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ANTISENSE RNA TECHNOLOGY IN FOOD BASED APPLICATIONS

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ABSTRACT
Antisense RNA technology is an important molecular approach for regulating gene expression through targeted inhibition of specific genes. The technology is based on the complementary base pairing of antisense nucleic acid sequences with target messenger RNA (mRNA), thereby reducing or preventing the production of specific proteins. Gene silencing and antisense technology have significant applications in plant biology, food crops and therapeutic research. In plants, antisense technology has been used to suppress genes associated with fruit ripening, softening, pigmentation and enzymatic discoloration. A notable application is the Flavr Savr tomato, in which suppression of the polygalacturonase gene reduced pectin degradation and delayed fruit softening, thereby improving post-harvest characteristics. Antisense approaches have also been investigated for suppressing disease-associated proteins, viral infections and oncogene products, demonstrating their potential in therapeutic applications. The review highlights the principle and mechanism of antisense technology, its role in gene silencing, major applications in food-based systems, and its therapeutic significance. Although antisense technology provides a targeted approach for modifying gene expression, challenges related to specificity, delivery, off-target effects and the complex regulation of agricultural traits remain. Overall, antisense RNA technology represents a valuable molecular tool for improving crop quality, extending shelf life and developing targeted strategies for regulating gene expression.

KEYWORDS: Antisense RNA technology; Gene silencing; Antisense oligonucleotides; mRNA; Gene expression; RNA interference; Flavr Savr tomato; Fruit ripening; Post-harvest quality; Therapeutic applications.

INTRODUCTION

Diseases are often connected to the insufficient or excess production of certain “Proteins”. If the production of these proteins is disputed many diseases can be treated or cured. The world population has reached around 7.7 billion and it is projected to reach 9.7 billion by 2050 (Rajam, 2020). To satisfy the world food demand, improving the production and productivity of food crops is required (Saurabh et al., 2014). Antisense technology is a method that can dispute protein production. It may be used to design new therapeutics for diseases in whose pathology the production of a specific protein plays a circle role. Antisense technology is a tool that is used for the Inhibition of gene expression. The principle behind it is that an antisense nucleic acid sequence base pairs with its complementary sense RNA strand and prevents it from being translated into a protein. The complimentary nucleic acid sequence can be either a synthetic oligonucleotide, often oligodeoxy ribo nucleotides (ODN) of less than 30 nucleotides or longer antisense RNA (aRNA) sequences. An example of sense and antisense RNA is: - 5’ACGU3’mRNA and 3’ UGCA5’ Antisense RNA (Allison et al., 2006).
1990 Richard Jorgenson gave the phenomenon of 'co suppression of gene expression'. 1992 Romano and Macino gave the phenomenon of as Virus Induced Gene Silencing, set of such phenomenon was termed as post transcriptional gene silencing. 1994 Calgene california company brought the GM tomato crop with the as RNS technology. 1995 RNA silencing was first documented in animals by Guo and Kemphues and for this phenomenon they coined the term antisense mediated silencing.1998 Andrew Fire and Craig C.Mello coined the term RNA interference (RNAi). 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). 2001 Gregory Hannon 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 mRNA7. 2002 Effect named "short hairpin-activated gene silencing". 2004 Morris et al. observed that asRNA silences genes at the transcriptional level.

Protien Formation

Proteins are constantly being produced in the body. Protein production is triggered by various stimuli, such as hormones. The formation of the proteins occurs in the following two steps: Transcription and Translation.
Protein molecules are the expression of a gene. However, to get to 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 (Fig.1). 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.

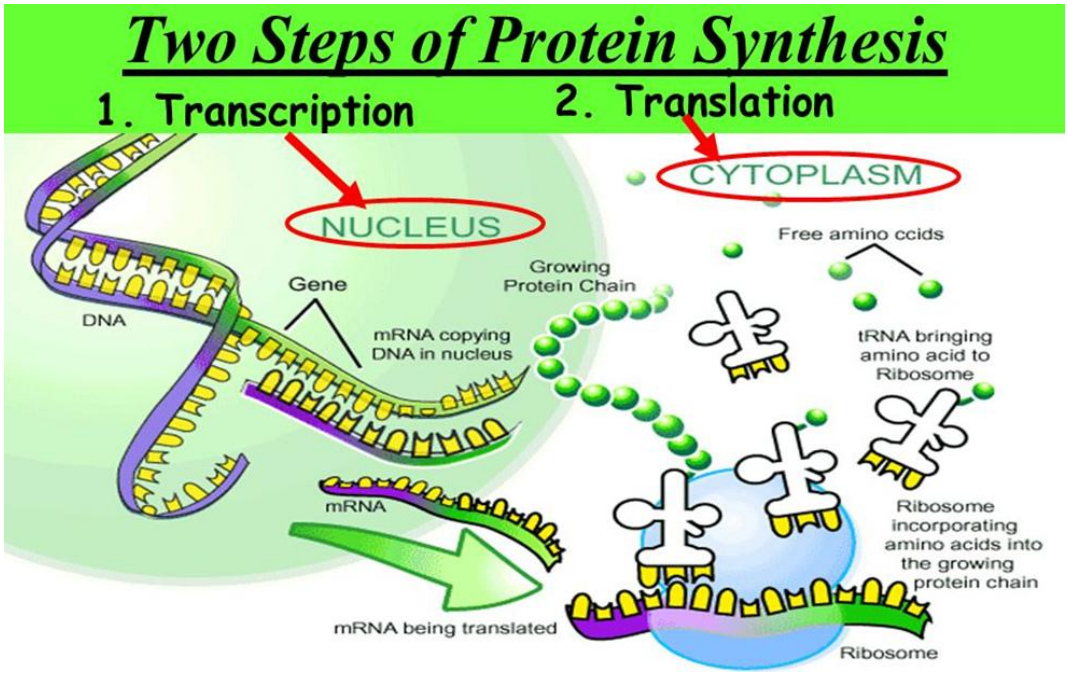


Fig.1 Two step of protein synthesis

Gene Silencing

Gene silencing is an effective molecular procedure that inhibits the expression of a speci c gene, and it serves as a regulatory mechanism for gene expression, determining cell fate and metabolic activities during an organism's lifespan. Gene silencing eliminates the effect of a gene by inhibiting its expression (Dash et al. 2015; El-Sappah et al. 2021; Woodward 2022). The presence of double-stranded RNA (dsRNA) in the cytoplasm is the primary factor that induces gene silencing (Fire et al. 1998; Mu et al. 2018). In plants, gene silencing is critical for protection against abnormal RNA, which can be fatal if it persists in the RNA pool of a cell (Dunoyer et al. 2006). This is achieved through nonsense-mediated mRNA decay (NMD) which could also be used for antisense therapeutic purposes (El-Sappah et al. 2021; Woodward 2022).

Antisense Technology

The idea of designing oligonucleotides to bind to specific sequences in target RNAs via Watson–Crick hydrogen bonding and the term "antisense" were introduced in 1978. As proposed, the idea was quite simple and the word antisense was nonspecific. The authors were agnostic as to the post-RNA-binding mechanisms that would ensue to alter the behavior and performance of a target RNA, the structure of drug administered (single or double strand) or the chemical modifications that might be required to introduce acceptable properties for therapeutic administration. "Antisense" included oligonucleotides of any structure or chemistry designed to bind target RNA via Watson–Crick hybridization. In practice, antisense is used to refer to ss ASOs, and siRNA refers to ds ASOs that act via AGO2 to cause degradation of target RNAs (Lauffer et al., 2024)

In Antisense technology, synthetically – produced complementary molecules seek out and bind to messenger RNA (mRNA), blocking the final step of protein production. mRNA is the nucleic acid molecule that carries genetic information from the DNA to the other cellular machinery involved in the protein production. By Binding to mRNA, the antisense drugs interrupt and inhibit the production of specific disease-related proteins. "Sense" refers to the original sequence of the DNA or RNA molecule. "Antisense" refers to the complementary sequence of the DNA or RNA molecules. The basic idea is that if an oligonucleotide (a short) RNA or DNA molecule complementary to a mRNA produced by a gene) can be introduced into a cell, it will specifically bind to its target mRNA 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 ribonucleotide.H. Therefore, the introduction of short chains of DNA complementary to m RNA will lead to a specific diminution or blockage, of protein synthesis by a particular gave. In effect, the gene will be turned off (Stuti et al., 2011).

Antisense Rna Technology

Antisense RNA Technology > tool used for inhibition of gene expression.
Single stranded RNA called oligo de oxy nucleotides.
Oligonucleotides are complementary to the mRNA, which physically bind to the mRNA.
Prevents the synthesis of specific protein.
Powerful weapon for Studying gene function and for discovering more specific treatments of diseases.
Gene silencing =Antisense RNA technology.

How Does Antisense Technology Work

Antisense technology is a molecular approach used to inhibit the expression of specific genes by preventing the production of their corresponding proteins. The therapeutic objective of antisense technology is to block the production of disease-causing proteins. This is achieved by creating a synthetic "antisense" or complementary nucleotide sequence of DNA or RNA that interacts with and binds to the "sense" or original mRNA sequence. The resulting mRNA–antisense complex can no longer be efficiently translated, thereby preventing the production of the disease-causing protein. Thus, antisense technology provides an effective strategy for regulating gene expression and modifying specific biological characteristics.

The degree of antisense RNA production in plant cells depends on several factors, including the type and strength of the promoter sequence selected. The antisense construct is incorporated into a plasmid and subsequently transferred into Escherichia coli (E. coli), which serves as a host for plasmid maintenance and multiplication. The hybrid gene can then be transferred from E. coli to Agrobacterium tumefaciens through triparental mating. This method facilitates the transfer of recombinant genetic information between bacterial hosts. Following this process, the gene of interest becomes incorporated into the A. tumefaciens Ti plasmid and is positioned adjacent to the T-DNA region. The T-DNA-associated construct can subsequently facilitate the transfer of the antisense gene into plant cells, resulting in the expression of antisense RNA and suppression of the target gene.

Nekhotiaeva et al. (2004) investigated the potential of antisense peptide nucleic acids (PNAs) to inhibit gene expression and bacterial growth in Staphylococcus aureus. Since conventional oligonucleotides have limited uptake into bacterial cells, the researchers used PNAs conjugated to the carrier peptide KFFKFFKFFK to facilitate cellular delivery. A xylose-inducible gfp reporter system was used to evaluate the antisense activity of the peptide–PNAs.

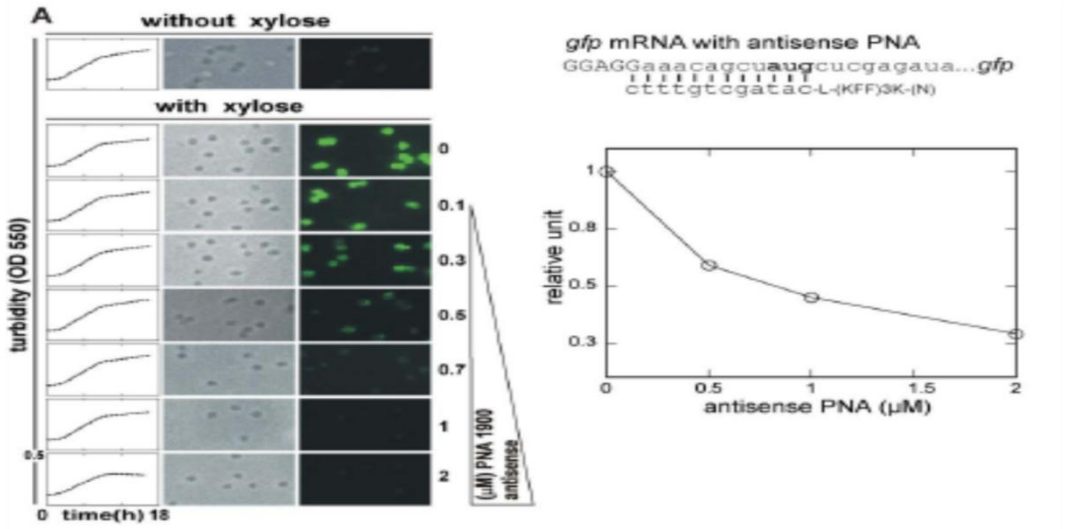
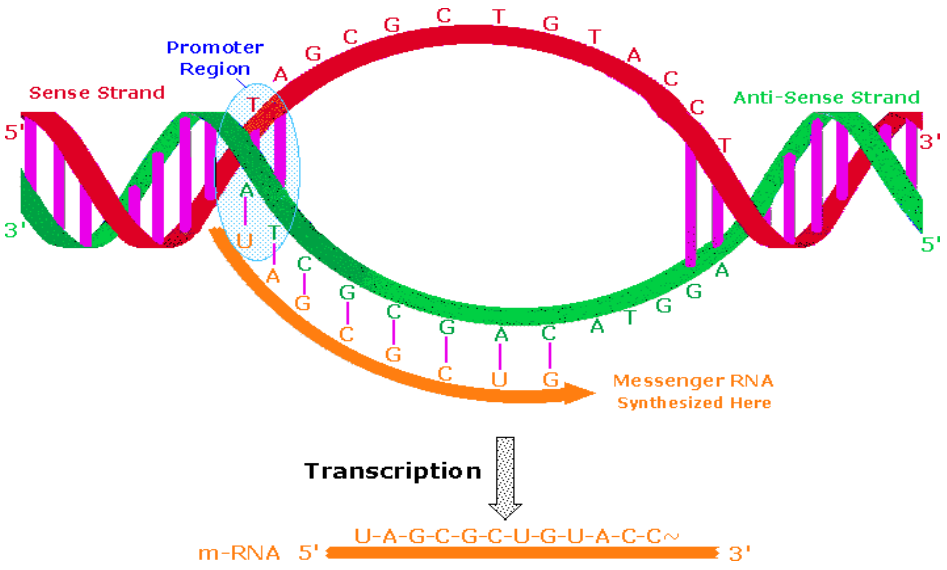


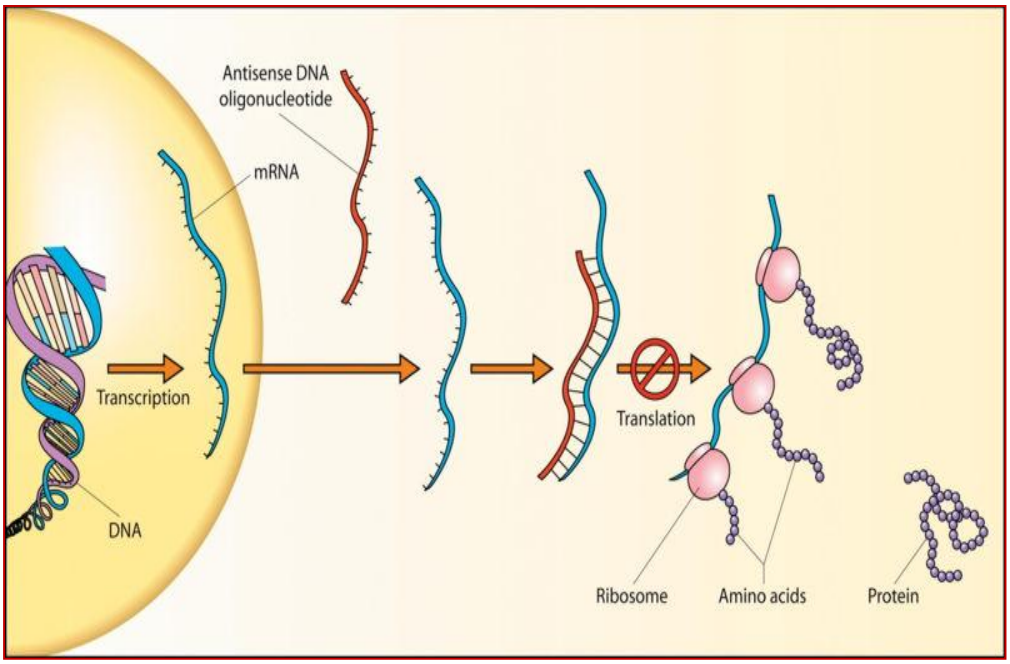
Fig. 1. Expression and inhibition of gfp using antisense peptide–PNAs in Staphylococcus aureus. After overnight growth in the presence or absence of PNA, S. aureus cells were visualized by light and fluorescence microscopy. The control cells grown without xylose showed no GFP expression. The graph on the right shows the relative levels of GFP in S. aureus cultures in the absence and presence of different concentrations of antisense peptide–PNA 1900.

The results demonstrated a clear dose-dependent and sequence-specific inhibition of GFP expression. As shown in Fig. 1, increasing concentrations of antisense peptide–PNA 1900 progressively reduced GFP fluorescence in S. aureus. At the highest concentration tested (2 μM), GFP expression was reduced to approximately 30 per cent of the untreated control, corresponding to about 70 per cent inhibition of GFP production. In contrast, untreated cells showed strong GFP fluorescence following xylose induction. The inhibitory effect was substantially reduced when a mismatched PNA was used, demonstrating that the effect was dependent on complementary base pairing between the PNA and its target mRNA. Naked PNA without the carrier peptide failed to produce significant inhibition, indicating that efficient cellular delivery was important for antisense activity.

The study further demonstrated that antisense peptide–PNAs could target endogenous chromosomal genes. PNAs directed against the phoB gene produced a dose-dependent reduction in alkaline phosphatase activity, whereas unrelated and mismatched PNAs showed considerably weaker effects. Importantly, PNAs targeting the essential genes fmhB, gyrA and hmrB produced growth inhibition, demonstrating that antisense PNAs could potentially be used not only for gene-function studies but also as antibacterial agents. The authors also observed that alterations in either the PNA sequence or its target mRNA sequence reduced or abolished inhibition, providing further evidence for sequence-specific antisense activity.

Overall, this study provided important evidence that peptide-conjugated PNAs can enter S. aureus cells and selectively suppress bacterial gene expression through an antisense mechanism. The findings established the feasibility of using antisense PNAs for functional genomics and suggested their potential as a new class of antibacterial agents. However, the study also highlighted the importance of improving intracellular delivery and carrier-peptide design before PNAs can be developed into effective therapeutic antibacterial agents. Subsequent work has extended this approach to clinically relevant S. aureus targets, including the essential transcription factor σ70 (rpoD) in methicillin-resistant S. aureus.





Antisense Therapies In Development

The therapeutic applications of antisense technology have expanded with increasing understanding of the relationship between specific genes and disease development. Antisense-based approaches have been investigated as potential therapeutic strategies for selectively regulating gene expression and suppressing the production of disease-associated proteins. Several antisense therapies have progressed through different stages of clinical development, including clinical trials, while some antisense-based therapeutics has received regulatory approval for clinical use. The continued development of antisense technology has highlighted its potential as a targeted approach for the treatment of genetic and acquired diseases (Takaha et al., 1998).

Antisense And How It Applies To Flavr Savr

One of the important applications of antisense technology in crop improvement was the development of the Flavr Savr tomato by Calgene. The approach was designed to suppress the expression of the polygalacturonase (PG) gene, which plays an important role in fruit softening during ripening. Polygalacturonase contributes to the degradation of pectin in plant cell walls, resulting in the softening of ripe fruits. Reduction of polygalacturonase activity was therefore expected to delay softening and improve the post-harvest characteristics of tomato.

The development of the Flavr Savr tomato involved the preparation of complementary DNA (cDNA) corresponding to the PG gene. A specific approximately 730-base-pair region, including a non-coding sequence, was isolated and cloned into a plasmid. The selected fragment was subsequently incorporated into an expression construct downstream of the cauliflower mosaic virus 35S (CaMV 35S) promoter in an antisense orientation. The resulting construct was introduced into Agrobacterium tumefaciens, which was used as a vector for transferring the recombinant DNA into tomato cells. The transformed cells were then regenerated into plants expressing antisense RNA directed against the PG transcript.

Flavr Savr Tomato And Fruit Ripening

The development of Flavr Savr tomato was intended to address an important limitation associated with conventional tomato harvesting and post-harvest handling. Conventional tomatoes are often harvested before complete ripening to withstand transportation and handling. They can subsequently be exposed to ethylene, a plant hormone that promotes ripening and development of the characteristic red color. However, harvesting before full ripening and subsequent artificial ripening may affect the development of desirable sensory characteristics.

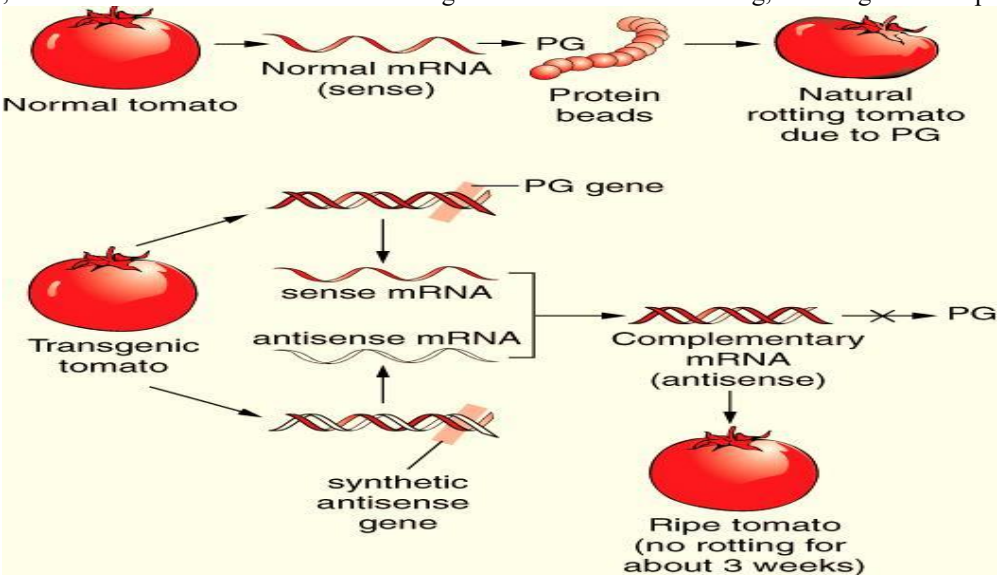
The Flavr Savr approach aimed to reduce the activity of polygalacturonase and thereby slow the degradation of pectin in the cell walls. The expected outcome was delayed fruit softening, allowing tomatoes to remain firm for a longer period while retaining desirable color and flavor characteristics. Reduced softening could also potentially decrease physical damage and susceptibility to post-harvest deterioration.

Flavr Savr tomato was developed by Calgene and became a notable milestone in the history of genetically modified food crops. It was reported as the first genetically modified whole food approved by the U.S. Food and Drug Administration (FDA), with approval granted in 1994. The development represented an early demonstration of how antisense technology could be used to modify a specific biochemical pathway associated with an economically important crop trait.

Performance And Limitations Of Flavr Savr Tomato

Although the antisense PG strategy successfully reduced polygalacturonase activity and contributed to an increase in shelf life, the expected improvement in fruit firmness was not fully achieved. The reduction in PG expression did not completely prevent softening because fruit texture is a complex characteristic controlled by several enzymes and biochemical processes.

Consequently, the Flavr Savr tomato still faced challenges associated with harvesting, handling and transportation.



The development nevertheless demonstrated the potential of antisense technology for modifying post-harvest characteristics through targeted suppression of gene expression. Subsequent improvements in flavor and consumer acceptance were also explored through conventional breeding approaches involving Flavr Savr and better-tasting tomato varieties.

Significance Of Flavr Savr In Antisense Technology

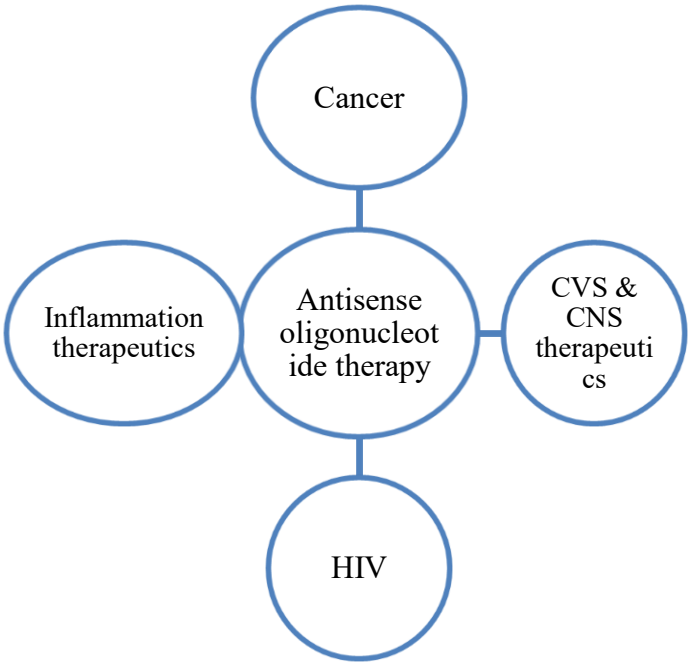
The Flavr Savr tomato represents an important case study in the application of antisense technology to crop improvement. Rather than introducing a new protein-producing trait, the strategy focused on reducing the expression of an endogenous gene involved in fruit softening. This demonstrated the potential of gene suppression for improving shelf life and post-harvest quality.

The Flavr Savr experience also highlighted the complexity of translating molecular-level gene suppression into desirable whole-plant characteristics. Although suppression of the PG gene influenced fruit softening and shelf life, it did not

produce the complete improvement in firmness originally anticipated. This illustrates the importance of understanding the interaction of multiple genes and biochemical pathways when developing genetically modified crops.
Overall, antisense technology has contributed significantly to the development of targeted approaches for regulating gene expression in both therapeutic and agricultural applications. Its use in Flavr Savr tomato provided an early example of applying molecular biotechnology to post-harvest quality improvement. At the same time, challenges related to specificity, delivery, off-target effects and the complex genetic control of agricultural traits continue to influence the development and application of antisense-based technologies.

Prevention Of Discoloration

The post-harvest discoloration of fruits and vegetables is a considerable problem for the food industry. A lack of acceptance of discolored foods by consumers has been dealt with by the food industry through the use of food additives in a wide range of foods. However, recently, the safety of some of these food additives, in particular sulfites, has been questioned. The enzymes that are thought to be responsible for the initial step in the discoloration of fruits and vegetables, the oxidation of monophenols and o- diphenols to o-quinones are polyphenol oxidases. These nucleus-encoded enzymes with a molecular weight of around 59,000 Daltons are localized in chloroplast and mitochondrial membranes. The contention that inhibition of the enzyme polyphenol oxidase would decrease the extent of discoloration has been tested with transgenic potatoes carrying a number of different polyphenol oxidase cDNA constructs. Vectors were constructed with either the full-length or partial potato polyphenol oxidase cDNA in either the sense or antisense orientation under the control of the cauliflower mosaic virus 35S promoter, the granule-bound starch synthase promoter, or the patatin type I promoter. The latter two promoters are specific for the potato tuber. The two commercial varieties of potato that were transformed with these constructs are considered to have a good level of resistance to black spot (enzymatic discoloration), so an increase in black spot resistance by genetic manipulation would be greater than what could be attained by traditional breeding techniques Transgenic plants with the polyphenol oxidase cDNA constructs were deliberately bruised, and then the extent of black spot damage was assessed. Most of the transgenic potato plants with an antisense version of the polyphenol oxidase gene under the control of either the cauliflower mosaic virus 35S promoter or the granule bound starch synthase promoter were significantly more resistant to black spot than the non-transformed potatoes. The patatin promoter, which may not be fully active in potato tubers, did not prevent polyphenol oxidase accumulation. The sense constructs all synthesized increased amounts of



polyphenol oxidase and showed larger amounts of black spot than non-transformed control plants. Similar antisense constructs are likely to reduce enzymatic discoloration in wide range of commercially important plants (Krit et al., 2007).
A second application of this technology and one that is potentially of more immediate relevance to the practicing physician, in the use of this technology in therapy. In principle, antisense oligonucleotides complementary to viral RNAs can suppress a wide variety of viral infections, a tremendous amount of research is ongoing in this area, similarly, antisense oligonucleotides directed towards the products of oncogenes can play a role in reducing the growth of cancer cells, and this leads in being hotly pursued. Perhaps the most widely discussed application of antisense technology lies in its applications to gene therapy. In this case, a variety of vectors is used to introduce antisense encoding genes into a large number of cells in a patient or animal to produced long term inhibition of a protein.
For example, in animal models the introduction of vectors encoding antisense angiotensin II receptor sequences results in long term norm tension in spontaneously hypertensive animals. These are but a few of the possible application of antisense technology. As familiarity with the relevant chemistry increases, it is likely that more effective

oligonucleotides and gene vector will be developed thereby providing the ability to interfere at will with the translation of specific mRNA (Behrooz et al., 2007).

Antisense Therapy

Genetic disorder or infections.

A complementary mRNA strand is synthesized on the basis of the known pathogenic sequence, and which switch 'off' the pathogenic gene by activating the degrading enzyme RNase H.

Antisense drugs are being researched to treat cancers, HIV and CMV etc.

Formivirsen is the first antisense antiviral drug developed to treat CMV. It was licensed by FDA in Aug, 1998 (Behrooz et al., 2007).

Indian Contribution

NIPGR (National institute of Plant Genome Research) in Feb, 2010 has developed a tomato by antisense technology which can last long up to 45 days.

NIPGR scientist had silenced the expression of two important genes which are responsible for loss in firmness and textures during ripening.

The two gene silenced are alpha-man and beta-hex of Glycosyl hydrolase, a kind of enzyme that breaks the chemical bond holding a sugar to either another sugar or some other molecule, like a protein.

Applications In Food Crops

Enhancing nutritional values and quality

It is well known that antisense technology is capable of increasing the nutritive value of crops (e.g., amino acids, fatty acids, fiber), removing/decreasing undesired toxic compounds, creating male sterility for crop breeding, enhancing shelf life, and modifying many other traits (Auer & Frederick, 2009). Improving protein quality has been reported using antisense RNA technology. Zeins are proteins that are specifically expressed during seed development and act as a reservoir of free amino acids. In maize, RNA silencing has been successfully applied to produce high lysine maize variants by knocking out the expression of 22-kD maize zein storage proteins (Song et al., 2001). Corn proteins are largely (60%) made up of zein, storage proteins, that barren essential amino acids like lysine and tryptophan. Reducing the zein content by RNA silencing technology results in the production of high lysine and tryptophan content maize (Frizzi & Huang, 2010)

Tomatoes are the source of carotenoids and flavonoids, which are highly beneficial for human health. The suppression of genes (DET1) encoding biosynthetic enzymes has led to tomatoes with improved carotenoids or flavonoids. Fruit specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes (Davuluri et al., 2005).

Glutenin is a major protein found in most food crops including rice, wheat, and maize. Glutenin is essentially liable for the functional properties of dough that increase the viscoelasticity of the dough from these crops (Zhaojun Wang et al., 2017). Gliadins contribute mainly to the extensibility and viscosity of gluten and dough, with the polymeric glutenins being liable for elasticity. The extent of glutenin could be reduced using RNA silencing technology producing a spread of crops called LGC (low glutenin content). Silencing the expression of specific ɣ [gamma]-gliadins by RNAi has demonstrated the feasibility of systematically silencing specific groups of gluten proteins (Gil- Humanes et al., 2008).

CONCLUSION

Antisense RNA technology is a powerful molecular tool for the specific regulation and suppression of gene expression. By targeting complementary RNA sequences, it provides an approach for reducing the production of specific proteins and modifying desired biological characteristics. Its applications in food crops have demonstrated considerable potential, particularly in controlling fruit ripening, softening and enzymatic discoloration. The Flavr Savr tomato is an important example of the successful application of antisense technology for improving post-harvest characteristics through suppression of the polygalacturonase gene. The technology also has potential therapeutic applications in the treatment of genetic disorders, viral infections and diseases associated with abnormal protein production. However, challenges such as delivery, specificity, off-target effects and the complex interaction of multiple genes and biochemical pathways need to be addressed. Continued advances in molecular biology and antisense-based approaches may further improve the efficiency and applicability of this technology. Overall, antisense RNA technology has significant potential in both food-based applications and therapeutic research.

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