Review article

A Review on Role of Post-transcriptional Gene Silencing in Crop Improvement

Manisha Sharma[1], Neha Sharma[1]*, Shuchi Kaushik[2] and Rajesh Singh Tomar[1]

[1]*Amity Institute of Biotechnology, Amity University, M.P., Gwalior, 474005, India*
[2]*State Forensic Science Laboratory, Sagar, M.P. 470001, India*

Curr. Appl. Sci. Technol. 2024, Vol. 24 (No. 6), e0259786; https://doi.org/10.55003/cast.2024.259786

Received: 17 October 2023, Revised: 8 March 2024, Accepted: 24 April 2024, Published: 2 August 2024

Keywords

RNA inference;

Dicer-like proteins;

Argonaute proteins;

RNA-dependent RNA polymerase;

post-transcriptional gene silencing

Abstract

Gene silencing is an important tool for increasing the yield of crops and plays an important role in revolutionizing agriculture. In agriculture, gene silencing is done by post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) technology. RNA interference (RNAi) is a well-known technique of post-transcriptional gene silencing and is widely utilized in crop improvement through gene regulation. In the present review, we discussed various aspects of RNA interference gene silencing viz RNA-dependent RNA polymerase (RdRp), Argonaute proteins (AGOs) and Dicer-like proteins (DCLs) and their applications in the optimization of agricultural yield.

1. Introduction

A new global report has claimed that almost half of the world's population is getting poor nutrition [1]. The growth rate of agricultural products has been higher than the population growth rate, which is an indicator of success on the agricultural front. Plants exhibit two different types of gene-silencing phenomena: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS), which are caused by sequence-specific RNA degradation and reduced RNA synthesis due to promoter methylation, respectively. Therefore, employing a gene-silencing approach is among the crucial methods for examining how different genes work [2]. Scientists originally discovered gene silencing when experimenting with transgenic petunia plants that expressed the chalcone synthase (CHS) gene, a gene thought to be involved in the pathway that produces the flavonoid anthocyanins that give flowers their color [3]. It was noticed when the CHS gene was overexpressed in plants that produced colored flowers, the CHS gene produced a different phenotype, and white color flowers were produced. This process is known as co-suppression. After some time, it was

*Corresponding author: E-mail: nsharma2@gwa.amity.edu

reported that co-repression arises from the suppression of the process of transcription or the post-transcriptional destruction of transcripts [4].

Transcriptional gene silencing is the suppression of the transcription process involving changes, e.g., chromatin remodeling or histone methylation of promoter sequences at the DNA level which leads to switch-off transcription. The transcription gene silencing process takes place in the nucleus and displays stable inheritance [4, 5]. TGS does not involve the process of systemic silencing, whereas the PTGS pathway is found in many cellular regulatory networks [6, 7]. Contrary to TGS, PTGS takes place in the cytosol and involves specific sequence mRNA degradation and exhibits the phenomenon of systemic silencing. It silences the unfamiliar DNA in transposons (jumping genes), and transgenes (artificial genes). Viruses comprise similar mechanisms called RNAi in animals and quelling in fungi [8, 9]. Such procedures were initially identified through research involving viral vectors [10], transgenes [3, 11] as well as injected double stranded RNAs [12] where the unrelated RNA and DNA had been premised upon the expression of endogenous genes. These procedures are instances of natural viral defense mechanisms. These defense mechanisms were focused against these endogenous RNAs because of the presence of alien nucleic acids, causing the organism to exhibit a phenocopy of the linked genes' loss-of-function (LOF) mutations [12, 13]. Regulation of gene expression by sequence can be inhibited both at the level of post-transcriptional gene silencing (PTGS) and transcription gene silencing (TGS), RNAi is known to be an important biological process of PTGS [14].

To enhance crop productivity, gene silencing mechanisms via a variety of necessary components, like DCL proteins, AGO proteins, and RDR proteins can be used [15]. Therefore, RNA gene silencing is an important platform to improve crop quality, reduce plant disease and remove harmful pathogens [16]. RNA interference strategies have been used in a variety of plant types to combat different insects, diseases, and pests (Table 1) [17].

2. Post-transcriptional Gene Silencing

Post-transcriptional gene silencing occurs in the cytoplasm and involves the degradation and precise targeting of mRNA transcripts of genes [18]. Post-transcriptional gene silencing (PTGS) takes place via biological degradation of messenger RNA. Post transcriptional gene silencing mechanisms is shown in Figure 1. Post transcriptional gene silencing as well as transcriptional gene silencing are related mechanically.

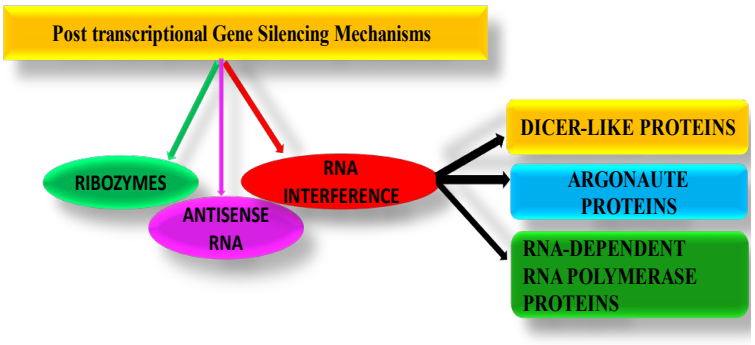

Figure 1. Mechanism of post-transcriptional gene silencing including ribozymes; antisense RNA and RNA interference. Adapted from Hammond *et al*. [18].

Table 1. Implementation of RNA interference technique in numerous plant species to combat various insects, pests, and pathogens.

S. No.	Experimented Plant	Insect/Pathogen	Aim of the Experiment	Genes to be Targeted	Ref.
1	*Arabidopsis thaliana*	*Meloidogyne species*	RNA interference was used to suppress the parasitism gene.	16D10	[19]
2	*Oryza sativa* L.	*Magnaporthe grisea*	A rice homolog of SS12 (OsSS12) was functionally analyzed for disease resistance.	OsSSI2	[20]
3	*Prunus domestica* L.	Plum Pox Virus (PPV)	PTGS (RNAi) was used to develop viral tolerance in a perennial woody plant.	PPV coat protein gene	[21]
4	*Gossypium hirsutum*	*Helicoverpa armigera*	Plant-mediated RNAi was used to silence the P450 gene CYP6B6 from the cotton bollworm monooxygenase gene.	Cytochrome P450 gene (CYP6AE14)	[22]
5	*Oryza sativa* L.	*Nilaparvata lugens*	RNA interference-mediated knockdown of midgut genes in dsRNA-transgenic plants.	NIHT1, NIcar, NItry	[16]
6	*Nicotiana rustica*	*Bemisia tabaci*	Enhanced white fly resistance through RNA expression.	v-ATPase	[23]
7	*Zea mays*	*Diabrotica virgifera*	RNA interference-based coleopteran insect pest control	Genes encoding proteins	[24]
8	*Citrus aurantifolia*	Citrus tristeza virus (CTV)	Transgenic plants created by transformation that possess the CTV coat protein gene	CTV (citrus tristeza virus)-CP (coat protein)	[25]
9	*Juglans regia* L.	*Agrobacterium tumefaciens*	Utilizing oncogene silencing technology to create crops resistant to crown gall	Tryptophan mono-oxygenase (iaaM) and isopentenyl transferase (ipt)	[26]
10	*Genus Malus*	*Venturia inaequalis*	Employing a hairpin vector strategy to significantly add resistance to *V. inaequalis*	GFP transgene and tri-hydroxy-naphthalene reductase gene (THN)	[27]

2.1 Ribozymes

Ribozymes are catalytic RNA molecules that cleave mRNA with high specificity and are used to block gene expression (Figure 2). The RNase H family can be found in nearly all microorganisms from bacteria to eukaryotes and targets RNA of a DNA-RNA hybrid. Ribozymes are usually concerned with mRNA degradation. Ribozymes are catalytic RNA molecules that carry out gene silencing and are employed to suppress the expression of genes. By cleaving mRNA molecules, these chemicals virtually shut down the genes (no protein) from which these mRNAs were generated post-transcriptionally. Ribonuclease P ribozyme is a type of ribonuclease enzyme which breaks RNA molecules and group II intron ribozymes [28, 29].

There are various types of ribozyme motifs such as hammerhead ribozyme (HHR) motifs, short hairpin ribozyme (self-cleaving) motifs, hepatitis delta virus (HDV) motifs, group I introns motifs, group II introns motifs, and ribonuclease P ribozyme (RNaseP) motifs (Figure 3). The hammerhead ribozymes (HHR) motifs, short hairpin ribozymes motifs, and hepatitis delta virus (HDV) ribozyme motifs are commonly found in some viroids. Group I intron motifs (self-splicing) and group II intron motifs are generally found in bacteria, bacteriophages, and eukaryotes. The *E. coli* RNase P ribozyme is well recognized for its capacity to combine with a protein cofactor and break the phosphodiester bonds of several transfer precursors [28-30].

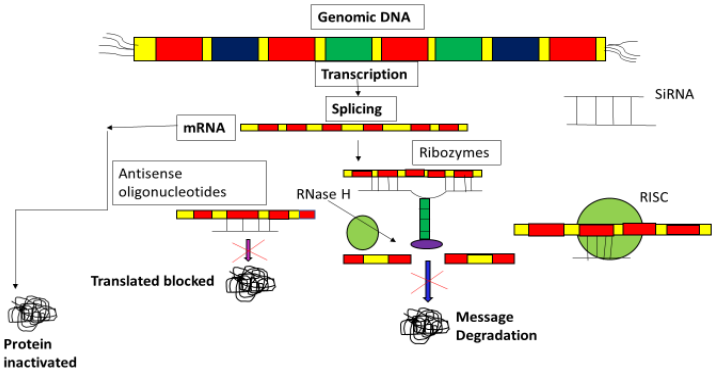

Figure 2. Schematic representation of ribozyme mechanisms. Adapted from Phylactou *et al.* [28].

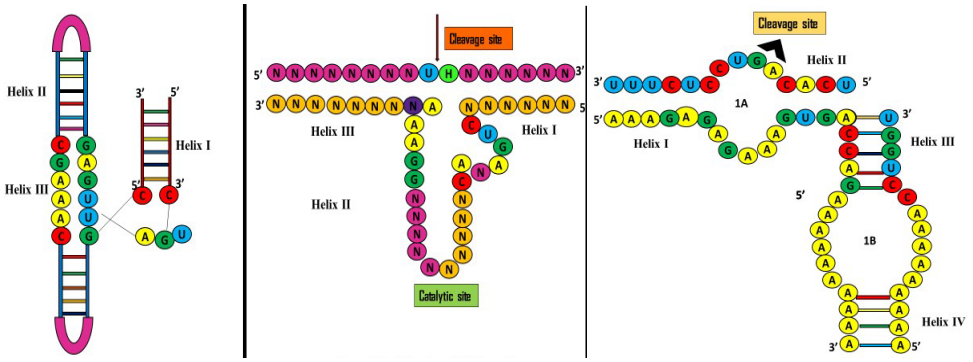

Figure 3. Schematic representation of ribozyme mechanisms. Adapted from Phylactou *et al.* [28].

2.2 Antisense technology

The term "antisense RNA" (asRNA), also known as "antisense transcript", "antisense oligonucleotides", and "NAT" (natural antisense transcript), refers to a single-stranded ribonucleic acid which is complementary to a protein-coding messenger ribonucleic acid with which it is hybridized and thereby prevents that messenger RNA (mRNA) from being translated into a protein [31-33]. Prokaryotes and eukaryotes both contain antisense RNA, which can be divided into long ncRNAs (>200 nt.) and short ncRNAs (200 nt. non-coding RNAs). The sense strand, the strand of the normal gene that is not transcribed becomes oriented in the 5'→ 3' direction concerning its promoter and antisense strand, and the strand of the normal gene is transcribed since now its orientation is 3'→5' direction (Figure 4). The primary purpose of antisense RNA is to control gene expression by inhibiting the translation process. Antisense RNA may also be produced artificially and is extensively used as a research tool for improving crop productivity [33-35].

2.3 RNA interference

Cells frequently employ the RNAi method to control gene expression. The gene silencing process comes into existence due to the presence of double-stranded RNA molecules in the cells which interact with various RNA processing proteins [17]. The dsRNA molecule is next fragmented into tiny dsRNA pieces by an enzyme known as Dicer. These small dsRNAs fragments, including interfering RNA and micro RNAs, are approximately 21-23 nt. in length [36].

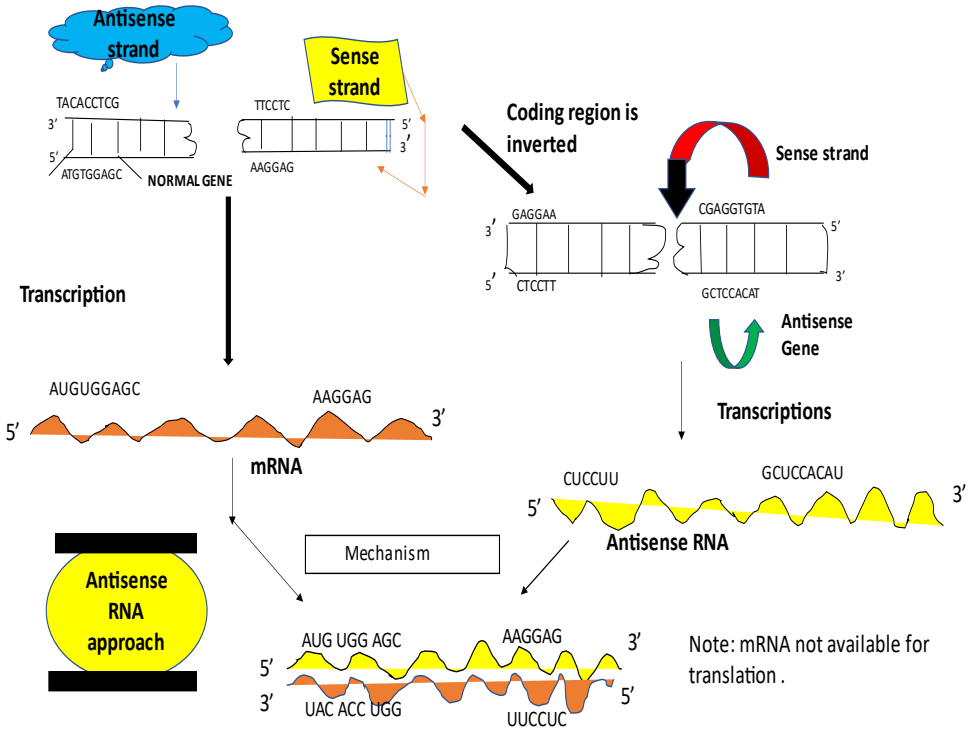

Figure 4. Antisense RNA technology. Adapted from Saberi *et al*. [32].

The AGO (Argonaute) proteins (Figure 5), which are crucial elements of the RNA interference pathways, are integrated into many subunit proteins known as the RNA-induced silencing complex (RISC), which also include tiny double-stranded RNA fragments [37]. A natural phenomenon that silences genes called RNAi is triggered by dsRNA molecules that stop specific genes from being expressed (Figures 5 and 6).

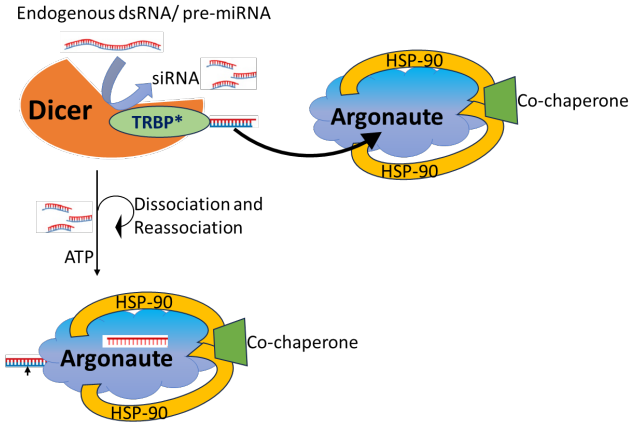

Figure 5. Mechanism of Dicer and Argonaute proteins.
***TRBP**- Transactivation response element RNA-binding protein, ensures efficient Dicer processing of precursor [9].

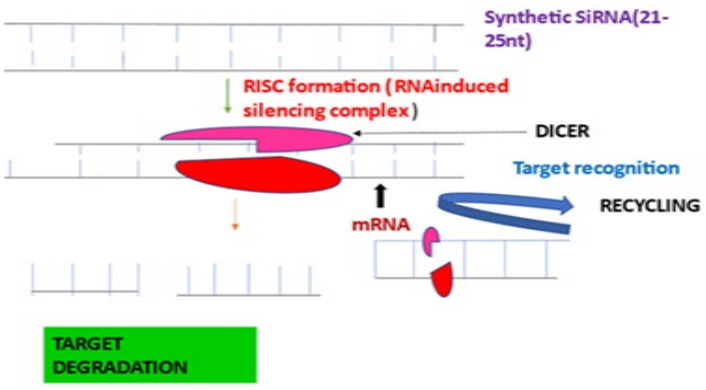

Figure 6. RNA interference leading to gene silencing. Adapted from Sijen *et al*. [17].

2.3.1 Dicer-Like proteins

Dicer-like proteins belong to the ribonucleases III family of endoribonucleases containing DExDbox RNA Helicase-C, PAZ (Piwi/Argonaute/Zwille), DUF283 (unknown functions), ribonuclease III, and double-stranded ribonucleic acid-binding domains [38, 39]. Dicer-like proteins cleaves dsRNA. Dicer enzyme-1 conducts pre-miRNA recognition and miRNA production, whereas Dicer enzyme-2 cleaves dsRNA to produce siRNA, although it also has non-canonical functions such as induction of apoptosis, DNA repair, and chromatin remodeling (Figure 7). Dicer-like proteins perform the

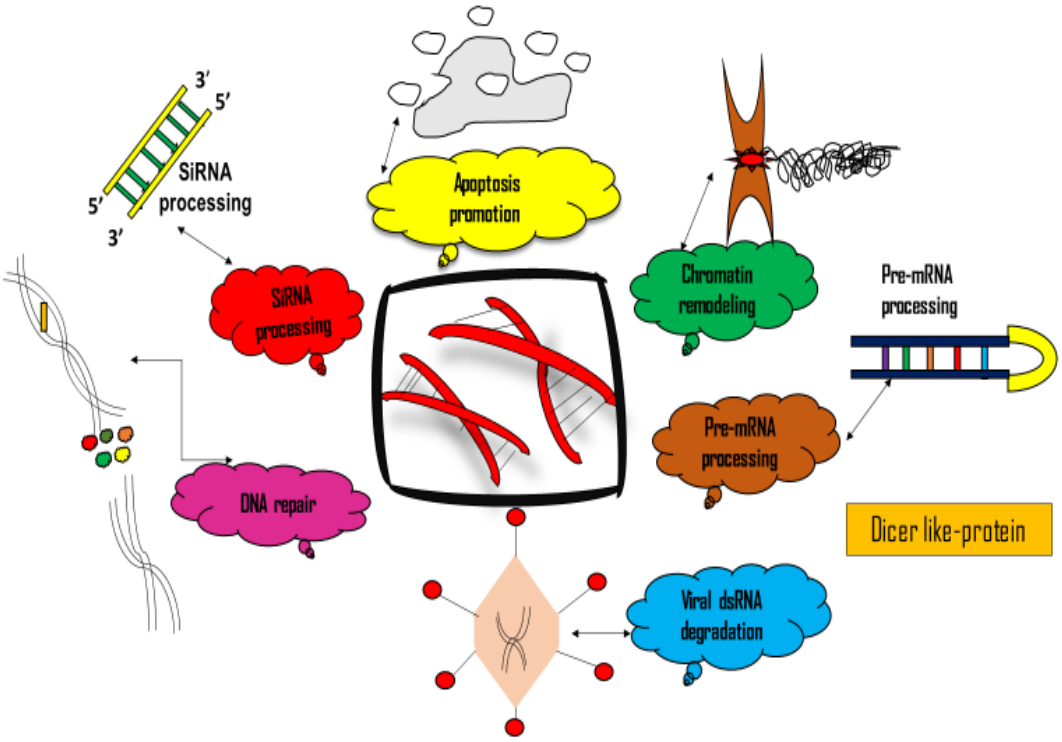

Figure 7. Mechanism of action of Dicer-like protein. Adapted from Montavon *et al*. [40].

first step of the RNA interference mechanisms, in which cleavage of double-stranded RNA produces short RNA, which is 21-24 nucleotides in length [38-40]. Piwi-Argonaute-Zwille domain has phosphate-binding pockets made of arginine (Arg) constituents that are necessary for the breakdown of double-stranded RNAs and identification of the 5'-phosphorylated end of pre-miRNA [41, 42].

2.3.2 Argonaute (AGO) proteins

Argonaute proteins are quite precise small ribonucleic acid-binding molecules that are important components of RNA-induced gene silencing complex (RISC) in a silencing pathway [8, 43]. The effector phase of silencing is carried out by Argonaute proteins, which are associated with sRNAs generated initially to direct RNA sequence-specific regulation of gene expression. Argonaute proteins have a crucial function in alternative splicing, gene activation and inhibition, gene integrity and mRNA cleavage (Figure 8).

There are three conserved domains exist in Argonaute proteins, according to structural research, which are the Piwi-Argonaute-Zwille domain, the Middle domain and the Piwi domain. The NTD (N-terminal domain) consists of the N-terminus site and Piwi-Argonaute-Zwille domain which, respectively, make it easier for sRNAs to be separated and bind the thirty ends of the bound sRNA. The C-terminus lobes contain the Middle domain and Piwi-Argonaute-Zwille domain, and the binding pocket that connects these domains at their intersections (5’ ends of sRNAs) [44]. However, the ribonuclease H enzymes that cleave ribonucleic acid strands behave in the same way as the Piwi-Argonaute-Zwille domain [45].

2.3.3 RNA-dependent RNA polymerase (RDR) proteins

RNA-dependent RNA polymerase proteins carry out RNA amplification of silencing in many stages of RNA interference cascades, which include the process of converting single-stranded RNA to double-stranded RNA (reverse transcription). A new cycle of RNA interference is initiated when proteins that act as dicers further process double-stranded RNA.

First, a strand of the dsRNA molecule, known as the guide strand, binds to the RNA-inducing silencing complex, whereas the second strand referred to as the "passenger strand" or antisense strand, is degraded. The part of the fragment that continues to be coupled to the RNA-induced silencing complex, either the guide strand or the passenger strand, directs the sequence-specific silencing of the target messenger RNA molecules (Figure 9) [36].

In sRNA, the phase and repeat-associated siRNA are found on RNA-dependent RNA polymerase proteins for biogeny, while micro-RNAs and short hairpin-derived small RNAs are RNA-dependent RNA polymerase proteins independent RNA [46].

RNA-dependent RNA polymerases were first studied in plants, animals, and viruses. Small RNA was identified as a component of the genome of less evolved creatures and was identified as a new RdRP catalytic domain [47]. The RdRp catalytic domain is a member of the structural protein classification, which was initially used to identify a viral gene replication enzyme that was present in RNA viruses. The RNA-dependent RNA polymerase catalytic domain plays a role in maintaining genome integrity, messenger RNA-template formation, post-transcriptional gene silencing, and defense against extracellular ribonucleic acids and deoxyribonucleic acids [48, 49]. Genetic engineering strategies for biotic and abiotic resistance enhanced self-life and quality improvement in horticultural crops [50] (Figure 10). Figure 10 illustrates the potential applications of PTGS in agriculture and crop improvement.

PTGS has practical implications for agriculture with potential benefits for farmers. PTGS is a mechanism that regulates gene expression by degrading messenger RNA (mRNA) molecules, thereby preventing the translation of specific genes. This technology has several applications in agriculture, offering crop protection, improved crop quality, increased yield, enhanced stress tolerance, extended shelf Life, delayed senescence, and biofortification [15, 49, 50].

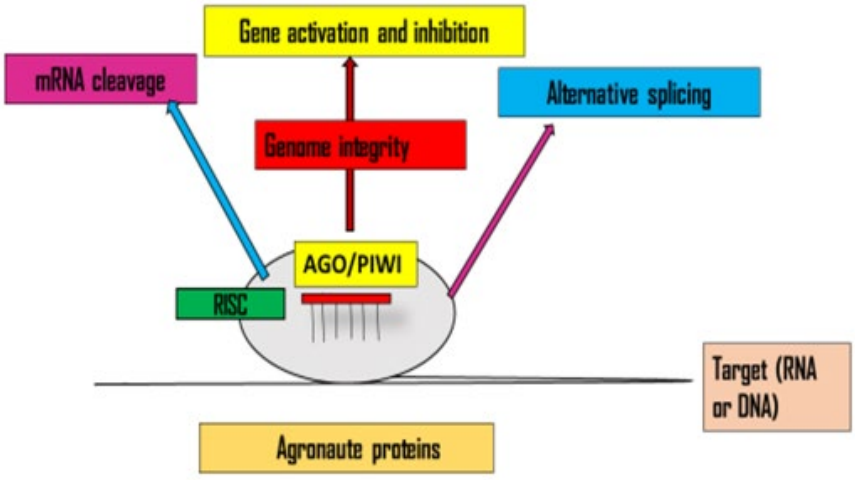

Figure 8. Role of Argonaute proteins. Adapted from Simon *et al*. [45].

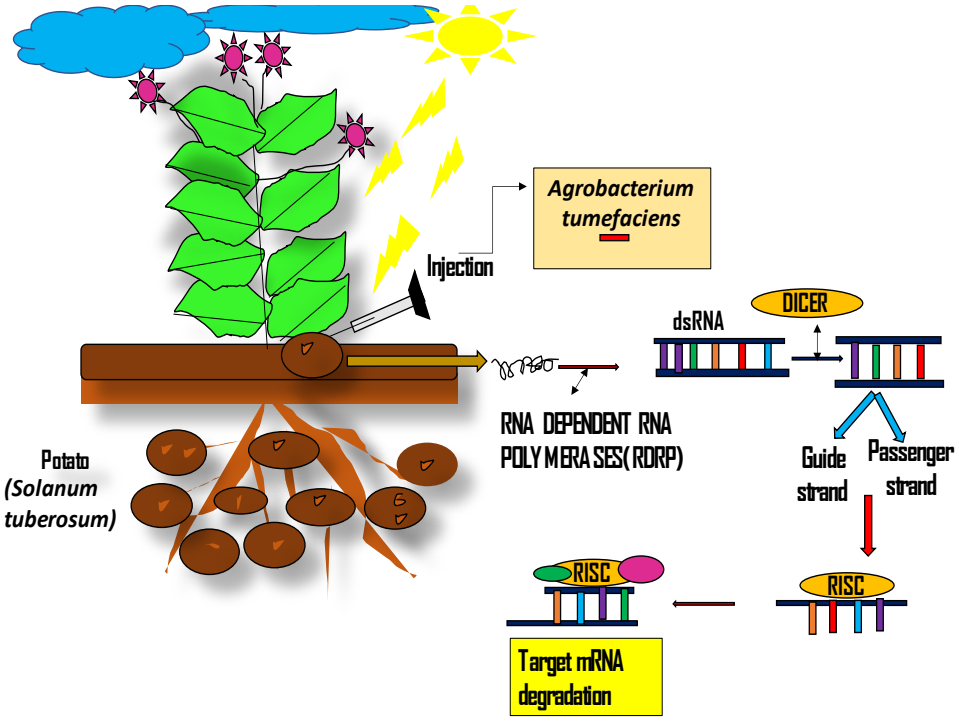

Figure 9. RNA dependent RNA polymerase proteins. Adapted from Wilson and Doudna [36].

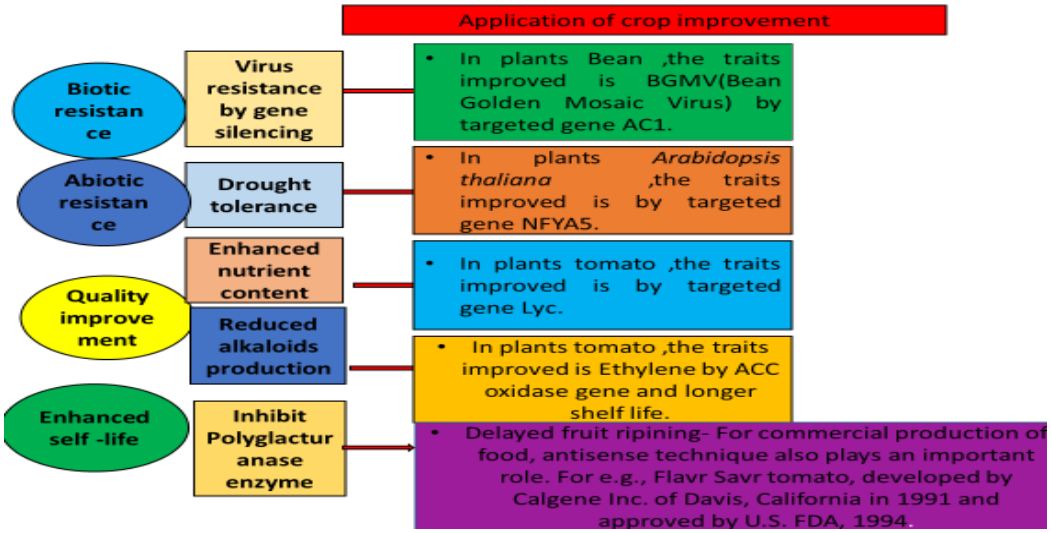

Figure 10. Application of gene silencing technology. Adapted from Uluisik *et al*. [50].

3. Limitations

While post-transcriptional gene silencing (PTGS) methods, such as RNA interference (RNAi), hold great promise for various applications, they also face several challenges and constraints. Some of the key challenges associated with PTGS methods include:

3.1 Off-target effects

One of the major challenges is that the process of silencing molecules (e.g., siRNAs) may inadvertently target and suppress the expression of unintended genes. This lack of specificity can lead to unpredictable consequences and impact the interpretation of experimental results or the safety of therapeutic applications.

3.2 Duration and stability of silencing

The transient nature of PTGS effects can be a limitation. Maintaining sustained gene silencing over an extended period, especially in therapeutic applications, is challenging. Additionally, the stability of RNAi molecules can be influenced by factors such as degradation by nucleases and dilution over time.

3.3 Ethical and regulatory considerations

The use of PTGS methods, especially in therapeutic contexts, raises ethical considerations related to genetic manipulation and potential long-term effects. Regulatory frameworks must address safety concerns and ensure the responsible and ethical application of PTGS technologies.

3.4 Cost and scalability

The cost of developing and implementing PTGS methods, particularly for therapeutic purposes, can be a limiting factor. Ensuring cost-effectiveness and scalability for widespread use is essential for the practical application of these technologies. Researchers and biotechnologists continue to address these challenges through ongoing research and technological innovations aimed at improving the precision, safety, and efficiency of PTGS methods. Advances in delivery technologies, molecular design strategies, and a deeper understanding of the underlying biology are expected to contribute to overcoming these constraints over time. All the recently available transcriptional gene silencing and post-transcriptional gene silencing methods have their benefits and limitations. Careful selection of the target sequences and regulation of gene expression of small interference RNAs of specific length may aid in reducing the selection of off-target genes. Numerous forecasting algorithms have been created using cutting-edge in-silico technology to help in the selection of target gene areas that minimize off-target gene silencing. We discussed various aspects of RNA interference gene silencing pathways like RNA-dependent RNA polymerase (RDRp), Argonaute proteins (AGOs) and their applications in the improvisation of agricultural yield. Addressing these challenges requires ongoing research and development to improve the design, delivery, and specificity of PTGS methods while considering the unique characteristics of each target gene and experimental system.

4. Conclusions

Our review article is mainly focusing on gene silencing, which is the most important technology in agriculture for improving crop productivity. Post-transcriptional gene silencing (PTGS) research has been an active area of investigation that has been primarily focused on RNA interference (RNAi) pathways and related mechanisms. The potential future developments or areas of research that may have been explored or have evolved in the field of PTGS include: precision targeting and delivery systems; CRISPR-Cas technologies integration; and therapeutic applications. The easy characterization of abiotic and biotic stress has been accomplished with the use of gene silencing relying on the PTGS approach. RNAi is a popular method used for improving nutritional traits. RNAi can also be used for advanced genetics. RNAi is a very fast and powerful method of gene silencing. The recent advances in post-transcriptional gene silencing are popularly used for gene function analysis. Today agricultural technology needs more molecular and genomics techniques. Gene silencing develops viral resistance in plants and the production of male sterile plants. In addition, the latest techniques of gene silencing with increased accuracy provide more benefits for improving crop quality.

References

[1] Chen, C., Chaudhary, A. and Mathys, A., 2020. Nutritional and environmental losses embedded in global food waste. *Resources, Conservation and Recycling*, 160, https://doi.org/10.1016/j.resconrec.2020.104912.

[2] Pandey, P., Mysore, K.S. and Senthil-Kumar, M., 2022. Recent advances in plant gene silencing methods. In: K.S. Mysore and M. Senthil-Kumar, eds. *Plant Gene Silencing. Methods* in Molecular Biology. Vol. 2408, New York: Humanan, pp. 1-22.

[3] Napoli, C., Lemieux, C. and Jorgensen, R., 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *The Plant Cell*, 2(4), 279-289.

[4] Ashfaq, M.A., Kumar, V.D., Reddy, P.S.S., Kumar, C.H.A., Kumar, K.S., Rao, N.N., Tarakeswari, M. and Sujatha, M., 2020. Post-transcriptional gene silencing: Basic concepts and applications. *Journal of Biosciences*, 45, https://doi.org/10.1007/s12038-020-00098-3.

[5] Li, B., Carey, M. and Workman, J.L., 2007. The role of chromatin during transcription. *Cell*, 128(4), 707-719.

[6] Cogoni, C., Romano, N. and Macino, G., 1994. Suppression of gene expression by homologous transgenes. *Antonie Van Leeuwenhoek*, 65, 205-209.

[7] Ghildiyal, M. and Zamore, P.D., 2009. Small silencing RNAs: an expanding universe. *Nature Reviews Genetics*, 10(2), 94-108.

[8] Vaucheret, H., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B. and Vernhettes, S., 1998. Transgene-induced gene silencing in plants. *The Plant Journal*, 16(6), 651-659.

[9] Sharp, P.A., 1999. RNAi and double-strand RNA. *Genes and Development*, 13(2), 139-141.

[10] Ruiz, M.T., Voinnet, O. and Baulcombe, D.C., 1998. Initiation and maintenance of virus-induced gene silencing. *The Plant Cell*, 10(6), 937-946.

[11] Van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N and Stuitje, A.R., 1990. Flavonoid genes in petunia: The addition of a limited number of gene copies may lead to a suppression of gene expression. *The Plant Cell*, 2(4), 291-299.

[12] Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391(6669), 806-811.

[13] Ratcliff, F.G., MacFarlane, S.A. and Baulcombe, D.C., 1999. Gene silencing without DNA: RNA-mediated cross-protection between viruses. *The Plant Cell*, 11(7), 1207-1215.
[14] Muhammad, T., Zhang, F., Zhang, Y. and Liang, Y., 2019. RNA interference: a natural immune system of plants to counteract biotic stressors. *Cells*, 8(1), https://doi.org/10.3390/cells8010038.
[15] Koeppe, S., Kawchuk, L. and Kalischuk, M., 2023. RNA Interference past and future applications in plants. *International Journal of Molecular Sciences*, 24(11), https://doi.org/10.3390/ijms24119755.
[16] Younis, A., Siddique, M.I., Kim, C.-K. and Lim, K.-B., 2014. RNA interference (RNAi) induced gene silencing: a promising approach of hi-tech plant breeding. *International Journal of Biological Sciences*, 10(10), 1150-1158.
[17] Sijen, T., Vijn, I., Rebocho, A., van Blokland, R., Roelofs, D., Mol, J.N. and Kooter, J.M., 2001. Transcriptional and posttranscriptional gene silencing are mechanistically related. *Current Biology*, 11(6), 436-440.
[18] Hammond, S.M., Caudy, A.A. and Hannon, G.J., 2001. Post-transcriptional gene silencing by double-stranded RNA. *Nature Reviews Genetics*, 2(2), 110-119.
[19] Dinh, P.T.Y., Brown, C.R. and Elling, A.A., 2014. RNA interference of effector gene Mc16D10L confers resistance against *Meloidogyne chitwoodi* in Arabidopsis and potato. *Phytopathology*, 104(10), 1098-1106.
[20] Ma, S., Song, Q., Tao, H., Harrison, A., Wang, S., Liu, W., Lin, L., Zhang, Z., Ai, Y. and He, H., 2019. Prediction of protein–protein interactions between fungus (*Magnaporthe grisea*) and rice (*Oryza sativa* L.). *Briefings in Bioinformatics*, 20(2), 448-456.
[21] Ravelonandro, M., Scorza, R. and Briard, P., 2019. Innovative RNAi strategies and tactics to tackle Plum Pox Virus (PPV) genome in *Prunus domestica*-Plum. *Plants*, 8(12), https://doi.org/10.3390/plants8120565.
[22] Lim, Z.X., Robinson, K.E., Jain, R.G., Chandra, G.S., Asokan, R., Asgari, S. and Mitter, N., 2016. Diet-delivered RNAi in *Helicoverpa armigera* progresses and challenges. *Journal of Insect Physiology*, 85, 86-93.
[23] Thakur, N., Upadhyay, S.K., Verma, P.C., Chandrashekar, K., Tuli, R. and Singh, P.K., 2014. Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of *v-ATPase* A gene. *PloS One*, 9, https://doi.org/10.1371/journal.pone.0087235.
[24] Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. and Roberts, J., 2007. Control of coleopteran insect pests through RNA interference. *Nature Biotechnology*, 25(11), 1322-1326.
[25] Domínguez, A., Guerri, J., Cambra, M., Navarro, L., Moreno, P. and Peña, L., 2000. Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Reports*, 19(4), 427-433.
[26] Walawage, S.L., Leslie, C.A., Escobar, M.A. and Dañdekar, A.M., 2014. *Agrobacterium tumefaciens*-mediated transformation of walnut (*Juglans regia*). *Bio-protocol*, 4(19), https://doi.org/10.21769/BioProtoc.1258.
[27] Williams, E.B. and Kuc, J., 1969. Resistance in malus to *Venturia inaequalis*. *Annual Review of Phytopathology*, 7(1), 223-246.
[28] Phylactou, L.A., Kilpatrick, M.W. and Wood, M.J.A., 1998. Ribozymes as therapeutic tools for genetic disease. *Human Molecular Genetics*, 7(10), 1649-1653.
[29] Shampo, M.A., Kyle, R.A. and Steensma, D.P., 2012. Sidney Altman-Nobel laureate for work with RNA. *Mayo Clinic Proceedings*, 87(10), https://doi.org/10.1016/j.mayocp.2012.01.022.
[30] Dong, X., Qu, G., Piazza, C.L. and Belfort, M., 2020. Group II intron as cold sensor for self-preservation and bacterial conjugation. *Nucleic Acids Research*, 48(11), 6198-6209.

[31] Magistri, M., Faghihi, M.A., St Laurent, G. and Wahlestedt, C., 2012. Regulation of chromatin structure by long noncoding RNAs: focus on natural antisense transcripts. *Trends in Genetics*, 28(8), 389-396.
[32] Saberi, F., Kamali, M., Najafi, A., Yazdanparast, A. and Moghaddam, M.M., 2016. Natural antisense RNAs as mRNA regulatory elements in bacteria: a review on function and applications. *Cellular and Molecular Biology Letters*, 21(6), https://doi.org/10.1186/s11658-016-0007-z.
[33] Wahlestedt, C., 2013. Targeting long non-coding RNA to therapeutically upregulate gene expression. *Nature Reviews Drug Discovery*, 12(6), 433-446.
[34] Larivera, S., Neumeier, J. and Meister, G., 2023. Post-transcriptional gene silencing in a dynamic RNP world. *Biological Chemistry*, 404(11-12), 1051-1067.
[35] Pelechano, V. and Steinmetz, L.M., 2013. Gene regulation by antisense transcription. *Nature Reviews Genetics*, 14(12), 880-893.
[36] Wilson, R.C. and Doudna, J.A., 2013. Molecular mechanisms of RNA interference. *Annual Review of Biophysics*, 42, 217-239.
[37] Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, KD., Lellis, A.D., Zilberman, D., Jacobsen, S.E. and Carrington, J.C., 2004. Genetic and functional diversification of small RNA pathways in plants. *PLoS Biology*, 2(5), https://doi.org/10.1371/journal.pbio.0020104.
[38] Carmell, M.A. and Hannon, G.J., 2004. RNase III enzymes and the initiation of gene silencing. *Nature Structural and Molecular Biology*, 11(3), 214-218.
[39] Margis, R., Fusaro, A.F., Smith, N.A., Curtin, S.J., Watson, J.M., Finnegan, E.J. and Waterhouse, P.M., 2006. The evolution and diversification of Dicers in plants. *FEBS letters*, 580(10), 2442-2450.
[40] Montavon, T., Kwon, Y., Zimmermann, A., Hammann, P., Vincent, T., Cognat, V., Berydoll, M., Michel, F. and Dunoyer, P., 2018. Characterization of DCL 4 missense alleles provides insights into its ability to process distinct classes of ds RNA substrates. *The Plant Journal*, 95(2), 204-218.
[41] Kandasamy, S.K. and Fukunaga, R., 2016. Phosphate-binding pocket in Dicer-2 PAZ domain for high-fidelity siRNA production. *Proceedings of the National Academy of Sciences*, 113(49), 14031-14036.
[42] Park, J.-E., Heo, I., Tian, Y., Simanshu, D.K., Chang, H., Jee, D., Patel, D.J. and Kim, V.N., 2011. Dicer recognizes the 5' end of RNA for efficient and accurate processing. *Nature*, 475(7355), 201-205.
[43] Mallory, A. and Vaucheret, H., 2010. Form, function, and regulation of ARGONAUTE proteins. *The Plant Cell*, 22(12), 3879-3889.
[44] Parker, J.S., 2010. How to slice: snapshots of Argonaute in action. *Silence*, 1(1), 1-10.
[45] Simon, B., Kirkpatrick, J.P., Eckhardt, S., Reuter, M., Rocha, E.A., Andrade-Navarro, M.A., Sehr, P., Pillai, R.S. and Carlomagno, T., 2011. Recognition of 2'-O-methylated 3'-end of piRNA by the PAZ domain of a Piwi protein. *Structure*, 19(2), 172-180.
[46] Schiebel, W., Pélissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sänger, H.L. and Wassenegger, M., 1998. Isolation of an RNA-directed RNA polymerase–specific cDNA clone from tomato. *The Plant Cell*, 10(12), 2087-2101.
[47] Mourrain, P., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., Rémoué, K., Sanial, M., Vo, T.A. and Vaucheret, H., 2000. Arabidopsis SGS2 and SGS3 genes are required for post-transcriptional gene silencing and natural virus resistance. *Cell*, 101(5), 533-542.
[48] Liang, G., He, H., Li, Y. and Yu, D., 2012. A new strategy for the construction of artificial miRNA vectors in Arabidopsis. *Planta*, 235, 1421-1429.
[49] Schwab, R., Ossowski, S., Riester, M., Warthmann, N. and Weigel, D., 2006. Highly specific gene silencing by artificial microRNAs in Arabidopsis. *The Plant Cell*, 18(5), 1121-1133.

[50] Uluisik, S., Chapman, N.H., Smith, R., Poole, M., Adams, G., Gillis, R.B., Besong, T.M.D., Sheldon, J., Stiegelmeyer, S., Perez, L., Samsulrizal, N., Wang, D., Fisk, I.D., Yang, N., Baxter, C., Rickett, D., Fray, R., Blanco-Ulate, B., Powell, A.L.T., Harding, S.E., Craigon, J., Rose, J.K.C., Fich, E.A., Sun, L., Domozych, D.S., Fraser, P.D., Tucker, G.A., Grierson, D. and Seymour, G.B., 2016. Genetic improvement of tomato by targeted control of fruit softening. *Nature Biotechnology*, 34(9), 950-952.