimilation and Rubisco content in C_4 plants and it was observed that the inhibition of Rubisco led to increase in CO_2 concentration and its leakage in bundle sheath.

The importance of peptide transport gene AtPTR2-B from Arabidopsis was evaluated by producing transgenic with antisense AtPTR2-B gene, the transgenics had altered phenotype, delayed flowering and no seed set, suggesting a major role of the gene in growth and development.

Other examples of the antisense RNA technology being utilized for elucidating gene functions include the transgenic tobacco-expressing antisense ascorbate peroxidase (APX) gene leading to increased susceptibility of transgenics to ozone injury, suggesting the major role of APX in oxidative stress tolerance. Antisense inhibition of biotin carboxylase gene in tobacco led to severe retardation of growth, reinforcing the importance of biotin for plant growth.

The antisense RNA technology has also been used for crop improvement, besides being used to gain knowledge in the basics of plant development. The technology has been used in modifying seed oil composition of Brassica seed oil. The desaturase enzyme gene was inhibited, leading to production of seeds with high-stearate oil content without there being any decrease in the seed lipid content.

The antisense RNA technology has recently been reported to generate Hsp70 mutant in A. thaliana and this brought forward the protective role of HSp70 in thermo tolerance and

a regulatory effect on heat shock transcription factors leading to auto regulation of the heat shock response.

The antisense gene strategy has been applied to inhibit the expression of an allergen gene during seed maturation in rice, and inhibition persisted in the progeny of the transformed plants.

The antisense RNA technology has certain basic lacunae which need to be overcome before its full potential is exploited. Firstly, there seems to be no single universal mechanism of antisense action, each system has to be studied independently for its mechanistic aspects of inhibition.

The degree of inhibition is quite variable even when single copy of antisense gene is present, this aspect needs to be studied further in correlation with copy number and positional effect on the antisense gene expression. Besides these, the long-term effects of expression of antisense gene or dosage of antisense oligonucleotides, their degradation and effects of nonspecific binding need to be investigated.In conclusion, the antisense RNA technology holds promise both for the plant systems as well as the animal systems but before its extensive use, the basics of the technology have to be elucidated and the technology accordingly modulated so that it may be exploited to its full potential.

Small interfering RNA (Si RNA)/ RNAi-

In this techniuque double stranded RNA molecule (ds RNA) is cleaved to form 21-23 bp double stranded fragments called short interference RNA (Si RNA).

Si RNA is unbounded by helicase activity associated with the multi-protein complex known as RNA induced silencing complex (RISC). The antisense RNA with RISC binds to its corresponding m RNA which is cleaved by the enzyme slicer rendering it inactive.

Advantages

- Specifically target a gene
- Varying levels of gene silencing using the same ihpRNA in different lines
- The timing and extent of the gene silencing can be controlled
- Great degree of flexibility in the field of functional genomics
- To protect the genome from viruses

Limitations

- For the use of RNAi the exact sequence of the target gene is required
- Delivery methods for the dsRNA is a limiting step for the number of species which RNAi based approaches can be used easily
- It does not knockout a gene for 100 per cent
- Expensive
- Ethical problems

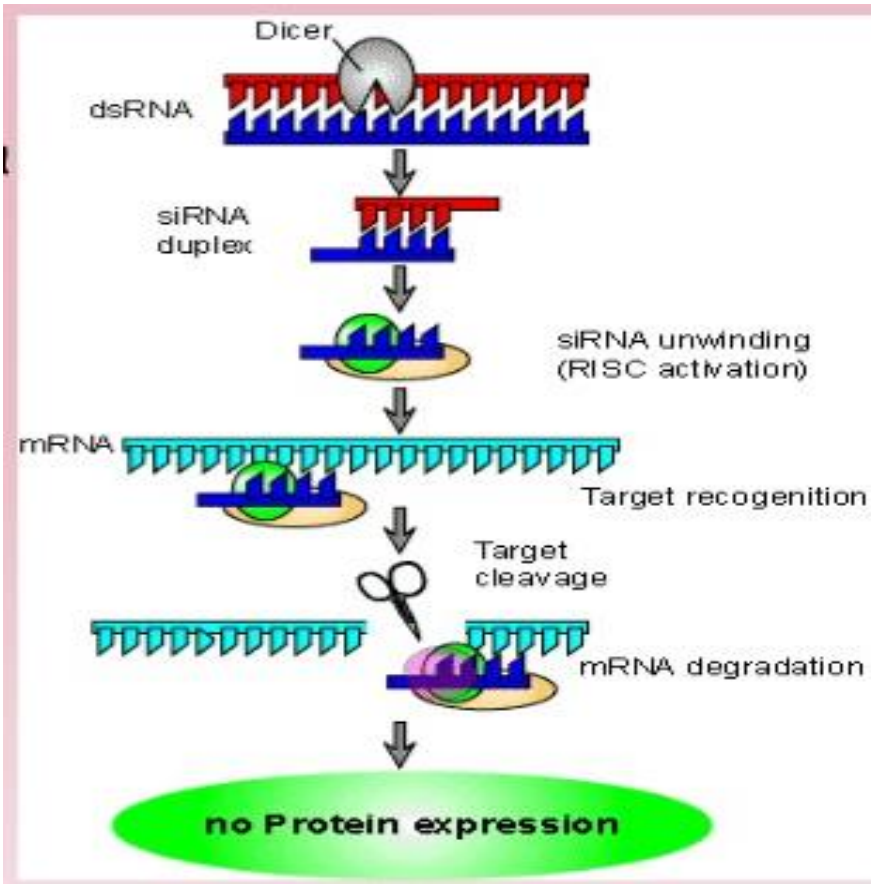


Fig- 3 Source: http://www.scq.ubc.ca/wp.content/siRNA.gif

Online tools for RNAi resources

S.No.	**Purpose**	**Online tools**
1.	Resources on RNAi	http://srna-tools.cmp.uea.ac.uk/plant/
2.	Computation model to predict gene function	http://www.sciencedaily.com/releases/2010/01/100131142436.html
3.	Target finder	http://bioinfo3.noble.org/psRNATarget/
4.	RNAi design tool	https://rnaidesigner.invitrogen.com/sirna/ http://biotools.idtdna.com/rnai/
5.	siRNA selection	http://jura.wi.mit.edu/siRNAext/register.php
6.	Find restriction sites	http://tools.neb.com/NEBcutter2/
7.	miRNA database	http://www.mirbase.org/
8.	For careful selection of an insert gene sequence	http://bioinfo2.noble.org/RNAiScan/RNAiScan.html

Flaver savr Tomato –a case study

Fruit-specific RNAi-mediated suppression of SLNCED1 increases both lycopene and β-carotene contents in tomato fruit.

The Vit A is an essential nutrition that occur in several forms – RETINOIDS. Plant sources contain pro-vit A caroteniods most important of which is β-Carotene. Dietary caroteniods essentially require for humans where β-carotene is most potent dietary precursor of vit A, deficiency of which leads to blindness, premature death. The biosynthetic pathway begins with the formation of phytoene from two molecules of geranyl geranyl diphosphate (GGPP) in the central isoprenoid pathway. Therefore, a key step in ABA biosynthesis, 9-cisepoxycarotenoid dioxygenase (NCED), was targeted for inhibition via RNAi in tomato fruit.

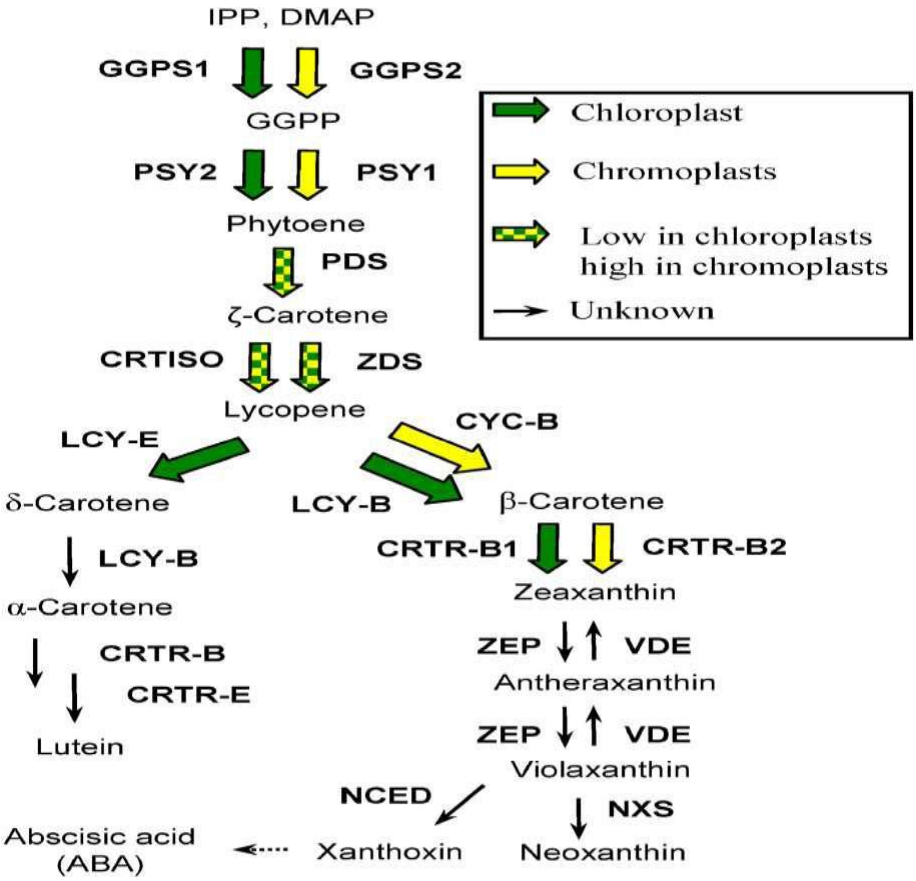
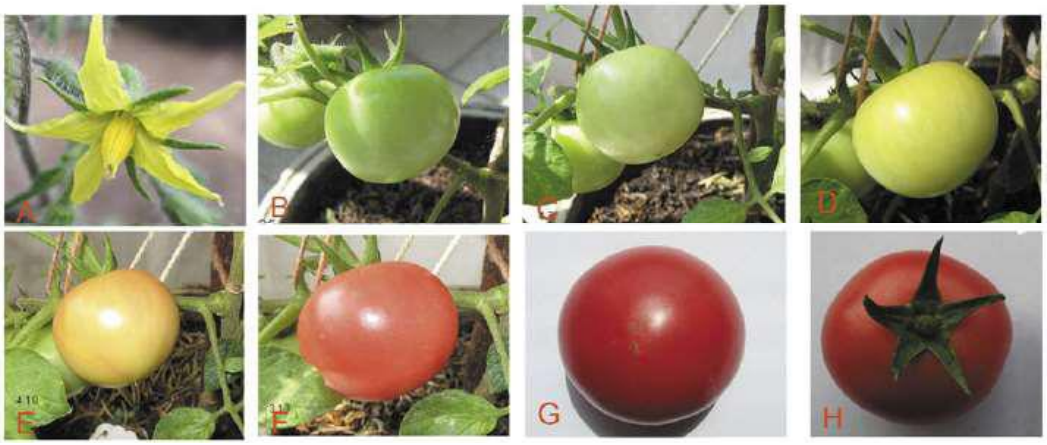


Fig- 4 Key step of ABA biosynthesis via RNAi in tomato fruit.

Fruit development and ripening of SlNCED1-RNAi line and Control fruit



SlNCED1-RNAi line

Control fruit

Application of anti sense RNA technology in crop improvement

S.No.	Characters	Examples
1.	Alteration of plant architecture	Plant height, short branching, leaf & inflorescence morphology
2.	Abiotic stress tolerance	Drought, flood, low, salinity & high temperature
3.	Biotic stress tolerance	Insects, nematodes, virus Fungal & bacterial diseases
4.	Nutritional improvement	Vitamin A, Zinc, Iron, Carotenoids
5.	Removal of toxic compounds	Caffeine, cyanogenic glycosides, gossypol

6.	Prolongation of shelf life	Tomato
7.	Engineering of secondary metabolites	Morphine, Ginsenoside, artemisinin
8.	Seedless fruit development	Tomato
9.	Development of male sterile plants	Rice

FUTURE THRUSTS OF ANTISENSE RNA :

- Manipulating new RNAi pathways, which generate small RNA molecules to amend gene expression in crops, can produce new quality traits.
- Applying RNA silencing in single cell organisms like *Chlamydomonas*, an algae close to plants, will enable high-throughput plant gene function identification.
- RNAi can prove its potential for inhibition of photorespiration to enhance the productivity of C_3 plants.
- Agronomically superior cultivar can be engineered for additional plant fitness (e.g. stress tolerance) by using RNAi technology.

CONCLUSION

- Anti sense RNA has become a major focus of molecular biology around the world.
- Anti sense RNA is coming increasingly into centre of attention through a combination of genetic engineering and biochemical studies related to silencing pathways.
- Anti sense is found to be very promising technique to prove function of any gene.
- It is even better than conventional transgenic technologies where they generally need the expression of whole genes, whereas, Anti sense requires comparatively small transgene for silencing, permitting multiple genes to be targeted in a single construct.
- Thus, Anti sense is accurate on our platforms of expectation for genetic improvement of cereal crops.

REFERENCES-

1. J.N.M. Mol, A.R. van der Krol, A.J. van Tunen, R. van Bfokland, P. de Lange and A.R. Stuitje (1990) Regulation of plant gene expression by antisense RNA, *Federation of European Biochemical Societies* Volume 268, number 2, 427-430.
2. Robert, L.S., Donaldson, P. A. Ladaigue, C., Altosaar, I., Amison, P.G. and Fahijanski, S.F. (1989) *Plant Molecular Biology*, 13, 399-409.
3. Stuti Gupta, Ravindra Pal Singh, Nirav Rabadia, Gaurang Patel, Hiten Panchal, (2011) *International Journal of Pharmaceutical Sciences* Review and Research, 9(2), page 38.
4. Rusk N, 2015. Understanding noncoding RNAs. *Nat Methods*, 12(1):35.
5. Qi P, Du X, 2013. The long non-coding RNAs, a new cancer diagnostic and therapeutic gold mine. *Mod Pathology*, 26(2): 155-165.

6. http://www.biologydiscussion.com/plant-biotechnology-2/types-of-antisense-method plants/72258
7. Einset J, Pratt C (1956). 'Giant' sports of grapes. *Proc Am Soc Horticult Sci* 63: 251–256.
8. R. Hofgen, B. Laber, I. Schuttke, A. K. Klonus, W. Streber, and H. D. Pohlenz, (1995) *Plant Physiology*, 107(2): 469–477.
9. Mauro Maccarrone, A. Rossi, Alessandro Finazzi Agrò, (2000) Repression of Acetolactate Synthase Activity through Antisense Inhibition (Molecular and Biochemical Analysis of Transgenic Potato (*Solanum tuberosum* L. cv Desiree), *Book Transgenic Crops I* (pp.325-337).
10. BirdCR, Ray JA, Fletcher JD, Boniwell JM, Bird As, Teulieres C, Blain I, Bramley PM, Schuch W. **(**1991**)** Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. *BioTechnology***,** 9,635–639.
11. MayerMP, Nievelstein V, Beyer P. (1992) Purification and characterisation of a NADPH-dependent oxidoreductase from chloroplasts of *Narcissus*—a redox mediator possibly involved in carotene desaturation. *Plant Physiology and Biochemistry*, 30,389–398.