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RNA INTERFERENCE: RECENT ADVANCES, APPLICATIONS, AND FUTURE PROSPECTS IN CROP IMPROVEMENT

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ABSTRACT

The mechanism of gene silencing by RNA interference (RNAi) is one of the most ancient and evolutionarily conserved pathways, present across all kingdoms of life. It likely originated during the early stages of molecular evolution, in the 'RNA world'—a period when RNA functioned both as genetic material and as a catalyst for enzymatic reactions. In this review, we provide a detailed explanation of the RNAi mechanism, with a particular focus on its application in plant-integrated pest management. RNAi-mediated gene silencing holds vast potential, especially in the management of plant diseases, offering a promising avenue for improving crop yields.

With the exponential growth of the global population and the increasing challenges posed by climate change and environmental stressors, there is an urgent need for high-yielding, resilient crop varieties. The recent advancements in our understanding of RNAi and its molecular mechanisms have transformed our approach to RNA-mediated gene regulation. RNAi is now widely used as a research tool to modulate gene expression across various model organisms, including *Arabidopsis*, tobacco, wheat, maize, *Drosophila*, and mice etc.

Beyond gene silencing, RNAi also plays critical roles in regulating developmental genes and in epigenetic modifications, such as DNA and chromatin remodelling through methylation. This review highlights the promising applications of RNAi as compare to CRIPER technology in plant pest & disease control and crop productivity enhancement, and discusses its future potential in sustainable agriculture.

***Key words*:** RNAi, dsRNA uptake mechanism, Drosha, Argonaute, RISC, post-transcriptional cgene silencing, RNAi-based insecticides, SmartStax Pro, Plant-Incorporated Protectant (PIP).

Introduction

The RNAi or co-suppression method is a very ancient, ubiquitous and natural antiviral process and existed even before the deviation of plants and animals. During the origin and evolution of Earth some four billion years ago, RNA may be the first genetic material and involved in several enzyme catalysed reactions (Gilbert, 1986; Hannon, 2003; Mao *et al*., 2007; Kumar *et al*., 2012). The process of RNAi is a highly regulated, enzymatic mechanism of gene silencing in response to foreign nucleic acids and provides resilience individually to endogenous parasites along with exogenous pathogenic nucleic acids. They are involved in a variety of processes, *viz*., control and expression of transposable elements, repetitive sequences, genomic information, gene function, regulation of RNA, post-transcriptional gene silencing (PTGS), protein expression and therapeutic interventions in plant pest control thus used as a tool for high crop yield (Artymovich, 2009). Therefore, RNAi has considerable potential for insect and other pest & disease control. This natural mechanism of gene silencing can be utilised in a variety of ways, *viz*., silencing of disease causing and yield reducing insect and pest genes in plants (Joga *et al*., 2016; Mamta and Rajam, 2017), thus improved plant varieties can be generated to feed the rising world population. As the world population is rising exponentially and will be nine billion by the end of 2050. Furthermore, climate changes, *viz*., abiotic and biotic stresses, along

with global warming and unpredictable natural calamities impose a great threat on both subsistence and marketable plant crops (Brodersen and Voinnet, 2006; Duan *et al*., 2012; Joga *et al*., 2016). Additionally, the RNAi technology is a proven, invaluable tool and can be harnessed to functionally validate the novel plant genes responsible for stress and disease resistance (Eschen-Lippold *et al*., 2012; Niu *et al*., 2018). The technology of RNAi mediated gene suppression has been extensively utilized to control agriculturally important insect pests. Several RNAi mediated insecticides have been generated successfully in past such as against *Bemisia tabaci's AChE* and *EcR* genes in Tobacco (Malik *et al*., 2016), *Helicoverpa armigera's Chitinase* gene in Tobacco and Tomato (Mamta *et al*., 2016), *Helicoverpa armigera's Arginine Kinase* gene in *Arabidopsis thaliana* (Liu *et al*., 2015), *Helicoverpa armigera's Chitin synthase, Cytochrome* P450 *monooxygenase* and *V-ATPase* in Tobacco (Jin *et al*., 2015), *Myzus persicae's hunchback* gene in Tobacco (Mao and Zeng, 2014), *Nilaparvata Lugen's* O-Hydroxyacedysone gene in Rice (Yu *et al*., 2014), *etc*. In spite of having done a lot of research in RNAi mediated insecticides, wide range of challenges still need to be addressed particularly at field level. For successful adoption of RNAi in pests control the first most challenge that needs to be addressed is protection of ingested dsRNA chemical hydrolysis (from nucleases) which initiates the entire RNAi process. For instance, when polygalacturonase dsRNA showed immediate down regulation upon in ingestion in the midgut, this might be due to the nucleases present in saliva (Bolognesi *et al*., 2012; Allen and Walker, 2012; Garbutt *et al*., 2013). The second challenge that needs to be addressed is the quantity and the optimal dose of the dsRNA molecule, which is influenced by a variety of internal and external factors, *viz*., insect species, its developmental stage, way of dsRNA delivery, rate of transcription of the target gene, length of the dsRNA molecule, and efficiency of dsRNA to spread inside host, *etc* (Xu *et al*., 2016; Miller *et al*., 2008; Araujo *et al*., 2006; Baum *et al*., 2007; Shakesby *et al*., 2009). It was found nitropin-2 RNAi efficacy is more in young insect at second instar larva stage due to physiological and genetic factors (Kumar *et al*., 2012). Likewise, RNAi induced silencing effect in *Helicoverpa armigera* showed that both long and short dsRNA was equally effective against target gene or pest (Saleh *et al*., 2006; Attardo *et al*., 2012).

RNAi :- RNAi is a natural gene regulation mechanism in eukaryotes. It involves sequence-specific recognition of mRNAs and then repressing their expression either by ribonuclease-directed degradation or by preventing their translation. RNA silencing is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (TGS) or by activating a sequence- Specific RNA degradation process [PTGS/ RNA interference (RNAi)] (Agrawal *et al*., 2003). The silencing effect was first observed in plants in 1990, when the Jorgensen laboratory introduced exogenous transgenes into petunias in an attempt to up-regulate the activity of a gene for chalcone synthase, an enzyme involved in the production of specific pigments (Agrawal *et al*., 2003; Napoli *et al*., 1990). Unexpectedly, flower pigmentation did not deepen, but rather showed variegation with complete loss of colour in some cases. Two types of small single-stranded RNAs (≈21–24 nucleotides) are key players in the two main RNAi pathways in plants; these are the siRNAs and miRNAs. miRNAs are encoded by the plant genome and mostly function as regulators of gene expression; siRNAs may also be derived from the genome, but plants have evolved to have an RNA surveillance system that recognizes and degrades specific RNAs, resulting in the generation of siRNAs. In plants this RNA surveillance system is also a robust antiviral defence mechanism. The siRNA pathway in plants begins with recognition of RNAs for degradation. These RNAs are often dsRNAs or highly structured RNAs; they are recognized in the cell cytoplasm and cleaved by ribonuclease activity of Dicer-like proteins (DCLs). Different plants can contain different DCLs (Muhammad *et al*., 2019); for example, *Arabidopsis thaliana* has four DCLs (DCL 1, 2, 3 and 4) (Gasciolli *et al*., 2005), which cleave ds RNAs or the structured RNAs into small RNA duplexes (typically 21, 22, 23 or 24 nucleotides). The RNAi response is maintained by the DCL generated small RNA duplexes, which is part of the RNA-induced silencing complex (RISC). One strand of the small RNA duplex is released (the passenger strand) and mostly degraded; the other is retained by RISC, serves as a guide RNA. When the RISC, carrying the guide RNA, encounters an RNA that is homologous to the guide RNA (by Watson–Crick base pairing), directs ribonuclease cleavage which results in degradation of the bound RNA. Furthermore, in plants, endogenous and virus-encoded RNA-dependent RNA polymerases can amplify the RNAi response (Muhammad *et al*., 2019; Baulcombe *et al*., 2019; Dalmay *et al*., 2000). Thus, the siRNAi activity is highly specific and robust. The majority of plant miRNAs originate from specific noncoding RNAs, also called primary microRNAs (pri-miRNAs), which fold into specific structures.

Short-Interfering RNAs (siRNAs):- Long dsRNA or short-hairpin RNA (shRNA) precursors, which are

homologous in sequence to the target gene to be silenced (Fire *et al*., 1998; Tuschl, 2001), initiate the process of RNAi. The entry of a virus, a genetic element like transposons or an introduced transgene provide long dsRNA can triggers the RNAi pathway. The dicer enzyme is recruited in the cell (Bernstein *et al*., 2001) leading to the cleavage of the dsRNA into siRNA (Hamilton and Baulcombe, 1999) that are short, 5 - phosphorylated dsRNAs (21–25 nt) with two nucleotide overhangs at the 3'ends (Bernstein *et al*., 2001; Elbashir *et al*., 2001). The recruitment of siRNA-induced silencing complex (siRISC) leads to degradation of sense strand of siRNA (having the same sequence as the target gene) The siRISC is then incorporated into the antisense strand of siRNA which in association with AGO and other effector proteins brings about cleavage of the target mRNA in sequencespecific manner. The activated RISC can repeatedly participate in mRNA degradation and protein synthesis inhibition resulting into PTGS. On the basis of origin and mechanism of biogenesis, sRNAs in plants exhibit a range of size classes, predominantly from 20 to 24 nt. Four Dicer-like nucleases (DCLs) have been reported in Arabidopsis (Henderson *et al*., 2006). Depending on their origin and structure, dsRNA triggers of RNAi are cleaved by one or more DCLs (Zamore *et al*., 2000; Bernstein *et al*., 2001). DCL4 and DCL2 are involved in processing of dsRNA with perfectly complementary to produce 20- to 22-nt long shortinterfering RNA (Hamilton and Baulcombe, 1999; Hamilton *et al*., 2002; Hannon, 2002; Tang *et al*., 2003; Bouche *et al*., 2006). MiRNAs that are 21-nt long are produced by cleaving of partially paired dsRNA precursors by DCL1 (Denli and Hannon, 2003; Bartel, 2004). DCL3 is involved in biogenesis of 24-nt long repeat-associated sRNAs known as heterochromatic siRNAs (hc siRNAs; Xie *et al*., 2005). In a complex called RISC, siRNAs and miRNAs (20- to 21-nt long) in association with AGO proteins, direct sequence-specific cleavage of complementary target RNAs or interfere with their translation (Hammond *et al*., 2000; Hannon, 2002; Brodersen *et al*., 2008). Traditionally, sRNA species that are 24-nt long (derived from transposable elements and other repetitive sequences) are linked to transcriptional gene silencing via DNA methylation and repressive chromatin modification (Hamilton *et al*., 2002; Zilberman *et al*., 2003; Liu *et al*., 2004; Fagegaltier *et al*., 2009; Law and Jacobsen, 2010; Burkhart *et al*., 2011). SiRNA recruits several DNA- and histone-modifying proteins including the cytosine methyltransferase, chromomethylase 3 (Ossowski *et al*., 2008) which in turn mediate the formation of a transcriptionally inactive silent chromatin state. However, 21 nt long siRNAs may also participate in siRNA-dependent methylation of genomic loci (Pontier *et al*., 2012). AGO is an important protein that complexes with sRNA to form the core of the RISC. In Arabidopsis thaliana, 10 different AGO proteins are known to mediate the effects of several distinct types of sRNAs (Vaucheret, 2008). Endo nucleolytic cleavage (slicing) activity catalysed by the AGO protein (Huntzinger and Izaurralde, 2011) is an important step in post-transcriptional silencing. Guide RNA confers sequence specificity to any RNA silencing reaction whereas the precise nature of silencing is determined by the properties of the associated AGO protein, including its ribonuclease activity, interacting proteins, and subcellular localization. Movement of plant sRNAs falls into two main categories: cell-to-cell (short-range) and systemic (long-range) movement (Melnyk *et al*., 2011). Local movement is symplastic, *i.e.*, from the site of initiation to neighbouring cells through channels called plasmodesmata (Lough and Lucas, 2006). RNA silencing also spreads systemically over long distances through the vascular phloem tissue. This long distance or systemic movement of the silencing signal takes place over days (Voinnet *et al*., 1998) and it is generally from photosynthetic sources (i.e., leaves) to sucrose sinks (*i.e*., roots and growing points) through a bulk flow process. The systemic RNA silencing signal has been identified in plants by direct sampling of phloem sap (Yoo *et al*., 2004; Buhtz *et al*., 2008) and detection of RNAs in stocks and scions of grafted plants (Palauqui *et al*., 1997; Schwach *et al*., 2005; Brosnan *et al*., 2007; Dunoyer *et al*., 2010a; Molnar *et al*., 2010). Mobile silencing operates in a nucleotide-sequence-specific manner and its components include sRNA molecules (21–24 nt). Molnar *et al*., (2010) have reported mobile sRNAs of the 24-nt size class that are associated with DNA methylation of targeted loci. It was consistent with the analysis of viral suppressors of systemic silencing in Nicotiana benthamiana (Hamilton *et al*., 2002) and the presence of 24-nt sRNA in the phloem sap of oilseed rape (Buhtz *et al*., 2008) and pumpkin (Yoo *et al*., 2004). Dunoyer *et al*., (2010a, b) showed that the mechanically delivered, fluorescently labeled 21- and 24-nt siRNAs move from cell to cell and over long distances. Artificial miRNAs (amiRNAs) were shown to move short distances in leaves (Schwab *et al*., 2006) or between the pollen vegetative cell cytoplasm and the sperm cells (Slotkin *et al*., 2009). Endogenous 21-nt miRNAs (miR399) could also be mobile between shoots and roots (Pant *et al*., 2008) and within the roots (miR165a and miR166b; Carlsbecker *et al*., 2010). RNA interference can be used to achieve desirable traits in crops by manipulating the gene expression. RNAi mediated gene

silencing technique mainly involves identification of the target genes followed by generating RNAi construct with hairpin cassette (gene cloned in sense and antisense orientation flanking a spacer or intron), plant transformation and finally screening and evaluating the traits.

MicroRNA (miRNA):- An important arm of RNAi involves the microRNAs (miRNAs). These are endogenous duplexes that post transcriptionally regulate gene expression by complexing with RISC and binding to the 32 untranslated regions (UTRs) of target sequences via short stretches of homology, termed "seed sequences" (Bartel *et al*., 2004). The primary mechanism of action of miRNAs is translational repression, although this can be accompanied by message degradation (Bagga *et al*., 2005). The miRNA duplexes possess incomplete Watson-Crick base pairing, and the antisense strand cannot be chosen by cleavage of the passenger strand as it is for siRNAs; therefore, the antisense strand must be chosen by an alternative mechanism (12–14). miRNAs are endogenous substrates for the RNAi machinery. They are initially expressed as long primary transcripts (pri-miRNAs), which are processed within the nucleus into 60–70 bp hairpins by the Microprocessor complex, consisting of Drosha and DGCR8 (Lee *et al*., 2003; Han *et al*., 2004) into pre miRNAs. The pre-miRNAs are further processed in the cytoplasm by Dicer and one of the two strands is loaded into RISC, presumably via interaction with one of the Dicer accessory proteins (Lee *et al.*, 2006). Importantly, it is possible to exploit this native gene silencing pathway for regulation of gene(s) of choice. If the siRNA effector is delivered to the cell, it will "activate" RISC, resulting in potent and specific silencing of the targeted mRNA. Because of the potency and selectivity of RNAi, it has become the methodology of choice for silencing specific gene expression in mammalian cells. Plant miRNAs are a class of small regulatory RNAs (20–22 nt) that are encoded by endogenous miRNA genes and transcribed by RNA polymerase II into primary miRNAs (pri-miRNA) having partially double- stranded stem–loop structures (Jones-Rhoades *et al.,* 2006). Following transcription, pri-miRNA is cleaved by a DCL1 enzyme in a two steps process resulting in production of a pre-miRNA and a mature miRNA duplex (miRNA/miRNA"). The mature miRNA is incorporated into RISC and mediates the degradation of mRNA target. miRNA can down-regulate the level of protein of their target genes through either translational repression (Aukerman and Sakai, 2003; Chen, 2004; Brodersen *et al.,* 2008), through cleavage of transcript (Llave *et al.,* 2002a; Xie *et al*., 2003), or transcriptional inhibition (Bao *et al.,* 2004; Khraiwesh *et al.,* 2010). In animals, miRNA mostly act by translational inhibition, where they often bind motifs in the 3 UTRs of their targets, which show several mismatches to the miRNA. However, in plants target motifs of miRNA have few mismatches which are most often found in the coding sequences and act mostly by transcript cleavage through a mechanism closely related to RNAi. In animals, miRNA usually consist of 20–22 nucleotides where as in plants it is 20–24 nucleotides (Reinhart *et al*., 2002; Bartel, 2004). Plant miRNAs are less conserved compared to animal miRNAs. In plants, mostly the mature miRNAs are conserved whereas miRNA precursors are usually conserved in animals (Bartel, 2004). Similar to animlas, in Arabidopsis, a RNAse III-like enzymes carry out processing of miRNAs from primary precursors followed by incorporation into a protein complex named RISC. However, an additional step is included in the biogenesis of plant miRNAs, i.e., the methylation on the ribose of the last nucleotide of miRNA by the methyltransferase Hen1 (Park *et al*., 2002). Unlike animals, plant miRNAs show high degree of sequence complementarity to their target mRNAs (Rhoades *et al.,* 2002). Because of this fact, bioinformatics prediction of plant miRNA targets is much easier and has facilitated prediction and subsequent validation of many plants' miRNA targets. A majority of plant miRNA targets includes genes encoding for transcription factors (Rhoades *et al.,* 2002; Mitsuda and Ohme-Takagi, 2009), few code for hormone receptors (Navarro *et al*., 2006), some encode enzymes (Xie *et al.,* 2003; Fujii *et al*., 2005). Many plant miRNAs are known to regulate posttranscriptional gene expression and play critical roles in many developmental processes, abiotic stresses and pathogen responses (Xin *et al*., 2010), in nutrient homeostasis etc. Functional characterization of several plant miRNA and their target need highthroughput sequencing at global genome-level (Wang *et al.,* 2011). MiRNAs have been extensively studied in model plants such as rice, A. thaliana etc. Schwab *et al.,* (2006) have designed a series of amiRNAs targeting different endogenous mRNAs and compared their effects to those of natural miRNAs. amiRNAs can be generated by replacing the sequences of miRNA/miRNA*within the miRNA precursor without disrupting its structural features. Like natural miRNAs, amiRNAs had varying number of target mismatches and could silence both single and multiple target genes with high specificity as determined by genome-wide expression profiling. The direct targets of amiRNAs can be accurately predicted by parameters of target selection already determined for natural miRNAs (Schwab *et al.,* 2005). Thus, extensive base pairing with targets is required for plant miRNA

function. amiRNAs can be efficiently used for gene silencing, especially when there is need to down regulate several related, but not identical, target gene. Transgenic plants harbouring amiRNAs under constitutive and inducible promoters have shown specific and efficient down-regulation of target genes of interest with limited non-autonomous effect. Thus, amiRNAs have great potential for crop improvement.

Historical events related to the discovery of RNAi:- The journey toward the utilization of RNAi for plant disease management started well before there was any knowledge of its mode(s) of action. Early studies on virus-infected plants showed that in some cases plants were able to recover from symptoms and become immune to reinfection. These virus-specific responses were not understood at that time, but now we know that some are due to RNAi. In 1928, Wingard found that tobacco plants inoculated with Tobacco ringspot virus (TRSV) often recovered, and the newly expanded leaves appeared healthy instead of showing the typical ringspots visible on the inoculated leaves. Furthermore, when these asymptomatic leaves were challenged again with TRSV, they were immune to superinfection by TRSV. Ratcliff *et al*., 1997 further examined the specificity of plants showing recovery phenotypes. They showed that recovered leaves from plants inoculated with Tomato black ring virus (TBRV) were immune to subsequent inoculations with TBRV but were susceptible to other viruses unrelated to TBRV, and they provided evidence that the immunity was RNA sequence based (Ratcliff *et al.,* 1997). In 1929, McKinney observed another specific antiviral response in plants. He showed that plants infected with mild forms of Tobacco mosaic virus (TMV), which caused light green mosaic, were resistant/immune to superinfection by more virulent strains of the same virus, which caused yellow mosaic (McKinney, 1929). As with recovery, these plants were susceptible to subsequent infections by unrelated viruses. Thus, in both cases there was a specific response toward the infecting virus that resulted in resistance/immunity, and it was homology dependent (Ratcliff *et al.,* 1999). The recovery phenotype is not common for different plant virus infections, and it is somewhat difficult to imagine how it might be used for virus disease control. By contrast, several studies using different viruses and plants have shown that a virus could often protect plants against subsequent infections by the same or highly homologous strains of a virus. Various terminology, such as acquired immunity (Johnson, 1937; Salaman, 1933) super-infection exclusion, and cross protection, has been used to describe this interaction (Fulton, 1986; Ziebell, 2010). Regardless of the terminology used, cross protection seems to be more applicable than recovery in the pursuit of plant protection. Early attempts were made to gain a fundamental understanding of cross protection, and some suggested that the coat protein (CP) of the protecting virus might be the functional molecule(s).

Process of RNAi:- In general, RNAi is triggered by double stranded RNA, which may be produced naturally in a cell or may enter the cell exogenously. An enzyme, called Dicer, cuts the long double stranded RNA into small pieces of approximately 21 nucleotides length. These small pieces could be miRNA (micro-RNA; originating from endogenous long dsRNA) or siRNA (small interfering RNA; originating from exogenous sources). These RNAs then bind to the RNA-induced silencing complex (RISC). After binding, one strand of the double stranded RNA is removed, leaving the remaining strand available to bind to messenger RNA target sequences. This strand is complementary to the sequence of the target mRNA. RNA Induced Silencing Complex (RISC) cleaves mRNA or represses their translation by homology dependent mRNA degradation, which effectively silences the gene. The use of RNAi has been extensively reported for modifying plants to enhance their nutritive value, pathogen and pest resistance, decreasing amount of unwanted metabolite production, etc. Recently two RNAi based crops have been given regulatory approval for commercial production and sale. These are the non-browning Arctic apples and the non-browning Innate potatoes. The firms producing these crops claim that the idea behind producing the non-browning apples and potatoes is not only to improve the look of the product, but it is also intended to increase the consumption of the raw fruits along with reducing naturally occurring carcinogens (as in the case of innate potatoes). While the science behind both these products is a little complicated as both are RNAi based, in simple way it can be put as both apples and potatoes have certain genes suppressed. Both of them, though genetically modified, are grown the same way as conventional varieties. These products are likely to find a place of attraction in the freshcut product sales.

Mechanism of RNAi:- The mechanism of RNAi involves a complex set of multiple RNA-protein interactions involving four big steps, *viz*., formation of a complex of small interfering RNA (siRNA) with RNA induced silencing complex (RISC), beginning of RISC, target identification and target degradation (Nykanen *et al*., 2001; Mello and Conte, 2004; Xu *et al*., 2016). The overall efficiency of RNAi directed down regulation of gene expression and target recognition depends on activation and association of siRNA-RISC complex

(Zamore, 2000; Uhlirova *et al*., 2003; Kevin and Nikolaus, 2007). The mediators of RNAi are short nucleotide sequence of 21 to 22 base pairs (bp) which are gene specific in nature and are generated by a RNAase III-like enzyme catalysed reaction using long dsRNA as substrate, thus forming sense or antisense target RNA (Forstemann *et al*., 2007; Younis *et al*., 2014; Challa *et al*., 2019). The dsRNA enters into the cell *via* either of two processes, *viz*., the transmembrane channel-mediated uptake mechanism and an endocytosis-mediated uptake mechanism (Mette *et al*., 2000; Liu *et al*., 2009; Rangasamy and Siegfried, 2012; Tatiparti *et al*., 2017). In the nucleus, initial processing occurs with the help of Drosha, wherein precursor-micro RNAs (pre-miRNA) are produced and transported to cytoplasm, thereafter it is cleaved by Dicer into a mature micro RNAs along with siRNAs (Preuss, 2003; Rana, 2007; Allen and Walker, 2012; Darrington *et al*., 2017). Subsequently, these double stranded products associate with the Argonaute proteins in a way that one strand is selected, which guides in a sequence-specific manner, in gene silencing of complementary mRNAs by RISC (either suppression or degradation) (Zamore *et al*., 2000; Waterhouse *et al*., 2001; Ogita *et al*., 2003; Qiao *et al*., 2007; Sun *et al*., 2012; Sharma *et al*., 2013; Wu *et al*., 2017; Zhang *et al*., 2019).

- The entry of long double stranded RNA, such as an introduced transgene, a rogue genetic element or a viral intruder, triggers the RNAi pathway of cells. This results in the recruitment of the enzyme Dicer.
- Dicer cleaves the dsRNA into short, 20-25 base pairs long, fragments, called small interfering RNA (siRNA).
- An RNA induced silencing complex (RISC) then distinguishes between the two siRNA strands as either sense or antisense. The sense strands (with exactly the same sequence as the target gene) are degraded.
- The antisense strands on the other hand are incorporated to the RISC. These are used as guide to target messenger RNAs (mRNA) in a sequence specific manner.
- Messenger RNAs (mRNA), which codes for amino acids, are cleaved by RISC. The activated RISC can repeatedly participate in mRNA degradation, inhibiting protein synthesis.

RNAi as a Biotechnological Approach for Management of Biotic and Abiotic Stress:- RNA interference (RNAi) has emerged as a powerful biotechnological tool for addressing both biotic and abiotic stresses in plants. By enabling sequence-specific gene silencing, RNAi offers a targeted and environmentally friendly alternative to conventional breeding and chemical control strategies. This approach allows researchers and plant breeders to precisely manipulate gene expression, improving crop resilience and productivity.

Biotic stress, caused by pathogens such as viruses, bacteria, fungi, insects, and nematodes, significantly limits agricultural productivity. RNAi-based strategies can target essential genes in these organisms, thereby disrupting their life cycles without harming the host plant or surrounding ecosystems. Notably, transgenic crops expressing double-stranded RNA (dsRNA) targeting pest or pathogen genes have shown high efficacy in controlling infestations. For instance, Smart Stax Pro maize incorporates RNAi to suppress the expression of *DvSnf7*, a gene vital to western corn rootworm survival.

RNAi for Disease Control and Future Therapeutics: The ability to specifically target and silence disease-associated genes makes RNA interference (RNAi) a highly promising tool for future plant therapeutics. Since many plant diseases are driven by the activity of one or a few key genes, RNAi-based strategies offer an attractive and targeted method for disease control. This includes a wide range of plant pathogens, such as fungi, viruses, bacteria, and even viral-like organisms. Additionally, dominant genetic disorders in plants can potentially be mitigated through RNAi-mediated gene silencing, making it a versatile platform for both protection and crop improvement.

RNAi can be triggered through two primary approaches:

1. **RNA-based delivery**, wherein synthetic small interfering RNAs (siRNAs) are introduced directly into target cells. These siRNAs are typically 21-base pair duplexes and are delivered using carriers such as nanoparticles, liposomes, or other delivery systems. This method is straightforward and often results in rapid and effective gene silencing, but its effects are usually transient and may require repeated applications for sustained action.
2. **DNA-based delivery**, where the siRNA effectors are generated intracellularly from longer RNA hairpin precursors. In this approach, genes encoding short hairpin RNAs (shRNAs) are introduced into the plant genome or delivered via vectors. These shRNAs are transcribed in the nucleus, exported to the cytoplasm through the

endogenous miRNA pathway, and then processed by Dicer into functional siRNAs. This method offers the potential for stable and long-term gene silencing, particularly in gene therapy settings, where viral vectors can deliver the shRNA-encoding genes for sustained expression (Hannon *et al.*, 2004; Scherer *et al.*, 2003).

While RNA-based strategies provide quick and flexible control, DNA-based RNAi therapies hold promise for long-term and possibly heritable disease resistance in crops. As delivery technologies and our understanding of RNAi mechanisms continue to advance, the practical applications of RNAi in plant disease management and agricultural biotechnology are expected to expand significantly.

A. Biotic Stress Resistance:

Virus Resistance: Virus-induced gene silencing (VIGS) is a RNA-mediated PTGS mechanism that protects plants against foreign gene invasion (Beclin *et al.*, 2002; Ding, 2010). VIGS has emerged as an extremely powerful functional genomics tool for knocking out gene expression of target plant genes in some plants. The concept of pathogen derived resistance (PDR) has promoted research aimed at achieving plants resistant to virus through genetic engineering (Simon-Mateo and García, 2011). PDR is either protein mediated involving protein encoded by the transgene or RNA-mediated, i.e., by the transcript produced from the transgene. In order to achieve PDR, hairpin dsRNA including small hairpin RNA (shRNA), self-complementary hpRNA, and intron-spliced hpRNA were formed in vivo using inverse repeat sequences from viral genomes. Among these, PTGS with the highest efficiency was elicited by the method involving self-complementary hairpin RNAs separated by an intron (Smith *et al*., 2000; Wesley *et al*., 2001). High resistance against viruses has been observed in plants even in the presence of inverted repeats of dsRNA-induced PTGS (IR-PTGS; Beclin *et al.,* 2002; Pandolfini *et al*., 2003; Zrachya *et al*., 2007). Resistance to Potato Spindle Tuber Viroid (PSTVd) infection was achieved in transgenic tomato plants producing dsRNA against PSTVd sequences (Nora *et al*., 2009). Similar strategy was used to successfully engineer resistance in cassava plants against African Cassava Mosaic Virus (ACMV; Vanderschuren *et al.,* 2009). Virus resistance has been engineered successfully in plants by targeting the coat protein (CP) gene through RNAi. Powell-Abel *et al*., (1986) showed that transgenic tobacco expressing the CP gene of Tobacco Mosaic Virus (TMV) was resistant to TMV and that the resistance was due to the expressed CP. Later, this strategy was extended to generate resistance against many different viruses as potato resistant to Potato Virus Y (PVY; Missiou *et al.,* 2004), tobacco resistant to Beet Necrotic Yellow Vein Virus (BNYVV; Andika *et al.*, 2005), *Cucumis* cv. *melo* resistant to Papaya Ring Spot Virus type W (PRSV-W; Krubphachaya *et al.,* 2007), *N. benthamiana* resistant to Cucumber Green Mottle Mosaic Virus (CGMMV; Kamachi *et al.,* 2007), *N. benthamiana* and *Prunus domestica* resistant to Plum Pox virus (PPV; Hily *et al.,* 2007). The use of RNA silencing strategy to engineer resistance is not limited to RNA viruses but can successfully be applied to DNA viruses. For example, blackgram plants recovered efficiently from geminivirus Vigna mungo yellow mosaic virus (VMYMV) infection when inoculated with hpRNA construct containing the promoter sequence of VMYMV under the control of the 35S promoter (Pooggin *et al*., 2003). RNAi method has been used to generate common bean resistant to geminivirus Beans Golden Mosaic Virus (BGMV; Bonfim *et al.*, 2007). A broad-spectrum resistance has been developed against tospoviruses in tomato plants by targeting multiple regions of a viral gene (Bucher *et al.*, 2006). Zhou *et al.,* (2012) have used sequences from disease specific protein gene and CP gene from Rice Stripe Virus to develop resistance against Rice Stripe Disease. Many viruses express viral silencing repressors (VSRs) proteins to counter host antiviral RNA silencing (Burgyan and Havelda, 2011). One of the strategies to improve virus resistance involves targeting the miRNA against these VSR. Niu *et al*., (2006) developed Arabidopsis plants with specific resistance against Turnip Yellow Mosaic Virus (TYMV) and Turnip Mosaic Virus (TuMV) by expressing amiRNAs based on miR159 precursor of A. thaliana. These amiRNAs target the sequence of two VSRs, P69 of TYMV and HC-Pro TuMV. Transgenic tobacco resistant to Cucumber Mosaic Virus (CMV) was generated by targeting a VSR, 2b of CMV through expression of an amiRNA based on an A. thaliana miR171 precursor (Qu *et al.,* 2007). TGBp1/p25 of Potato Virus X (PVX) was targeted in tobacco by expression of amiRNAs based on an *A. thaliana* miR159a, miR167b, and miR171a precursors (Ai *et al.*, 2011). Singh *et al*., (2014) generated transgenic tobacco plants resistant to Tomato leaf curl New Delhi virus (ToLCNDV) by transformation with trans-acting siRNA generating constructs against RNAi suppressor proteins (AC2 and AC4) of the geminivirus. Another promising strategy to reduce the multiplication and spread of virus in the plant includes the use of amiRNAs targeting the viral genes involved in replication, transmission, and symptom development after viral infection. Vu *et al.*,

(2013) used two amiRNA targeting the middle region of the AV1 (coat protein) transcript (amiR-AV1-3) and the overlapping region of the AV1 and AV2 (pre-coat protein) transcripts (amiR-AV1-1) of a geminivirus, Tomato leaf curl virus (ToLCV). Transgenic tomato plants expressing amiR-AV1-1, were highly tolerant to ToLCNDV and could successfully propagate until the T2 generation.

Bacterial Resistance: Bacterial diseases are extremely difficult to control due to rapid rate of spreading. RNAi mediated supression of two genes of Agrobacterium tumefaciens involved in crown gall tumor formation (iaaM and ipt) could significantly reduce the production of tumors in Arabidopsis (Escobar *et al*., 2001; Dunoyer *et al*., 2006). This strategy could be further extended to other plants. Fatty acids and their derivatives are important signaling molecule reported to negatively regulate plant's resistance to bacterial disease (Li *et al*., 2008a; *Jiang et al*., 2009). Arabidopsis and soybean plants showing enhanced resistance to multiple pathogens were generated by RNAi mediated suppression of SACPD gene encoding a fatty acid desaturase (Jiang *et al.,* 2009). In Arabidopsis, miR393 was reported to repress auxin signaling by negatively regulating the F-box auxin receptors like TIR1, thereby restricting the infection by bacteria Pseudomonas syringae (Navarro *et al*., 2006). Transgenic Arabidopsis plants over-expressing miR393 had enhanced bacterial resistance but with some developmental alterations (Navarro *et al*., 2006). Two different miRNAs, miR398 (Jagadeeswaran *et al.,* 2009) and miR825 (Fahlgren *et al*., 2007) were reported to be downregulated by bacterial infections. In the case of miR398, the expression of miR398 targets coding for two Cu/Zn superoxide dismutases (CSD1 and CSD2) was analyzed, and CSD1 was up-regulated upon bacterial infection in accordance with the down-regulation of miR398 under biotic stress (Jagadeeswaran *et al*., 2009). Very recently, new insight into miRNA function was gained with the discovery, that several miRNA families target genes of plant innate immune receptors (NBS-LRR) in Legumes (Zhai *et al.,* 2011) and Solanaceae (Li *et al.,* 2012). MiR482/2118 family of miRNAs were shown to target numerous NBS-LRR mRNAs encoding disease resistance proteins in tomato (Solanum lycopersicum) and other members of the Solanaceae (Shivaprasad *et al.,* 2012). Viral and bacterial infection suppresses miR482- mediated silencing of R genes. Thus, these pathogens responsive miRNA are either up- or down-regulated in response to bacterial invasion and effect gene expression by suppressing negative regulators and inducing positive regulators of immune responses. Identification and characterization of the targets of these miRNAs would help decipher new players in the pathways of host defence. If these pathogen-regulated miRNAs serve as positive regulator of bacterial resistance, strategy to generate transgenics with enhanced bacterial resistance involves constitutive over expression of miRNA or amiRNA. When miRNAs act as negative regulators, transgenic plants over-expressing these miRNAs become more sensitive to bacteria. In these cases, up-regulation of their target genes might be an effective strategy for improving plant stress tolerance, which can be achieved by over-expressing a miRNA-resistant form of its target or using amiRNA target mimic (Franco-Zorrilla *et al*., 2007).

Fungal Resistance: Targeting the genes of fatty acid metabolism through RNAi has proved to be an important strategy to generate diseases tolerance in various crop plants. RNAi-mediated suppression of a rice gene OsSSI2 led to enhanced resistance to blast fungus *Magnaporthe grisea* and leaf blight bacterium *Xanthomonas oryzae* (Jiang *et al.,* 2009). Besides, enhanced disease resistance against *M. grisea* in rice was achieved by suppression of two genes namely OsFAD7 and OsFAD8 which are 3 fatty acid desaturases (Yara *et al.,* 2007). RNAi mediated targeting of genes involved in lignin production, led to enhanced resistance of soybean to phytopathogen *Sclerotinia sclerotiorum* due to reduced lignin content (Peltier *et al*., 2009). Very recently, 24 miRNAs were shown to be involved in responses to attack by the fungus *Blumeria graminis* f. sp. *tritici* (Bgt) in wheat which cause a devastating disease of wheat powdery mildew (Xin *et al*., 2010). A rice miRNA osa-miR7695 was found to negatively regulate a natural resistance-associated macrophage protein 6 (OsNramp6) in response to the blast fungus *Magnaporthe oryzae*. Improved resistance to rice blast infection was achieved by over-expression of Osa-miR7696 (Campo *et al*., 2013).

Insect pests and Nematode Resistance: RNA interference has also been applied to control insect pests which lead to substantial crop loss (Huvenne and Smagghe, 2010). The development of a new generation of insect-resistant crops involves feeding of dsRNA as a diet component to insect which was shown to efficiently down-regulate the targeted genes in insect (Price and Gatehouse, 2008). The strategy was used in corn plants by expression of dsRNAs for tubulin or vacuolar ATPase genes to develop western corn rootworm (WCR) resistant transgenic corn plants (Baum *et al*., 2007). Cotton bollworm (*Helicoverpa armigera*) larvae showed reduced growth when fed on plant material expressing dsRNA specific to cytochrome P450 gene (CYP6AE14;

Mao *et al.*, 2007). Silencing of genes involved in parasitism or housekeeping genes in the root-knot nematode by expression of dsRNA in host plant resulted in enhanced resistance to the nematode (Gheysen and Vanholme, 2007). Sindhu *et al.*, (2009) has targeted four genes involved in parasitism of sugar beet cyst nematode (*Heterodera schachtii*), having host A. thaliana by RNAi. Though complete resistance was not achieved but the number of mature nematode females in different RNAi lines were reduced to 23-64%. Ibrahim *et al.*, (2011) could successfully reduce the gall formation by *Meloidogyne incognita* in soybean roots by suppressing the genes encoding tyrosine phosphatase (TP) and mitochondrial stress-70 protein precursor (MSP). microRNAs were also reported to be involved in plant nematode interactions. In response to infection by the nematode, *H. schachtii*, miR161, miR164, miR167a, miR172c, miR396c, miR396a, b, and miR398a were down-regulated in Arabidopsis (Hewezi *et al.*, 2008; Khraiwesh *et al.*, 2012). Comparative analysis of miRNA profiling in soybean indicated that 101 miRNAs belonging to 40 families were responsive to the infection of the soybean cyst nematode (SCN; *Heterodera glycines*), the most devastating pathogen in soybean. It was further revealed that 20 miRNAs were differentially expressed between SCN resistant and susceptible soybean cultivars (Li *et al.*, 2012). Moreover, it has been suggested that nematode induced miRNAs and sRNAs likely to be involved in feeding site establishment and parasitism, respectively (Hewezi *et al.*, 2008). Over-expression of these nematode induced candidate miRNAs, and/or silencing of their corresponding targets, may provide clear insight about plant-nematode parasitism, and also may provide nematode resistance to crop plants. Nematode resistance can also be achieved by expressing amiRNA, containing known miRNA genes with the replaced seed region of the vital gene (parasitism or housekeeping) of plant parasitic nematode.

B. Abiotic stress:

Including drought, salinity, extreme temperatures, and oxidative stress—also severely impacts crop yield and quality. RNAi can be used to downregulate stress-responsive negative regulators or modulate metabolic pathways, enhancing plant tolerance to these harsh environmental conditions. Targeted silencing of genes associated with stress sensitivity can result in plants with improved adaptability and growth under adverse conditions.

Abiotic Stress Tolerance:

Drought and Salinity 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 downregulation 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 (Wang *et al.*, 2009). Transgenic rice plants tolerant to drought stress were developed by silencing of receptor for activated C-kinase 1 (RACK1; Li *et al.*, 2009). The ubiquitin ligase gene has been targeted for RNAi in rice to enhance drought tolerance. Rice knockdown of a RING finger E3 ligase gene-OsDSG1 leads to enhanced drought tolerance (Park *et al.*, 2010). Similarly, silencing of OsDIS1 (for *Oryza sativa* drought induced SINA protein 1), a C3HC4 RING finger E3 ligase by RNAi enhanced drought tolerance. Wang *et al.*, (2011) studied legume model plant, *Medicago truncatula* and identified several drought-responsive miRNAs. The already known and predicted targets of these miRNAs were found to be involved in wide variety of processes in plant cell including development, transcription, protein degradation, detoxification, etc. It was observed that miR393 was strongly upregulated by dehydration, cold, high salinity, and ABA treatments (Sunkar and Zhu, 2004). On exposing Arabidopsis to varying degrees of different abiotic stress, up regulation was observed in miR402, miR319c, miR389a, and miR397b. Xin *et al.*, (2010) identified and reported 12 miRNAs responsive to heat stress in wheat (*Triticum aestivum* L.). MiRNA responsive to various stresses (drought, cold, salinity, and ABA) were identified in rice seedlings (Jian *et al.*, 2010). Among many stresses responsive miRNAs discovered in rice, only two miR393 and miR169 were found to be responsive to abiotic stress like dehydration (Zhao *et al.*, 2007). In Arabidopsis, miR169 was down-regulated by drought stress, and its target nuclear factor YA5 (NF-YA5) was significantly induced upon drought stress. Transgenic plants over-expressing miR169a were more sensitive to drought stress with increased leaf water loss as compared to wild-type controls, and over-expression of NF-YA5 led to enhanced drought tolerance in transgenic plants (Li *et al.*, 2008a). Similarly, a newly characterized Soybean (*Glycine max* L.) gene GmNFYA3, a target of miR169, was demonstrated to positively modulate drought stress tolerance in transgenic Arabidopsis plants (Ni *et al.*, 2013). However, over expression of GmNFYA3 in Arabidopsis resulted in increased sensitivity to salinity stress and exogenous ABA (Ni *et al.*, 2013). These findings were contrary to the observation in tomato (*S. lycopersicum*), in which miR169 was induced by drought stress and its four targets NF-YA1/2/3 and multidrug resistance associated protein

gene 1 (MRP1) were all down-regulated by drought stress. Constitutive over-expression of tomato miR169c led to reduced stomatal openings, transpiration rate, and leaf water loss, thus enhanced drought tolerance in transgenic plants in comparison to wild-type controls (Zhang *et al.*, 2011a). It is notable that transgenic tomato plants had no noticeable morphological and developmental alterations under field conditions, indicating that miR169 or its targets could be potential candidate genes for genetic engineering to achieve enhanced abiotic stress tolerance in transgenic plants (Zhang *et al.*, 2011a). However, as mentioned above, miR169 positively regulates plant response to drought stress in tomato, but negatively in Arabidopsis. The opposite roles miR169 play in different plant species suggest that even conserved miRNAs might function in a species-specific manner and this should be taken into careful consideration when tailoring GM strategies for specific target species. In a recent study, transgenic creeping bent grass (*Agrostis stolonifera*) plants over-expressing a rice miR319 gene (Ossa miR319) exhibited enhanced tolerance to drought and salinity that was associated with increased leaf wax content and water retention but reduced sodium uptake. Gene expression analysis indicated that the enhanced abiotic stress tolerance can be attributed to a significant down-regulation of at least four putative turf miR319 target genes teosinte branched/cycloidea/proliferating factors (TCP)—AsPCF5, AsPCF6, AsPCF8, and AsTCP14 and a homolog of a rice NAC domain gene AsNAC60 (Zhou *et al.*, 2013). It is noticeable that over-expression of Osa-miR319 caused pleiotropic phenotypes including increased leaf size, enlarged stems and decreased tiller number, which are undesirable traits for creeping bent grass. To better utilize the miR319-based GM strategy, further functional characterization of miR319 targets or downstream genes in the related biological pathways needs to be undertaken. However, the mechanism of miR319-mediated plant abiotic stress response revealed from this study provides important information to develop novel strategies for plant genetic engineering and has the potential to be applied in other important crop species. MiR159 was reported to respond to hormone signalling and dehydration responses in Arabidopsis (Achard *et al.*, 2004; Reyes and Chua, 2007). Hu *et al.*, (2009) showed that most of the rice histone deacetylases genes are responsive to drought or salt stresses with specific patterns of expression and divergent developmental functions compared to closely related homologs in Arabidopsis. Trindade *et al.*, (2010) identified several conserved miRNAs in *M. truncatula* plants which are expressed differentially in water-deficit condition. MiR169 was shown to be down-regulated in roots, whereas miR398a/b and miR408 showed very high expression in shoots as well as roots. Hwang *et al.*, (2011) reported a drought stress-responsive miR171 family in potato plants, Solanum tuberosum which miR171a, miR171b, and miR171c. The RNAi-mediated silencing of farnesyl transferase genes (FTA or FTB) in canola resulted in reduced transpiration rate due to decreased stomatal conductance, thereby promoting yield (Wang *et al.*, 2005, 2009).

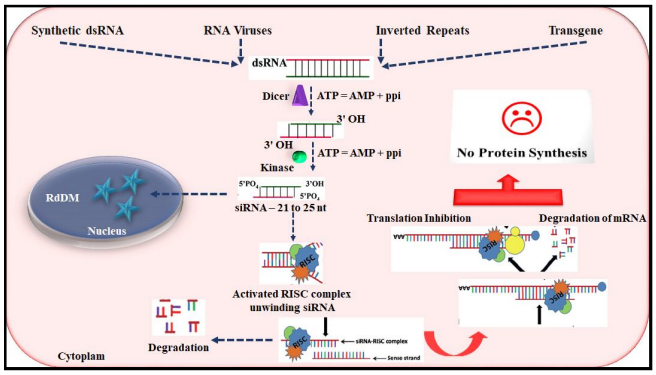

Fig. 1: Mechanism of RNA Interference.

Cold and Heat Stress Tolerance: The expression of miR319 was reported to change in response to cold stress in Arabidopsis (Sunkar and Zhu, 2004; Liu *et al.*, 2008), rice (Lv *et al.*, 2010), and sugarcane (Thiebaut *et al.*, 2011). Further transgenic studies using wild-type plants as controls indicated that over-expression of Osa-miR319 gene led to increased cold stress tolerance (4°C) after chilling acclimation(12°C) of plants (Yang *et al.*, 2013). However, miR319 transgenic rice plants displayed severe developmental delay. To avoid the pleiotropic effect of miR319, two RNAi lines for the miR319 targets, OsPCF5 and OsTCP21, were generated. These RNAi lines, upon chilling acclimation, also exhibited better cold tolerance than wild-type controls, but were phenotypically

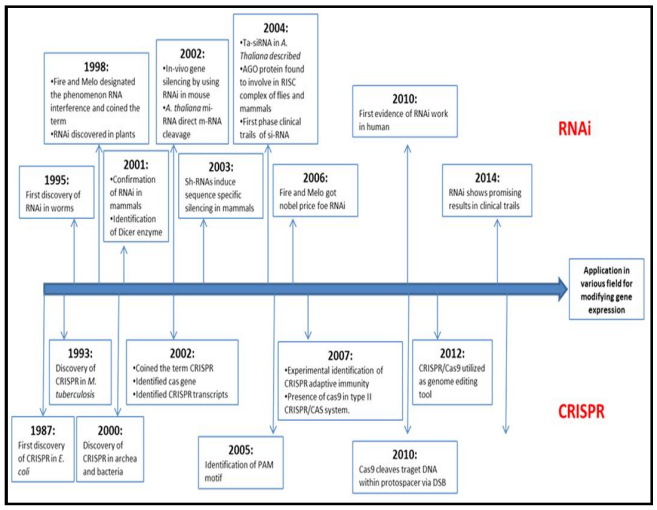

Fig. 2: Graphical representation of RNAi and CRISPER technology as blow diagram.

Table 1: Examples of novel plant traits engineered through RNAi.

Trait	Target Gene	Host	Application
Enhance-dnutrient content	Lyc	Tomato	Increased concentration of lycopene (carotenoid antioxidant)
	DET1	Tomato	Higher flavonoid and b-carotene contents
	SBEII	Wheat, Sweet potato, Maize	Increased levels of amylose for glycemic management and digestive health
	FAD2	Canola, Peanut, Cotton	Increased oleic acid content
	SAD1	Cotton	Increased stearic acid content
	ZLKR/SDH	Maize	Lysine-fortified maize
Reduced alkaloid production	CaMXMT1	Coffee	Decaffeinated coffee
	COR	Opium poppy	Production of non-narcotic alkaloid, instead of morphine
	CYP82E4	Tobacco	Reduced levels of the carcinogen nornicotine in cured leaves
Heavy metal accumulation	ACR2	*Arabidopsis*	Arsenic hyperaccumulation for phytoremediation
Reduced polyphenol production	s-cadinene synthase gene	Cotton	Lower gossypol levels in cottonseeds, for safe consumption
Ethylene-sensitivity	LeETR4	Tomato	Early ripening tomatoes
	ACC oxidase gene	Tomato	Longer shelf life because of slow ripening
Reducedall-ergenicity	Arah2	Peanut	Allergen-free peanuts
	Lolp1, Lolp2	Ryegrass	Hypo-allergenic ryegrass
Reduced production of lachrymatory factor synthase	lachrymatory factor synthase gene	Onion	"Tearless" onion

normal. It should be noted that improvement of plant cold tolerance in the miR319 over expression lines was more significant than that in the OsPCF5 and OsTCP21 RNAi lines (Yang *et al.*, 2013), most likely due to target function redundancy in the latter case. This is also a challenge when manipulating miRNA targets instead of miRNAs themselves for plant trait modification, because in some cases multiple targets have to be simultaneously down-regulated to achieve the same level of effect as over-expression of an individual miRNA gene. Guan *et al.*, (2013) discovered a novel plant thermotolerance mechanism, especially for the protection of reproductive organs. It involves induction of miR398 to downregulate its targets CSD (copper/zinc superoxide dismutase) genes, CSD1 and CSD2 as well as CCS (a gene encoding copper chaperone for both CSD1 and CSD2; Guan *et al*., 2013). They found that csd1, csd2, and ccs mutants displayed higher heat stress tolerance than wild-type plants associated with increased accumulation of heat stress transcription factors and heat shock proteins and reduced damage to flowers (Guan *et al.*, 2013). These results strongly suggest that manipulating miR398 or its targets can be an applicable strategy to increase heat tolerance in crop species, especially in corn, which suffers damage to its reproductive tissues by prolonged periods of high summer temperatures.

Oxidative Stress Tolerance: Plants tend to accumulate reactive oxygen species (ROS) in response to environmental stimuli such as high intensity light, extreme temperatures, UV radiation, heavy metals, salinity, drought stresses, and mechanical stresses (Khraiwesh *et al.*, 2012). Superoxide dismutases (SODs) in plants can detoxify superoxide radicals by converting them into molecular oxygen and hydrogen peroxide. Several studies have been conducted to improve plant stress tolerance by over-expression of superoxide dismutase (Cu/Zn-SODs) in transgenic plants which detoxify superoxide radicals (Tepperman and Dunsmuir, 1990; Pitcher *et al.*, 1991; Gupta *et al.*, 1993; Perl *et al.*, 1993; Sunkar *et al.*, 2006). However, in some of the studies, the transgenic plants exhibited minimal or no increase in stress tolerance (Tepperman and Dunsmuir, 1990; Pitcher *et al.*, 1991). Sunkar *et al.*, (2006) offered an improved strategy to solve this problem by over-expressing miR398-resistant form of CSD2, which led to increased tolerance to high intensity light, heavy metals and other oxidative stresses (Table 1; Sunkar *et al.*, 2006). The exploration of interactions between miR398 and its targets (CSD1 and CSD2) also provided a possible explanation to the previously failed attempts. The expression of the introduced SOD transgenes containing the miR398 target sites were negatively impacted by

miR398- mediated gene regulation.

Overall, RNAi offers a precise, sustainable, and scalable solution for developing stress-resilient crop varieties. As the global climate continues to shift and the demand for food rises, integrating RNAi into crop improvement programs could be vital for achieving food security and sustainable agriculture.

Engineered plant viruses in a trans-kingdom silencing strategy against their insect vectors: The majority of plant-infecting viruses have single-stranded RNA as their genetic material and they replicate their genomes in the plant cell cytoplasm through dsRNA intermediates. Thus, a conceptual approach which might be used to confer resistance against plant viruses is to pre-program plants to have an active RNAi defence against a particular viral RNA before the plant is challenged by that virus. Then, when the plant is challenged by the virus, the already active RNAi would recognize and degrade the incoming viral RNA before the virus can replicate and become established. Fortunately, plants can be genetically engineered to contain and express new genes, so engineering plants for specific antiviral RNAi-based resistance can be done. Many plants can be genetically engineered by taking advantage of the common soil-inhabiting bacterium *Agrobacterium tumefaciens*, which naturally transfers genes into plant cells, causing the disease crown gall. *Agrobacterium tumefaciens* contains a large plasmid, the Ti plasmid. When *A. tumefaciens* encounters a wounded plant cell, it transfers and inserts a portion of the Ti plasmid, the T-DNA, into the plant cell chromosome; the transformed plant cell then expresses the genes on the T-DNA. The wild-type T-DNA contains genes encoding for plant hormones; expression of these genes in the transformed cells results in galls developing at the site of infection. But the Ti plasmid can be genetically engineered to remove the genes leading to gall formation (disarmed Ti plasmid) and modified instead to contain and express desirable genes; for antiviral resistance, this could mean engineering it to express RNA designed to induce RNAi activity toward the viral RNA genome. *Agrobacterium tumefaciens* can be used to transfer these genes into individual plant cells, and the individual transformed plant cells can be cultured *in vitro* to regenerate whole plants. As each cell of the plant came from the original transformed cell, all cells will now contain the transgene in their chromosomal DNA, and it can be stable for generations.

Role of RNAi for improvement of crop and plant nutritional value:

Plant Architecture and Biomass: RNA interference can be employed successfully to improve yield of crop and fruit plants by manipulating the basic agronomic traits of plant such as height, inflorescence, branching and size. RNAi mediated knockdown of a gene OsDWARF4 in rice resulted in shorter plants with erect leaf architecture leading to increased photosynthesis in the lower leaves. Such plant has potential for improved yields under dense planting conditions (Feldmann, 2006). Some plant materials show recalcitrance to a process of saccharification which causes major limitation for conversion of lignocellulosic biomass to ethanol. The problem of recalcitrance of plant cell wall to bioconversion can be overcome by genetic reduction of lignin content (Chen and Dixon, 2007). Independent down-regulation of each of six lignin biosynthesis enzymes in transgenic alfalfa lines, yielded nearly twice as much sugar from stem's cell walls as compared to wild-type plants (Reddy *et al.*, 2005). Down-regulation of lignin genes like cinnamate 4-hydroxylase (C3H), shikimate hydroxycinnamoyl transferase (HCT) and 4-coumarate-coA ligase (4CL) in plants reduced total lignin content, improved accessibility of cellulases for cellulose degradation and increased dry matter degradability (Hisano *et al.*, 2009). This lignin modification also facilitated bypassing the need for acid pre-treatment (Chen and Dixon, 2007). Over-expression of the maize Corngrass1 (Cg1) miRNA that belongs to the MIR156 family caused prolonged vegetative phase and delayed flowering (Chuck *et al*., 2011) resulting in increased biomass. The transgenic plants showed up to 250% more starch and improved digestibility (Chuck *et al.*, 2011). It was reported that the degrees of morphological changes and biomass yields were related to the expression level of an exogenous rice miRNA Osa-miR156b (Fu *et al.*, 2012). Transgenic switch grass plants with relatively high levels of miR156 showed highly stunted growth, whereas those with moderate and low levels of miR156 expression had 58–101% more biomass production than wild-type controls as a result of increases in tiller numbers. It was also observed that over-expression of rice miR156 could improve the yield of solubilised sugar as well as forage digestibility. Over-expression of miR156 led to increased biomass in other plant species including Arabidopsis and rice (Schwab *et al.*, 2005; Xie *et al.*, 2006, 2012). Cg1 over-expressing, poplar transgenics plants showed significant increase in the growth of axillary meristem, shortening of internode length, and reduction in stem lignin content.

Grain yield and grain content: Plant architecture can be manipulated in order to achieve increased grain yield like in rice plants (Jiao *et al.*, 2010; Miura *et al.*,

Table 2: SOWT Analysis of RNA Interference (RNAi) and CRISPR/ Cas9 Technology for crop Improvement.

RNA Interference (RNAi):	CRISPR/Cas9:	Remarks
Mechanism: RNAi targets RNA transcripts. Because of the RNAi involves the use of small RNA molecules (like siRNA or miRNA) to silence gene expression by targeting and degrading mRNA or blocking its translation.	**Mechanism:** CRISPR targets DNA sequence. Because of the CRISPR /Cas9 utilizes a guide RNA (gRNA) to direct the Cas9 enzyme to a specific DNA sequence, where it can induce a double-strand break. This break can be repaired by the cell, leading to gene knockout, or be used as a template for gene editing.	Wider used application of CRISPR system is yet to be established at a genome scale. But to selecting the best gene silencing method due to RNAi.
Applications: RNAi is widely used in functional genomics research, therapeutic applications (e.g., targeting viral RNAs), and crop improvement. RNAi is the post-transcriptional gene silencing mechanism generally found in eukaryotes. RNAi is having a unique space in diverse genetic applications.	**Applications:** CRISPR is a versatile tool for gene editing, including gene knockout, gene activation, and targeted DNA modifications. It has applications in research, medicine, and agriculture. CRISPR is the new age genome editing tool that naturally serves as defence barrier found in prokaryotes. the increasing advantages of CRISPR/Cas9 may dominate over RNAi in near future.	But the increasing advantages of CRISPR/Cas9 maydominate over RNAi in near future. CRISPR is the biggest threatto RNAi.
Strengths: RNAi is relatively easy to implement and can achieve transient	**Strengths:** CRISPR offers high precision and efficiency in gene editing, with the	In near future predictably, CRISPR/Cas9 will rule
gene silencing. It is mostly used for research and therapeutic purposes.	potential for durable changes. It is an important gene editing tool that cleaves DNA sequences.	molecular biology lab for modifying gene expression, while RNAi will likely to be cornered with restricted domains of applications.
Weaknesses: RNAi can suffer from off-target effects (affecting genes other than the intended target) and can be less efficient than CRISPR in some contexts.	**Weaknesses:** CRISPR can have off-target effects, although they are generally considered lower than RNAi. There are also ongoing efforts to improve the delivery and specificity of CRISPR components.	

2010; Springer, 2010; Wang *et al.,* 2012). RNAi-mediated suppression of GA 20-oxidase (OsGA20ox2) gene resulted in semi-dwarf plants from a taller rice variety QX1. This RNAi transgenic exhibited significant increase in panicle length, increased number of seeds per panicle and higher test (1000 grain) weight (Qiao *et al.,* 2007). OsSPL14 (Souamosa promoter binding protein-like 14) was reported to be the target of OsamiR156 in rice (Jiao *et al.,* 2010; Miura *et al.,* 2010) and was shown to positively regulate the rice plant architecture leading to enhanced yield of rice grain (Jiao *et al.,* 2010). Higher expression of OsSPL14 could modify the rice plant architecture resulting in decreased tiller number and increased grain yield (Jiao *et al.,* 2010; Miura *et al.,* 2010; Wang *et al.,* 2012). Wang *et al.,* (2012) showed OsSPL16 to be a positive regulator of cell proliferation with increase in grain width and yield in over-expressing rice plants. However, elevated expression of OsSPL16 resulted in decrease grain appearance quality. This was expected as grain quality and yield are usually negatively correlated. But, decreased expression of OsSPL16 resulted in slender grains with better quality. Thus, miR156 and its targets OsSPL14 or OsSPL16 can be used for modifying plant architecture to create superior rice cultivars with greater yield. Recently, Guo *et al.,* (2013) showed interactions of OsmiR444a regulated OsMADS57, OsTB1 (TEO-SINTE BRANCHED1) and D14 (Dwarf14) in controlling rice tillering. Thus, manipulating OsmiR444a and its targets by genetic engineering can prove to be an important strategy to achieve high grain yield (Guo *et al.,* 2013). Zhang *et al.,* (2013) reported that over-expression of OsmiR397, which is expressed naturally in young panicles and grains of rice promotes panicle branching and enlargement of grain size, causing an increase in overall grain yield of up to 25% in a field trial. OsmiR397 increases grain yield by down-regulating its target, OsLAC coding for a laccase-like protein that is involved in the sensitivity of plants to

Table 3: The following traits targeted gene through CRISPR technology (Meenakshi Rajput *et al*., 2021).

Trait(s)	Crop Used	Targeted Gene(s)
Drought tolerance	*Z. mays* (Maize)	*ARGOS8*
Turnip mosaic virus (TMV) resistance	*A. thaliana*	*eIF(iso)4E*
Cucumber vein yellowing virus (CMYV) resistance	*Cucumis sativus*	*eIF4E*
Drought tolerance	*S. lycopersicum*	*SlMAPK3*
Cold tolerance	*O. sativa*	*OsAnn3*
Parthenocarpic fruit development	*S. lycopersicum*	*SlIAA9*
Chilling stress tolerance	*S. lycopersicum*	*SlCBF1*
Tomato yellow leaf curl virus (TYLCV) resistance	*S. lycopersicum*, *N. benthamiana*	*Coat protein* (*CP*) *Replicase* (*Rep*)
Cauliflower mosaic virus (CMV) resistance	*A. thaliana*	*CaMV CP*
Rice tungro spherical virus (RTSV) resistance	*O. sativa*	*eIF4G*
Salt tolerance		*OsRR22*
Male-sterile development	*T. aestivum*	*Ms1*
Heat stress tolerance	*S. lycopersicum*	*SlMAPK3*
Drought and salt stress tolerance	*A. thaliana*	*DAP4 SOD7*
Drought tolerance		*AREB1*
Wheat dwarf virus (WDV) resistance	*Hordeum vulgare*	*CP Rep/Rep4*
Yield improvement	*B. napus*	*BnaMAX1*
Yield improvementStress tolerance	*O. sativa* (Nippobare)	*OsPIN5b GS3 OsMYB30*
Yield improvement	*O. sativa*	*Cyt P450 homeologs OsBADH2*
Drought and stress tolerance		*OsDST*
Tomato yellow leaf curl virus (TYLCV) resistance	*S. lycopersicum*	*rgsCaM*
Soyabean mosaic virus (SMV) resistance	*Glycine max*	*GmF3H1 GmF3H2 GmFNSII-1*

brassinosteroids. Since, miR397 is highly conserved across different species; this strategy can be extended to other cereal crops for increasing grain yield.

Flower colour modification by RNAi-mediated gene silencing: Gentians, *Gentiana triflora, Gentiana scabra* and their interspecific hybrid, are one of the most popular floricultural plants in Japan, and more than half of gentian production is from the Iwate prefecture. Gentians come into bloom from early summer to late autumn in Japan, and are often used as ornamental cut flowers. Genetic engineering approaches are being applied to several ornamental plants (Forkmann *et al.,* 2001; Tanaka *et al.,* 1998; Tanaka *et al*., 2005). For example, Florigene Ltd. and Suntory Ltd. have developed blue flowered carnations using genetic engineering, and they are commercialized in North America, Australia and Japan (Tanaka *et al.,* 2005). We have also produced white-flowered transgenic gentians by suppressing the chalcone synthase (CHS) gene using antisense technology (Nishihara *et al.,* 2006). In this case, only 3 of 17 independent transgenic lines displayed white-flowered phenotypes, but other transformants did not lead to successful suppression of CHS gene expression. Moreover, no transgenic gentian plants with suppressed expression of other anthocyanin biosynthetic genes, such as dihydroflavonol 4-reductase (DFR) and flavonoid 3, 5-hydroxylase (F3 5 H) genes, have so far been obtained by antisense and sense suppression technology (Nishihara *et al.,* 2005). The low frequency of down regulation of the targeted genes was thought result from the use of both the cauliflower mosaic virus (CaMV) 35S promoter and antisense gene suppression technology. Gene silencing by RNAi was also utilized to modify the flower color in some plant species, including petunia, torenia and tobacco plants (Tsuda *et al.,* 2004; Nishihara *et al*., 2005; Nakamura *et al*., 2006; Nakatsuka *et al*., 2007a; Nakatsuka *et al.,* 2007b; Nakatsuka *et al.,* 2008).

Fruit nutritional value and edibility: Tomato (*Lycopersicon esculentum*) is one of the most economically important fruit minerals, fibres, and vitamins (Rajam *et al.,* 2007). RNAi has been utilized in development of tomato fruit with enhanced level of carotenoids and flavonoids which are highly beneficial for human health. RNAi in combination with fruit specific promoter has been used to suppress an endogenous DET1 gene in tomato, a photomorphogenesis regulatory gene involved in repression of several light controlled signalling pathways (Davuluri *et al.,* 2005). DET1 was specifically degraded in transgenic tomatoes with suppressed DET1, accompanied by an increase in the level of flavonoid and carotenoid. Abscisic acid (ABA) plays important roles during tomato fruit ripening. The SlNCED1 gene of tomato encoding 9-cis-epoxycarotenoid dioxygenase), an important enzyme in the ABA biosynthesis, was

suppressed by RNAi. The fruits from RNAi lines showed enhanced accumulation of upstream compounds in the pathway, chiefly lycopene and β carotene. Similarly, RNAi has been utilized to increase the carotenoid content of rapeseed (*Brassica napus*) by down regulating the expression of lycopene epsilon cyclase (ε-CYC) gene. The seeds obtained from transgenic Brassica showed increased in zeaxanthin, violaxanthin, β-carotene, and lutein content (Yu *et al.,* 2007). RNAi approach has also been used in apple to improve the fruit quality by enhanced self-life (Dandekar *et al.,* 2004), reducing the amount of a major apple allergen (Gilisen *et al.,* 2005) and silencing the leaf sorbitol synthesis which affects fruit quality such as starch accumulation and sugar-acid balance (Teo *et al.,* 2006).

Seedless Fruit Development (Parthenocarpy): Parthenocarpy or seedlessness is a process involving production of seedless fruits, developed from the ovary without pollination and fertilization. Parthenocarpy or seed lessness is a highly desirable agronomic trait for fruit which enhances its marketing values (Molesini *et al.,* 2012) because high yield can be achieved even under environmental conditions unfavourable for pollination and fertilization. In tomato, seedless fruits have been achieved by down-regulating a chalcone synthase, a gene involved in first step of flavonoid biosynthesis (Schijlen *et al.,* 2007). Phytohormones such as auxin and gibberellins are closely associated with the trait of parthenocarpy (Molesini *et al.,* 2012) which in turn are regulated by many miRNAs. Thus, manipulating the level of phytohormones by controlling activities of miRNAs or their targets can prove to be an important approach to achieve parthenocarpy. For example, expression of an aberrant form of auxin response factor 8 (ARF8), a target of miR167 (Ru *et al.,* 2006; Wu *et al.,* 2006; Molesini *et al.,* 2012), resulted in parthenocarpic fruit in both Arabidopsis and tomato (Goetz *et al.,* 2007). Seedless fruits were observed in tomato plants when ARF7 function was suppressed using RNAi (De Jong *et al.,* 2009). Parthenocarpic fruits were also observed in tomato in which genes of the AUCSIA family coding for 53-amino-acid-long (protein or peptide) were functionally suppressed by RNAi (Molesini *et al.,* 2009).

CRISPR/Cas9 system-mediated gene editing in crops: Both RNA interference (RNAi) and CRISPR technologies are powerful tools for manipulating gene expression, but they work through different mechanisms. RNAi primarily silences gene expression at the post-transcriptional level by targeting mRNA, while CRISPR/Cas9 enables targeted DNA editing and can be used for gene knockout or activation. While RNAi has been a prominent research tool for gene silencing, CRISPR has emerged as a leading technology for genome editing.

Application of RNAi Technology: RNA interference (RNAi) technology has revolutionized functional genomics and is now widely recognized as a versatile tool in agriculture, particularly for improving crop resilience, yield, and protection against pests and diseases. By enabling the targeted silencing of specific genes, RNAi offers a precise and environmentally sustainable alternative to chemical pesticides and traditional breeding techniques.

Crop Protection Against Insect Pests: One of the most impactful applications of RNAi in agriculture is pest control. RNAi can be used to silence essential genes in insect pests by delivering double-stranded RNA (dsRNA) molecules through genetically modified plants, topical sprays, or baits. Upon ingestion, the dsRNA is processed within the pest's cells, leading to gene knockdown and eventual mortality or reproductive failure. Commercial examples include SmartStax® Pro maize, which expresses dsRNA targeting the *DvSnf7* gene in the Western corn rootworm.

Disease Resistance in Plants: RNAi is also being employed to combat plant pathogens such as viruses, fungi, and bacteria. By targeting pathogen-specific genes, RNAi can prevent infection or suppress disease progression. Host-induced gene silencing (HIGS) is a prominent approach where plants are engineered to produce dsRNA targeting genes in pathogens, conferring resistance without the use of chemicals. For example, transgenic wheat expressing dsRNA against *Fusarium graminearum* genes has shown enhanced resistance to Fusarium head blight.

Weed and Nematode Management: RNAi holds potential in the management of parasitic weeds and plant-parasitic nematodes. Targeting essential genes in these organisms through plant-delivered dsRNA can disrupt their development and parasitism. This approach provides a species-specific and ecologically safe alternative to herbicides and nematicides.

Abiotic Stress Tolerance: Beyond biotic threats, RNAi can be harnessed to improve plant tolerance to abiotic stresses such as drought, salinity, heat, and cold. Silencing negative regulators of stress response pathways or modulating transcription factors involved in stress signaling enables crops to better withstand environmental extremes. This application is especially critical in the face of climate change and the need for sustainable food production.

Nutritional Enhancement and Quality Traits: RNAi can be applied to modify metabolic pathways in crops to enhance nutritional content, shelf life, or taste. For instance, silencing of the *polygalacturonase* gene in tomatoes delays fruit softening, improving post-harvest shelf life. Similarly, RNAi has been used to reduce allergens and toxic compounds in food crops, making them safer for consumption.

Functional Genomics and Gene Discovery: In research, RNAi serves as a critical reverse genetics tool to study gene function. By selectively silencing genes in model plants like *Arabidopsis thaliana*, scientists can infer gene roles in growth, development, and stress response. This knowledge aids in the identification of candidate genes for crop improvement.

RNAi technology has thus emerged as a cornerstone of modern plant biotechnology. Its precision, scalability, and broad applicability make it a vital component of sustainable agriculture and crop enhancement strategies.

- In plant system, it provides defence mechanism to protect against infection by viruses, transposons and other insertional elements.
- RNAi also plays a role in regulating development and genome maintenance.
- Development of male sterile plants in rice.
- Recently two RNAi based crops have been given regulatory approval for commercial production and sale. These are the non-browning Arctic apples and the non-browning Innate potatoes. The firms producing these crops claim that the idea behind producing the non-browning apples and potatoes is not only to improve the look of the product, but it is also intended to increase the consumption of the raw fruits along with reducing naturally occurring carcinogens (as in the case of innate potatoes). While the science behind both these products is a little complicated as both are RNAi based, in simple way it can be put as both apples and potatoes have certain genes suppressed. Both of them, though genetically modified, are grown the same way as conventional varieties. These products are likely to find a place of attraction in the fresh cut product sales.

Advantages of RNAi:

- **High Gene Specificity**: RNAi allows for precise targeting of specific genes, minimizing off-target effects and ensuring accuracy in gene silencing.
- **Efficient Gene Silencing**: It achieves high levels of gene knockdown, often sufficient to observe phenotypic effects and control undesirable traits or pathogens.
- **Rapid Screening**: RNAi enables faster identification of gene function in targeted plants, reducing the time required for functional genomics studies.
- **Inducibility**: RNAi can be designed to be inducible under specific conditions (e.g., stress, developmental stage), allowing controlled gene silencing when needed.

Disadvantages of RNAi:

- **Incomplete Gene Knockdown**: RNAi typically reduces gene expression but does not completely eliminate it, which may be insufficient in cases where total gene suppression is necessary.
- **Off-target Effects**: Small interfering RNAs (siRNAs) can sometimes trigger unintended silencing of non-target genes due to sequence similarities, leading to undesirable outcomes.
- **Activation of Unwanted Pathways**: In some cases, siRNA can unintentionally activate immune responses or other cellular pathways, potentially affecting overall plant health or development.

Conclusions and Future Perspectives

RNAi in Crop Improvement and Nematode Management: Current Advances and Future Prospects: RNA interference (RNAi) has emerged as a powerful and environmentally sustainable strategy for managing multiple pests and pathogens, including plant-parasitic nematodes. As agriculture moves toward reducing reliance on harmful chemical pesticides, RNAi offers a gene-specific, eco-friendly alternative for crop protection. However, nematode management presents unique challenges due to their obligate parasitism and dependence on living host tissue for survival.

Host-induced gene silencing (HIGS) has shown promising results in controlling root-knot nematodes (RKNs) and cyst nematodes (CNs). Advances in functional genomics, the availability of nematode genome sequences, and sophisticated bioinformatics tools have enabled the design of dsRNA constructs with minimized off-target effects. The stacking of multiple dsRNA sequences targeting different nematode genes has further enhanced the efficacy of RNAi-based nematode control strategies.

Beyond pest management, RNAi has become a key

tool in functional genomics and crop improvement. Small RNAs such as siRNAs and miRNAs are now routinely employed to silence or modulate gene expression in plants. This allows for the precise manipulation of both beneficial and detrimental genes, offering plant biologists a way to develop novel traits and improve existing ones. Artificial miRNAs (amiRNAs), overexpressed natural miRNAs, and gene-specific siRNAs have been utilized to enhance crop productivity, biomass, and grain yield.

In addition to yield-related traits, RNAi has been successfully applied to improve nutritional quality in crops. Examples include cereals, fruits, and vegetables enriched with essential minerals, vitamins, amino acids, and fatty acids. Furthermore, RNAi-based approaches have enabled the development of plants with enhanced abiotic stress tolerance (especially drought resistance) and resistance to biotic stresses such as viruses, bacteria, insects, and nematodes.

The growing body of research on small non-coding RNAs (sRNAs) continues to provide insights into post-transcriptional gene regulation and stress response mechanisms. The completion of numerous plant genome sequencing projects has accelerated the identification and functional analysis of RNAi-related pathways, allowing researchers to engineer crops with better resilience and agronomic performance.

An important distinction exists between traditional transgenic crops expressing heterologous proteins and RNAi-based crops expressing non-coding RNA constructs. Unlike protein-based traits, RNAi constructs do not produce proteins and thus bypass many safety concerns related to allergenicity and toxicity. According to the U.S. Food and Drug Administration (FDA, 1992), introduced nucleic acids themselves do not raise safety concerns. Furthermore, in its guidance on RNA-based traits, the FDA clarified that the use of antisense RNAs or other non-coding RNA strategies does not inherently pose safety risks. Therefore, transgenic crops engineered with RNAi constructs generally do not require acute oral toxicity or digestibility testing, in contrast to those producing novel proteins.

Nevertheless, biosafety concerns remain, particularly in relation to transcriptional gene silencing via chromatin modifications, which may lead to unintended heritable epigenetic changes. These concerns necessitate careful design of RNAi constructs and thorough risk assessment, particularly for food safety and environmental impact.

Despite a few limitations, RNAi-based crop improvement strategies hold enormous promise. By leveraging small non-coding RNAs for precise gene regulation, researchers can develop crops with increased yield, enhanced nutrition, and improved resilience to stress, thereby contributing significantly to global food security and sustainable agriculture.

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