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Ankit Kumar Sinha
Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi, India

Ishu Kumari
Department of Horticulture and Fruit Science, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Subhashree Subhasmita
Department of Vegetable Science, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Jitendra Rajak
Department of Horticulture Vegetable & Floriculture, Bihar Agricultural University, Sabour, Bihar, India

Atul Kumar Singh
Department of Horticulture and Post-Harvest technology, Institute of Agriculture, Visva Bharti University, Sriniketan, West Bengal, India

Corresponding Author:
Ankit Kumar Sinha
Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi, India

Exploitation of antisense RNA technology in horticultural crops

Ankit Kumar Sinha, Ishu Kumari, Subhashree Subhasmita, Jitendra Rajak and Atul Kumar Singh

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Abstract
Antisense RNA is a trimming technique that is rising in popularity in agricultural research. "Sense" refers to the original DNA or RNA molecular sequence. "Antisense" refers to the complementary sequence of DNA or RNA. Since it is the most practical and recent approach accessible, crop breeders employ antisense technology to create different crop species and variations. Long non-coding RNA (lncRNA), antisense RNA (asRNA), and a number of other enzymes and molecules are all included in the antisense technology category. Using asRNA technology, crops may have their nutritional value increased, undesirable toxic compounds reduced, male sterility created for crop breeding, shelf life extended, etc. The FLAVR SAVR tomato is the result of genetic engineering by the biotechnology company Calgene, which used the modified bacterial parasite *Agrobacterium tumefaciens* to transfer genetic material into Flavr Savr plant cells and slow down the ripening of tomatoes. Using RNAi technology, β-carotene and lutein levels in potatoes have been raised by inhibiting beta-carotene hydroxylase (BCH), which transforms β-carotene to zeaxanthins. Auxin response factor 7 (ARF7) was blocked by RNA silencing in *Solanum lycopersicum*, resulting in parthenocarpic fruits. Scientists have developed tobacco strains that are male sterile by reducing the expression of the TA29 gene, which is in charge of producing pollen. In order to make plants male-sterile again, RNA silencing is also essential. Because they limit or completely remove the expression of genes implicated in the synthesis of harmful compounds in food, antisense technologies are helpful for crop development.

Keywords: RNAi, antisense RNA, DNA, gene expression, genetic engineering, gene silencing

Introduction
In agricultural research, antisense RNA is a novel technology that is getting traction. The original DNA or RNA molecule sequence is referred to as "sense". The complementary sequence of DNA or RNA is referred to as “antisense”. Antisense RNA is a single-stranded RNA that is also known as an antisense transcript or antisense oligonucleotide that hybridises with a protein-coding mRNA and prevents the translation process, hence inhibiting protein formation. mRNA is a nucleic acid molecule that transports genetic information from DNA to another recipient cell that helps with the synthesis of protein. Antisense compounds attach to mRNA and prevent certain proteins from being produced.

Crop breeders use antisense technology to generate various crop species and varieties since it is the most convenient and new technique available. Antisense technology encompasses RNA interference (RNAi), long non-coding RNA (lncRNA), antisense RNA (asRNA) as well as a variety of other enzymes and molecules. RNA interference technology is gaining prominence in plant genetics and system biology these days because of its consistent transgenic expression. The tomato is the most studied crop so far, but study has previously been done on a variety of horticulture crops such as fruits, vegetables and flowers. RNA interference (RNAi) technology, which has proven to be a strong strategy for silencing genes to improve attributes in crops, can give genetic improvement in crops. Antisense RNA and RNA interference are utilized to develop new agricultural quality features and to protect crops from pests, nematodes and diseases. Antisense RNA technology is playing an important role in generating biotic and abiotic resistance, high nutrient value crops, stress tolerant crops, seedless fruits, and plant architectural modification, colour and texture altering attributes, and secondary metabolite enhancement (Williams *et al.*, 2004) [43]. Antisense technologies are useful for crop improvement because they restrict or eliminate the expression of genes involved in production of hazardous chemicals in food.

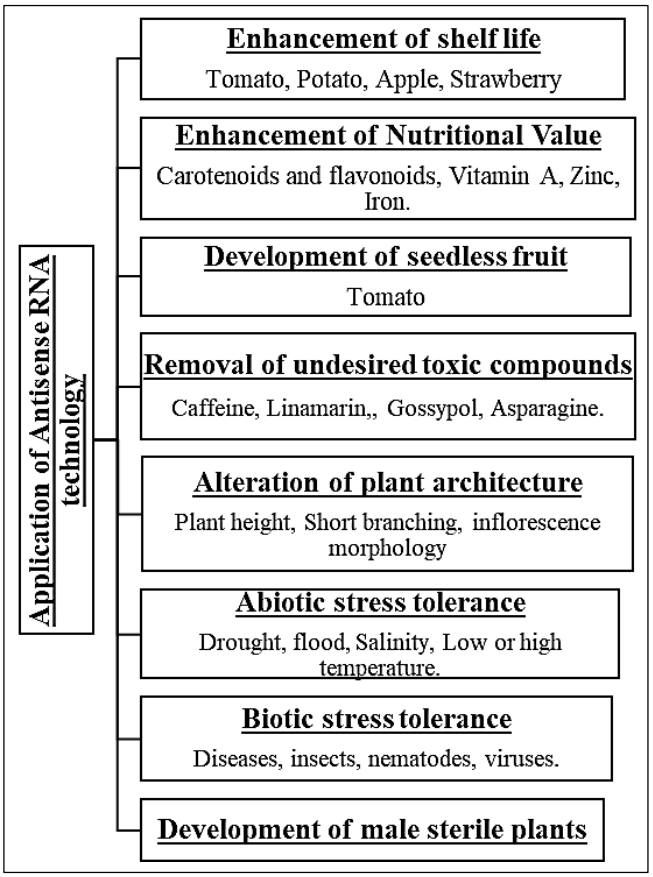

Fig 1: Showing Application of Antisense RNA technology

Uses of Antisense RNA (asRNA) technology

asRNA technology has the ability to improve the nutritional content of crops, reducing unwanted harmful substances, create male sterility for crop breeding, improve shelf life, etc. (Auer and Frederick, 2009) [1].

Enhancement of Shelf-Life and quality

Tomato is a climacteric fruit vegetable and has a limited shelf life. The softening process (ripening) can cause more fruit to be ruined during transhipment. Because tomatoes have such a short shelf life, they can spoil before reaching to consumers if plucked while ripe. To remedy this, tomatoes for shipping are frequently plucked while unripe, or "green" and then induced to ripen right before delivery using ethylene gas, which works as a plant hormone. The disadvantage of this method is that the tomato does not finish its natural growing phase, and as a result, the final flavour decreases.

Calgene (a biotechnology company) use genetic engineering to slow down the tomato's ripening process and prevent it from softening too soon, while still retaining the tomato's original flavor and color. This would permit it to properly ripen on the vine while still being able to be transported vast distances without becoming soft. Calgene scientists transferred genetic material into Flavr Savr plant cells using the modified bacterial parasite *Agrobacterium tumefaciens*. As part of its life cycle, the bacterium "infects" plants with foreign genes. The parasite genetic material in the bacterial T-plasmid was removed and substituted with the preferable genes. asRNA was used to modulate the activity of the polygalacturonase (PG) or pectin depolymerase in ripened fruit of *Lycopersicon esculentum*, resulting in the FLAVR SAVR tomato. This enzyme is found in abundance in ripe tomato fruit and it has long been assumed to be responsible for softening.

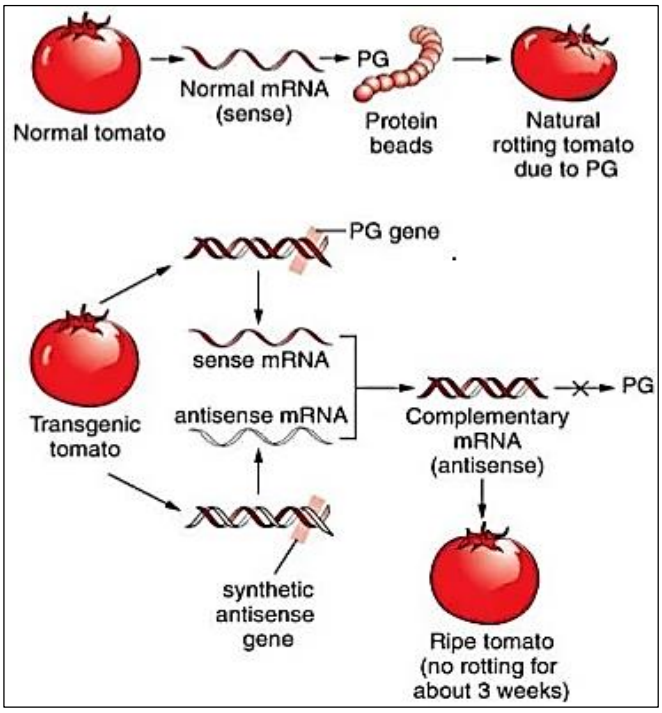

Fig 2: Application of Antisense technology in Flavr-Savr

Ethylene, the well-known ripening hormone, is responsible for starting, regulating and coordinating the expression of several genes involved in the ripening process. The commencement of ripening in high respiration rate fruits, i.e., tomatoes, is triggered by a surge in ethylene synthesis. RNAi technology was used to delay the ripening process in tomatoes by silencing the ACC synthase (ACS) gene during ripening. The chimeric RNA interference-ACC synthase product, which targets ACC synthase homologs, successfully inhibited ethylene production in tomato. Fruits from these lines ripened late and had a prolonged time span of 6 weeks with better juice quality (Gupta *et al.*, 2013) [17].

Other approaches included suppressing genes that codes cell wall-degrading proteins in transgenic *Lycopersicon esculentum* to reduce fruit softening. In tomatoes, N-glycans are said to make a significant contribution in ripening by dissolving the cell wall. α-Mannosidase (α-Man) and β-d-N-acetyl hexosaminidase (β-Hex) are the two known ripening-specific N-glycoprotein-modifying enzymes. Because of the slower rate of softening, suppressing these enzymes increased the fruit shelf life by about one month (Meli *et al.*, 2010) [25].

Strawberry (cv. Chandler) is fruit with high respiration rate and limited shelf life, owing to its rapid loss of firmness. To limit strawberry fruit softening, antisense regulation of the pectate lyase gene under the control of the CaMv35S promoter was used. Cell walls obtained from ripened genetically modified *Fragaria x ananassa* fruits showed lower levels of pectins and in vitro swelling, indicating greater firmness, which coincided with the drastically reduced pectate lyase gene expression level.

PPO catalyses the transformation of phenolic substances into quinones, which are then polymerized to produce brown pigments. Several studies have shown that suppressing PPO gene expression with antisense or hpRNA reduces browning in potatoes and apples (Bachem *et al.*, 1994; Murata *et al.*,

2001) [2, 28]. An artificial micro-RNA (amiRNA) method was recently employed to repress 4 members of the Polyphenol oxidase gene family, either separately or in group, as a result, potatoes with few DNA inserts are produced that are low in browning (Chi *et al.*, 2014) [5]. Therefore, now consumers are demanding potatoes and apples that do not brown during processing and consumption.

Two SEP-like subfamily genes, MaMADS1 and MaMADS2, were functionally identified in a banana study and inhibition of either gene resulted in late ripening and prolonged shelf-life traits (Elitzur *et al.*, 2016) [11].

When pineapples are exposed to higher temperatures, the activity of the enzyme polyphenol oxidase (PPO) is stimulated, resulting in the fruit defect known as blackheart. Blackheart-producing circumstances were used by Stewart *et al.* (2001) to clone a PPO gene from pineapple fruits. Transgenic plants are now being examined in the field and have the PPO gene silenced.

Enhancement of nutritional value

Tomatoes include carotenoids and flavonoids, and both are necessary for good health. Tomatoes with better carotenoids or flavonoids have been produced by silencing an endogenic photo morphogenesis regulating gene (DET1) that encodes biosynthetic enzymes. Genetically-engineered tomatoes exhibiting a DET1 hpRNA arrangement demonstrated gene-specific mRNA disintegration, as well as a considerable rise in flavonoid and carotenoid levels. On the other hand, other fruit quality measures were mostly unaffected. This study is especially intriguing since it shows that manipulating a plant regulatory gene can affect numerous plant nutrient biosynthesis processes at the same time, resulting in genetic enhancements in the nutritive value of plant-derived products (Q Guo *et al.*, 2016) [16].

By suppressing the production of beta-carotene hydroxylase (BCH), which converts β-carotene to zeaxanthins, RNAi technology has been utilised to increase β-carotene and lutein levels in potatoes (Van Eck *et al.*, 2007) [41].

When a desirable recombinant protein, such as albumin, was observed in genetically modified potatoes, it was found that the procedures involved in protein purification were important cost drivers due to patatin contamination. Patatin, a group of glycoproteins that make up to 40% of the tuber's total soluble proteins. By utilizing the hpRNA technique, patatin concentration was lowered by over 95% at both the proteins and mRNA levels in genetically modified potatoes particularly targeting the patatin gene, enabling for faster purification of additional potato glycoproteins or transgenically generated glycoproteins with less contamination.

Table 1: Show the improved traits RNA

Crops	Improved traits	RNA tools used	Targeted gene	References
Tomato	Reduce ethylene	RNAi	ACC synthase	Gupta *et al.*, 2013 [18]
	Seedless fruit improvement	RNAi	CHS	Schijlen *et al.*, 2007 [35]
	Male sterility	RNAi	SmTAF10/13	Toppino *et al.*, 2011 [40]
	Carotenoids and flavonoids	RNAi	DET1	Davuluri *et al.*, 2005 [7]
	Fusarium wilt resistance	RNAi	Polyamine (PA) biosynthesis gene	Singh *et al.*, 2020 [37]
Tobacco	Parthenocarpy	RNAi	TA29	Nawaz-ul-Rehman *et al.* 2007 [29]
	Tobacco mosaic virus resistance	asRNA	CP	Powell *et al.*, 1989 [33]
Cassava	Removing linamarin	RNAi	CYP79D1/D2	Meena *et al.*, 2017 [24]
Potato	Resistance to *P. infestans*	hpRNA	Syntaxin related 1 (SYR-1)	Eschen-Lippold *et al.*, 2012 [12]
	Beta-carotene	RNAi	BCH	Van Eck *et al.*, 2007 [41]
	Resistance to Potato virus Y (PV-Y)	hpRNA	Helper-component proteinase (HCPro) gene	Missiou *et al.*, 2004 [26]
	Reduced steroidal glycoalkaloids	RNAi	Sterol side chain reductase 2	Sawai *et al.*, 2014 [34]
Pepper	PMMoV resistance	RNAi	PMMoV replicase	Dalakouras *et al.*, 2020 [6]
Lettuce	Whitefly resistance	RNAi	v-ATPase	Ibrahim *et al.*, 2017 [18]
Brassica	Reduced erucic acid	RNAi	BnFAE1	Shi *et al.*, 2015 [36]
Arabidopsis	Nematode resistance	RNAi	Mi-msp2	Joshi *et al.*, 2019
Cotton	*Helicoverpa armigera* resistance	RNAi	CYP6AE14	Younis *et al.*, 2014 [44]
Banana	Enhanced shelf life	RNAi	*MaMADS1/S2*	Elitzur *et al.*, 2016 [11]
Apple	Apple scab fungus resistance	RNAi	GFP and THN	Fitzgerald *et al.*, 2004 [13]
Coffee	Decaffeinating	RNAi	CaMXMT1	Pathak and Gogoi, 2016 [32]

Development of parthenocarpic fruits

Parthenocarpy, or the development of seedless fruits in the absence of effective fertilisation, is a desirable characteristic for a number of key crop plants (George *et al.*, 1984) [15]. Consumers and farmers alike value the absence of seeds since it improves fruit quality and shelf life. It's also a favourable agronomic characteristic that raises fruit marketing value because it allows for high yields even in pollination and fertilization-challenged environments.

RNA silencing was used to inhibit the action of Auxin response factor 7 (ARF7) in *Solanum lycopersicum*, resulting in parthenocarpic fruits (De Jong *et al.*, 2009) [8]. Transgenic *Solanum lycopersicum* plants with the AUCSIA genes, which code for a short peptide produced only in the ovaries, were functionally repressed by hpRNA and produced parthenocarpic fruits after floral emasculation (Molesini *et al.*, 2009) [27].

The flavonoid biosynthesis system was downregulated via RNAi-mediated silencing of chalcone synthase (CHS), the major gene within the flavonoid system, to produce seedless tomatoes (Schijlen *et al.*, 2007) [35].

Removal of undesired toxic compounds

Plants are known to contain toxic compounds or poisons of various types, which can be a time-consuming and expensive process to remove. RNA silencing has been shown in numerous studies to be a powerful tool for removing toxins from plants. By inhibiting the activity of the gene encoding

theobromine synthase (CaMXMT1), researchers were able to lower caffeine concentration in genetically modified plants by up to 70%, proving that it is possible to make decaffeinated coffee beans (Ogita *et al.,* 2003) [30].

Cassava/Tapioca (*Manihot esculenta*) is a popular tropical staple food; however, its tuber contains deadly cyanogenic glucosides which defend the plant from herbivory and thievery (Siritunga and Sayre, 2003) [38]. Linamarin is a cyanogenic glucoside found in Tapioca. Linamarin is produced in leaf tissue by two CYP450 enzymes, CYP79D1 and CYP79D2, and then transferred to roots (Pandey *et al.,* 2019) [31]. Transgenic cassava plants with more than 90% reduction in cyanogenic glucoside levels in tubers were created by antisense downregulation of cytochrome P450 enzymes CYP79D1 and CYP79D2 (Siritunga and Sayre, 2003) [38].

Antisense RNA technology was also used to develop potatoes on a large scale with greatly lower asparagine content, one of the principal sources of neurotoxicant acrylamide, by simultaneously suppressing the StAs1 and StAs2 gene (Asparagine Synthetase genes). Similarly, significant levels of alpha-solanine and alpha-chaconine, these poisonous steroidal glycoalkaloids (secondary metabolites), accumulated in potato sprouts and immature tubers. According to latest report, sterol side chain reductase 2 is mainly responsible for production of these SGAs. The following genetic manipulation of SSR2 (sterol side chain reductase 2) downregulation by RNA silencing resulted in potato lines with approximately 10% drop in prevalent SGA amounts in contrast to non-transformed potato plants, without impacting plant development (Sawai *et al.,* 2014) [34].

Eady *et al.* (2008) [10] developed a tearless onion by utilising RNAi to silence the lachrymatory factor synthase gene, resulting in much lower amounts of tear-inducing lachrymatory factor when the onion was damaged. Propanthial S-oxide (lachrymatory factor LF), 1-propenyl methane thio-sulfinate, and di-propyl disulphide, the most common sulfoxide compounds found in *Allium cepa* (Block *et al.* 1992) [3]. Because of these compounds, tears are induced in humans. Imai *et al*. (2002) [19] found that an enzyme called lachrymatory factor synthase (LFS) is involved in the conversion of 1-propenyl sulfenic acid to LF. By lowering LFS and preventing the conversion of 1-propenyl sulfenic acid to the unwanted LF, resulting in considerably lower level of tear-inducing lachrymatory factor in *Allium cepa*.

Alteration of plant structure and blooming time

The molecular fundamentals of plant architecture have been studied through a number of studies on the genetic transformation of plant architecture by RNA silencing in tomatoes and petunia (Wang *et al.,* 2008) [42]. Such a biotechnological invention could have broad applications in horticulture crops, as demonstrated in the fields of mechanised fruit harvesting from tall trees and leaves picking in tea or mulberry plants. In model plants, CCD genes, i.e., it has been demonstrated that carotenoid cleavage dioxygenase genes are crucial in the regulation of branch growth (Drummond *et al.,* 2009) [9]. In genetically modified kiwi fruit plants over the course of two growing seasons, hpRNA-induced silencing of AcCCD8 was discovered to be correlated with an increase in the overall number of branches and a postponement of leaf senescence (Ledger *et al.,* 2010) [22]. Such an adjustment in plant architecture is expected to rise the number of blooms generated on kiwifruit vines by increasing the proportion of nodes with the ability to set fruit.

The prolonged juvenile phase that European pears typically require for flowering and fruit set. Early Flowering-Spadona (EF-Spa) is a transgenic line that exhibits a hpRNA cassette that targets the Terminal Flower 1 (TFL1) gene, a crucial gene in suppressing flowering and sustaining the flowering meristem by restricting the utterance of the Apetala 1 (AP1) and Leafy (LFY) genes (Freiman *et al.,* 2012) [14], offering an intriguing method to enhance pear breeding.

Enhancement of resistance to biotic stresses

Phytopathogens are the source of different plant diseases that cause severe crop loss and as a result, high economic loss. Various RNAi, asRNA and lncRNA techniques were developed to strengthen plant protective mechanism against biotic stresses like viral, bacterial, fungal pathogens, nematodes, and pests. Insufficient or excessive production of various "Proteins" is frequently linked to disease. Many diseases can be cured if the synthesis of these proteins is inhibited. Antisense technology can be used to disrupt protein production. It could be utilised to develop new treatments for diseases in which the production of a specific protein plays a crucial role in the pathogenesis. Antisense technology is a method for preventing gene expression from occurring.

Viruses are particularly challenging to control because they grow and spread through and across plants and are responsible for various diseases of horticultural crops. One of the earliest cases of complete immunity to potato virus Y (PV-Y) came from virus-resistant potato (*Solanum tuberosum* L.) plants transformed with vectors expressing both the sense and antisense transcripts of the viral helper-component proteinase (HCPro) gene. By producing hpRNA generated from the 3' terminal portion of the PVY coat protein gene, commercial potato cultivars resistant to three strains of PVY have been produced (Missiou *et al.,* 2004) [26]. Recently, genetically modified tomato plants resistant to the potato spindle tuber viroid (PSTVd) were developed by encoding hpRNA from PSTVd sequences.

Fusarium oxysporum, a filamentous fungal pathogen that affects a number of crop species, including tomato, is the source of fusarium wilt. According to a recent study, RNAi was used to control fusarium wilt by inhibiting the pathogen's important polyamine (PA) biosynthesis gene, ornithine decarboxylase (ODC), as PAs (putrescine, spermidine, and spermine) are required for the pathogen's proper development. The hairpin RNA construct was utilised to clone the target ODC gene fragment, which was then used to create transgenic tomatoes. Small interfering RNAs were produced by the RNAi transgene lines, which showed medium to high resistance to fusarium wilt in transgenic tomatoes (Singh *et al.,* 2020) [38].

Genetically modified *Solanum tuberosum* plants that produced hpRNA structure surveilling cell membrane-localized Syntaxin related 1 (SYR-1) showed improved resistance to the oomycete pathogen *Phytophthora infestans* (Eschen-Lippold *et al.,* 2012) [12]. In response to P. infestans infection, genetically modified potatoes had a continuous rise in salicylic acid and PR1 transcripts. This pathogen's infestation was associated with abnormal callose accumulation and reduced papilla development on cytological inspection, implying that syntaxins are involved in secretory adaptive immunity in potatoes.

RNA silencing technology is utilised to generate crops which are resistant to pest infestations. To lower plant insect pests, HD-RNAi must be developed because insects lacking the genes essential for the RNA-dependent RNA polymerase (RdRp) enzyme to duplicate siRNA molecules and carry out complete RNAi. Studies on RNA silencing technology have shown that it provides resistance to worms, fungi, bacteria, and mites in transgenic tobacco and Arabidopsis plants (Mansoor *et al.,* 2006) [23].

Root-knot nematodes are parasitic nematodes that attack numerous crops including Arabidopsis. HD-RNAi-mediated suppression of the effector gene Mi-msp2 promotes nematode resistance in Arabidopsis.

Apple scab is the most common and serious disease of apples, which is caused by an ascomycete fungus, *Venturia inaequalis*, that mainly affects their leaves and fruits. The endogenous gene trihydroxy naphthalene reductase (THN) and the green fluorescent protein (GFP) transgene were used to establish a gene silencing technique for *V. inaequalis*. Hairpin constructs for such GFP or THN genes delivered by *Agrobacterium tumefaciens* were used to induce high frequency gene silencing (Fitzgerald *et al*., 2004) [13].

More recently, hpRNA transgenes targeting the Colorado potato beetle's β-actin gene were developed to be produced in the chloroplast of potatoes, providing substantial protection against insect herbivory (Zhang *et al.,* 2015) [36]. Chloroplasts lack RNA silencing machinery like DCLs, allowing full-length hpRNA to accumulate at large levels, which is expected to account for chloroplast-expressed hpRNA's remarkable efficacy in regulating insect gene silencing.

The whitefly (*Bemisia tabaci*) is a sap sucking pest that causes significant damage to a variety of crops in tropical and subtropical areas. In whiteflies, RNAi-based plasmids with an interfering cassette engineered to produce dsRNAs targeting a novel v-ATPase transcript have proved successful (*Bemisia tabaci*). In whiteflies feeding on transgenic plants, quantitative reverse transcription PCR revealed a lower expression level of the endogenous v-ATPase gene (Ibrahim *et al.,* 2017) [18].

The inoculation of Pepper Mild Mottle Virus (PMMoV) in *Nicotiana benthamiana* plants with in vitro synthesized 997 bp dsRNAs targeting the PMMoV replicase viral infections was reduced (Dalakouras *et al*., 2020) [6].

Induction of male sterility

In hybrid seed production, male sterility is a significant characteristic. RNA silencing strategies are used to cause male sterility. By suppressing the expression of the TA29 gene, which is responsible for pollen formation, scientists have created male sterile tobacco strains. RNA silencing is also vital for restoring male-sterility in plants (Meena *et al.,* 2017) [24]. Male-sterile characteristics are caused by mitochondrial genome rearrangement and show maternal inheritance patterns. In terms of agriculture, this type of male sterility is beneficial to the hybrid seed industry since it allows for the production of cross-pollinated seed without the need for intensive labour. During microspore development, TA29 is only articulated within the anthers. In one study, ten of thirteen tobacco lines modified with a hairpin RNAi structure encoding TA29 sequences were male sterile. The SlTAF10 and SlTAF13 genes of tomato (*Solanum lycopersicum*) are suppressed or inactive by the microRNAs. Inducing the expression of the tomato TAF genes is expected to increase SmTAF10 and SmTAF13 activity, restoring male fertility (Toppino *et al*., 2011) [40].

Conclusion

There has been some significant advancement in the study of antisense RNA, which has continued to expand. The development of biotic and abiotic resistance, high-nutrient value crops, stress-tolerant crops, seedless fruits, and plant architectural alteration, colour and texture modifying properties, as well as secondary metabolite augmentation, are all made possible by antisense RNA technology. Because they limit or completely remove the expression of genes implicated in the synthesis of harmful compounds in food, antisense technologies are helpful for crop development. Additionally, significant advancements have been achieved in the use of antisense RNA in biology. Only a few in vivo antisense RNA activities have been proven in organisms to yet, particularly in the case of piRNAs and lncRNAs. The underlying processes of antisense RNA functions, however, are still poorly understood. In the near future, more antisense RNA molecules will be investigated in-depth by scientists. More significantly, the understanding of short RNAs and their roles has fundamentally changed how we think about how genes are regulated.

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