Potential applications of antisense RNA technology in plants

Ratna Kumria, Rashmi Verma and Manchikatla Venkat Rajam*

Plant Genetic Manipulation Group, Department of Genetics, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110 021, India

The antisense RNA technology involves the cloning of a particular gene in reverse orientation with respect to the promoter, such that the coding strand acts as template strand and vice versa. The antisense gene when transcribed gives rise to RNA which is complementary to sense mRNA, thus inhibiting the target gene expression by forming RNA duplex which is unstable. Besides, antisense oligonucleotides can also be administered exogenously to manipulate the expression of a particular gene. The concept of involvement of antisense RNA in gene regulation in natural systems is not new, however, recently only the antisense strategies have been used both in plant and animal systems for ascertaining gene function, manipulation of gene expression and in therapies. Though many potential applications of this technology have been put forward, several questions still remain unanswered, especially the stability and mechanism of action of antisense RNA and the relationship between the levels of inhibition and concentration of antisense RNA. The current status, including potential applications of this emerging field in plants is briefly reviewed here.

THE study of genetics for identifying a gene and its function traditionally involves the use of naturally occurring or experimentally induced mutants. This has certain disadvantages as any gene may be mutated and identification of a mutant gene can be tedious. Besides, it cannot be ascertained whether the mutant phenotype is the result of mutation in a particular gene. This particular disadvantage has been overcome by the technique of site-directed mutagenesis, yet in cases where mutation in a particular gene is lethal or a particular gene is present in multiple copies, the generation of mutants is futile.

In cases where a gene has been identified and assigned a particular phenotype, additional approaches are often required to exactly probe the function of gene.

Such hurdles in gene identification and manipulation can be overcome by antisense RNA technology. It involves the cloning of a gene in reverse orientation with respect to the promoter such that the coding strand acts as a template and the sequence of mRNA is the same as the opposite strand or the coding 'sense' strand. The gene cloned in reverse orientation or the antisense gene when transcribed gives rise to mRNA having the sequence complementary to the sense mRNA. The RNA–RNA binding of the sense–antisense RNA strands leads to inhibition of sense mRNA expression (Figure 1). Besides antisense RNA, sequence complementary to sense mRNA can also be administered exogenously to be able to manipulate gene expression. The antisense oligonucleotides bind to complementary mRNA and prevent its transport to cytoplasm or translation into protein. DNA–RNA hybrid serves as substrate for ribonuclease RNAase-H which specifically degrades RNA strand in a DNA–RNA hybrid.

The first effort that definitely demonstrated the blockage in translation due to the use of antisense RNA in cell-free extracts (CFEs) was carried out by Singer *et al.*[1]. They showed that synthesis of polyphenylalanine in CFE with polyuridylic acid as template was completely inhibited when polyadenylic acid was added to the translation mixture.

This article briefly describes the basic and applied aspects of antisense RNA technology, particularly in plant systems.

Natural antisense RNA regulation of gene expression

Naturally occurring antisense RNA was involved in gene

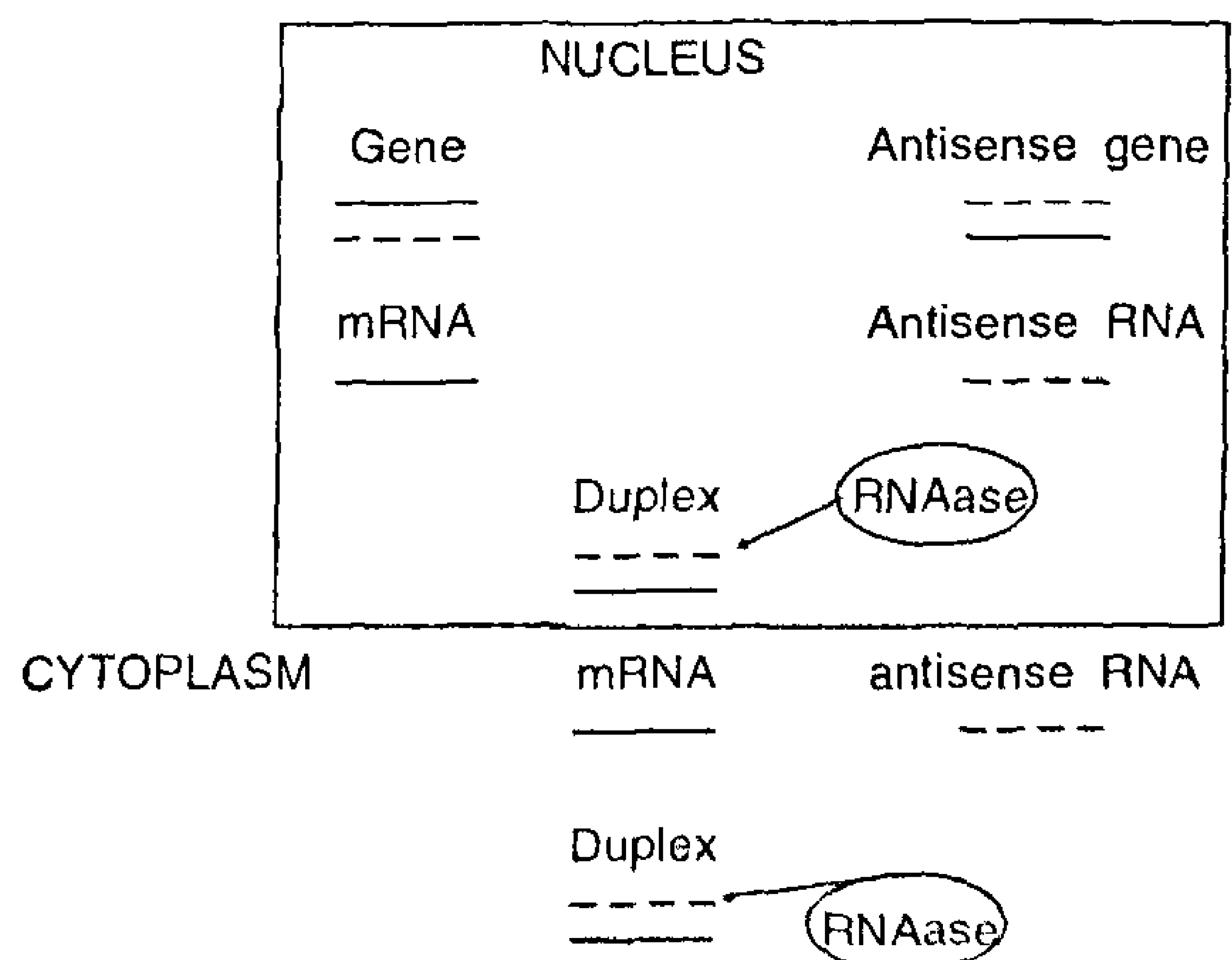
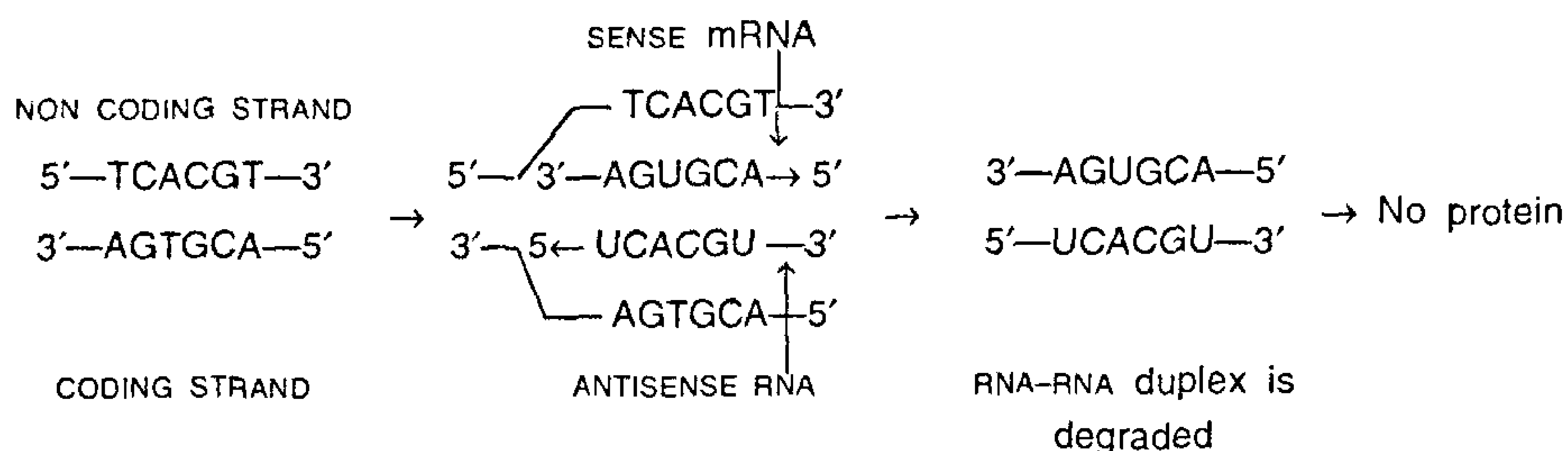


Figure 1. Proposed mechanisms of action of antisense RNA.

*For correspondence.



Antisense RNA refers to the RNA synthesized with the coding strand as the template. Since antisense RNA is complementary to the sense mRNA, it forms a RNA duplex, and hence it inhibits further processing and translation of mRNA into protein. Antisense RNA is known to regulate the sense gene expression in natural systems and is also being utilized to modulate gene function artificially, and therefore offers many potential applications.

regulation and this was demonstrated during the study of replication in *E. coli* ColEl plasmid. The replication of *E. coli* plasmid ColE1 involves formation of a RNA primer which is processed by RNAase-H while bound to the DNA template. Antisense RNA binds the primer inhibiting the processing of RNA primer and replication of the plasmid, hence the plasmid copy number may be regulated[2]. Likewise, *Staphylococcus aureus* plasmid (pT181) replication and copy number appear to be controlled by antisense RNA[3]. Translation of *E. coli* Tn10 transposase mRNA is inhibited by antisense mRNA. Besides prokaryotes, in eukaryotes antisense RNA is involved in splicing of H-RNA as it involves small nuclear ribonucleoproteins which have a RNA component complementary to the splice site[4,5]. Antisense small nucleolar RNAs encoded by introns have also been reported to play a role in rRNA methylation[6].

Naturally occurring antisense RNAs are known to regulate gene expression in plants too. These include antisense RNA transcripts to barley α-amylase mRNA[7], antisense mRNA complementary to *niv* gene encoding for enzyme of flavanoid pathway, chalcone synthase (CHS)[8]. Rogers identified two antisense transcripts in barley, both were imperfectly complementary to α-amylase gene whereas in case of *niv* gene, antisense transcripts arose due to an inverted duplication of untranslated leader sequences. Therefore a tentative mechanism has been proposed for the generation of antisense transcripts, antisense RNA arises when transcription of a gene proceeds in the strand opposite to template in absence of a strong transcription termination site in the short intergenic region[8]. Antisense transcripts have also been identified in *Brassica* for the S locus receptor kinase gene which controls self-incompatibility in *Brassica*[9]. In case of animal systems, naturally occurring antisense RNA has been reported in mice and *Xenopus* oocytes[10]. In *Xenopus* oocytes antisense RNA binds to fibroblast growth factor RNA and thus causes degradation of the target mRNA. Antisense RNA to myelin basic protein (MBP) gene causes severe reduction in MBP leading to characteristic shiverer phenotype[11].

The effectiveness and the exact regulatory role of naturally occurring antisense RNA can be determined only on the isolation of the antisense gene and whether the antisense RNA exist in a cell simultaneously with the sense mRNA. The transcription of the antisense gene would play a major role in regulating the amount of antisense RNA and further the expression of the gene, complementary to antisense RNA.

The regulation by antisense RNA involves certain basic mechanisms, on the basis of which they have been classified into three classes[12].

Class I – antisense RNAs are directly complementary to coding region or the SD sequence, resulting in direct inhibition of translation or mRNA destabilization.
Class II – RNAs include those that bind to non-coding regions of the target RNA, resulting in indirect effects produced by, e.g. alternative secondary structure formation that sequesters the ribosome-binding site.
Class III – antisense RNAs regulate transcription of the target mRNA by a mechanism similar to transcriptional attenuation.

Artificial antisense RNA regulation of gene expression

Antisense RNA has also been used to artificially modulate gene expression in plants and animals. Earlier model systems for the study of effectiveness of antisense RNA used circular plasmids carrying antisense gene which were either coinjected with the sense gene or the target gene would be a stably expressing one in the cultured cells (e.g. mouse cell lines and carrot protoplasts). To

letermine the optimum requirements for an efficient intisense RNA regulation varied lengths as well as egions of the sense gene β-galactosidase were targeted[13]. Such experiments led to certain general conclusions:

- The antisense RNA must be complementary to 5′ end of sense mRNA and a functional ribosome-binding site on the 5′ end[14].
- A significant correlation exists between the concentration of antisense RNA and sense mRNA. To produce maximum inhibition the molar ratios between antisense RNA and the sense mRNA vary from 50 : 1 to 600 : 1. It was 150 : 1 in case of inhibition of β-galactosidase synthesis[13]. Although there exist examples where significant inhibition is produced with molar ratio of 1 : 1 between sense RNA and antisense RNA, there is competition between ribosome and antisense RNA for binding to the ribosome-binding site of sense mRNA. Hence the factors that contribute to the increase in concentration of antisense RNA help in producing stronger inhibition[15].
- The factors that increase the rate of synthesis of antisense RNA as well as increase the half-life contribute towards the effectiveness of antisense RNA. Thus the promoter for antisense gene should be a strong one or antisense gene should be present in high copy number plasmid for high concentration of the antisense RNA. The length of antisense RNA and its configuration influence the stability and hence the effectiveness of the RNA[16].

Mode of action of antisense RNA

The study of natural antisense regulation as well as artificial antisense inhibition does not point towards the existence of any single mechanism of gene inactivation. Several modes of action are suggested by the evidences accumulated. The first stage at which a target gene can be inhibited is transcription but as yet no evidence has been brought to light which supports inhibition of transcription as the rate of transcription of both sense and antisense genes is unaffected by the expression of antisense gene[17]. The second stage where antisense transcript can interfere is the RNA-processing stage, it was shown by Tieman *et al.*[18] that when the two introns in pectin methylesterase gene were placed in antisense orientation, they were not spliced out. The formation of a duplex between antisense and sense transcripts is a factor contributing to inhibition, it has been hypothesized that a RNA–RNA duplex is unstable and susceptible to nucleases but no direct evidence for duplex formation has been found perhaps due to degradation of the duplex[19]. Therefore the formation of such a duplex would hinder the processing or transport of the sense mRNA across the nuclear membrane or would lead to degradation of antisense–sense duplex by nucleases making the sense transcript unavailable for translation[19].

Inhibition may also occur at the translational stage. The antisense transcript would compete with the ribosomes to bind 5′ end of the sense RNA, hence inhibiting the translation. It has been observed by Pestka *et al.*[13], that if the antisense RNA is not complementary to the 5′ end of mRNA, then the extent of inhibition is significantly reduced though a lower but significant level of inhibition remains; this shows that antisense RNA–mRNA duplex formation occurs but the ribosome which binds the 5′ end is capable of stripping the antisense RNA. Thus once the ribosome binds to mRNA, mRNA–antisense RNA duplex formation is greatly reduced and so is the inhibition[13].

Factors influencing antisense RNA regulation of gene expression

While making antisense constructs for specifically regulating the expression of a particular gene, certain factors have to be taken into account. The presence of antisense transcript much in excess of target mRNA is a prerequisite for effective inhibition, therefore the choice of promoter is important. Cauliflower mosaic virus (CaMV) 35S RNA promoter is a constitutive one and is the most widely used one. Other commonly used promoters include nopaline synthase promoter, chlorophyll *a/b*-binding protein gene promoter, and CHS gene promoter. Identical promoters and terminators may be employed for both sense and antisense constructs but usually an excess of antisense transcripts is required, thus antisense gene is cloned along with constitutive promoters[20]. Some workers have utilized tissue-specific promoters for antisense regulation of a particular function in a specific tissue. Van der Meer *et al.*[21] have used modified CaMV 35S RNA promoter for expression of antisense CHS gene in anthers. The inhibition of pigment synthesis in anthers led to male sterility showing a definitive role of flavanoids in male gametophyte development. Also the use of polymerase III promoters provides an advantage over polymerase II promoters as polymerase III transcripts are not capped and polyadenylated and are short in length. Such transcripts are thus more specific and accessible to bind to target sequences. Aiming to increase the stability and hence half-life of the antisense transcripts, Bourque and Folk[19] fused the methionine tRNA from soyabean to CAT gene and observed five-fold higher inhibition.

Since the formation of duplex of sense and antisense transcripts is the critical step for inhibition, the degree of homology and homology in certain specific regions is important. Though some heterogeneity is tolerated, e.g. starch synthase antisense gene from cassava could suppress the starch synthase in potato[22], antisense apple

ACC oxidase has been reported to inhibit ethylene production in tomato[23] yet low inhibition is seen in cases where degree of homology is low. Transgenic tobacco plants carrying antisense gene for tomato ACC oxidase showed variable inhibition in different parts and physiological states; this may be due to varying degree of homology or fluctuations in different tissues[24].

The insertion of gene during transformation is quite random, even the number of copies varies. The position of insert is quite important for its expression as varying degrees of inhibition is seen in different transformants having single gene insertions. The copy number of gene may or may not increase the inhibition. In case of inhibition of polygalacturonase enzyme, single copy insertion produced 50–95% inhibition depending on the site of insertion. However, two copies of antisense gene produced 99% inhibition[25].

Antisense oligonucleotides

Transient inhibition of a specific gene expression can be achieved by using antisense oligonucleotides, which are short (14–18 bases) DNA molecules complementary to the 5′ leader sequence or 3′ end of mRNA. In cases where oligonucleotide is complementary to the coding sequence, the 5′-fragment can be translated to generate a truncated polypeptide. The binding of antisense deoxy oligonucleotide and target mRNA leads to formation of a DNA–RNA duplex which is unstable and is recognized by RNAase-H which selectively degrades the RNA strand in a DNA–RNA duplex, thus inhibiting translation. In case of blockage of RNAase-H activity, oligonucleotide directed to cap sites can only inhibit translation as it inhibits binding of 40S subunit of ribosome[26].

Another class of oligonucleotides exists which are called the code blockers or triplex-forming oligonucleotides[27], these bind in the major groove of DNA target sequence, thus inhibiting transcription. They can either inhibit the binding of transcription factors by binding upstream to the coding region or can inhibit movement of RNA polymerase. Triplex formation requires the target site to have a homopurine or homopyrimidine sequences and third strand binds by hoogsteen base pairing, triplex strategy though is quite effective, especially in case of actively transcribing genes, yet it has its disadvantages like the requirement of a homopurine/homopyrimidine target sequence and further the problem of triplex instability under physiological conditions[28].

The effectiveness of oligonucleotides depends on several factors like position of target site against which they are directed, length of the oligonucleotide and further the presence or absence of secondary structure at the binding site. Stability of oligonucleotide is very essential for effective inhibition, and thus in case of unmodified oligonucleotides poor uptake and nuclease degradation were the limiting factors. Therefore various types of modifications have been developed which render the oligonucleotide resistant to nucleases. The modifications are either of the phosphate backbone (e.g. methylphosphonates, phosphorothioates, and phosphoroselonates)[29] or the oligomer ends may be modified (α-oligo decarboxynucleotide).

The oligomers can be coupled with intercalating agents and reactive metal agents like EDTA-Fe, phenanthroline-Cu, etc. Synthetic polymers like PAMAM dendrimers have also been investigated to function as delivery system for targeted gene modulation[30]. Novel methyl phosphonate oligonucleotides have been designed which have an internal non-nucleotide based linker moiety due to which the complementary unpaired base of RNA becomes sensitive to cleavage, hence site-specific cleavage of target RNA is possible[31]. Besides stability of the oligonucleotide, another critical factor remains, which is the cellular uptake of oligonucleotides. Oligo-nucleotides are taken up by endocytosis and achieving high concentrations within the cell is not plausible, hence various methods have been developed to improve cellular uptake. It has been reported that oligonucleotides conjugated with polyaminolipids like spermidine–cholesterol or spermine–cholesterol showed better cellular uptake compared with oligonucleotides alone[32]. C-5 propynyl pyrimidine-2′-deoxyphosphorothioate modified oligonucleotides have been checked for their potency in HeLa cell line and it was found that 11 bp oligonucleotide with modification has 66% of potency of parent 15 bp unmodified oligonucleotide. Though mismatches decreased the potency, expression level of target gene had no effect on their potency[33]. The specificity and easy uptake of oligonucleotides and their derivatives conjugated to carrier molecules presents a very promising future for antisense oligonucleotide technology in the field of therapeutics. As a matter of fact, oligonucleotides have been tested against a number of viruses in infected animal cells[34,35] and they have a good potential as antiviral agents and also in cancer therapy. But in case of therapeutics many aspects have yet to be studied like cellular uptake, specificity of binding, general effects on growth and long-term effects before their use as novel drugs.

Applications of antisense RNA technology

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

Antisense RNA provides an opening in the study of

regulation of vital genes, as an antisense inhibition can be taken to be a leaky mutation which would be useful in studying genes, mutations in which are lethal and their partial inhibition also leads to a significant change in phenotype. Such partial inhibition was used to create a tobacco mutant deficient in NADH-hydroxypyruvate reductase to study the role of photorespiration in stress protection[36]. Besides unravelling the vital gene functions, antisense RNA inhibition has been used to observe various steps in metabolic pathways. Majeau *et al.*[37] modified the activity of carbonic anhydrase which had no significant impact on CO_2 assimilation but it brought forward the effect of decline in carbonic anhydrase activity on stomatal conductance and susceptibility to water stress.

Antisense mutants of tobacco with drastic decrease in Rubisco content resulted in low photosynthetic rate; however, the leaf development was normal and independent of Rubisco content though leaf development was delayed[38]. The biochemical target of various herbicides is acetolactate synthase, and this was confirmed by raising transgenic potato plants expressing antisense acetolactate synthase which were inviable without amino acid supplementation, thus an *in vivo* model for herbicide action was put forward[39].

Similarly, the effect of ethylene on shoot morphogenesis was studied via the production of transgenic mustard plants expressing antisense 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase gene, and such plants showed marked increase in regeneration potential and corresponding decrease in ethylene production[40]. Cotton fibre protein genes have also been characterized using antisense RNA inhibition of a particular gene[41]. Antisense inhibition has been utilized to work out the role of lipoxygenase (LOX) in lentil protoplast by the introduction of antisense LOX gene[42].

The antisense RNA technology has formed the basis for elucidating the flavanoid biosynthetic pathway, and as a matter of fact, CHS gene was the first endogenous gene targeted by antisense RNA in plants. Antisense CHS petunia plants produced flowers with pale corolla pigmentation but the steady state levels of mRNA of other flavanoid-specific genes were not affected[43]. S-adenosylmethionine (SAM) is a common precursor for both ethylene biosynthesis as well as biosynthesis of polyamines (spermine and spermidine), and the antisense inhibition of SAM decarboxylase gene expression in potato transgenic plants provided a molecular approach to study the effect of manipulation of polyamine levels on growth and development[44]. The potato plants expressing the antisense SAM decarboxylase gene constitutively showed aberrant phenotypes due to the depletion of cellular polyamines and elevated levels of ethylene[44]. Further, potato transgenics were also produced with SAM decarboxylase antisense gene driven by an tetracycline-inducible promoter, and these transgenics provide an opportunity for further investigations into the inter-relationship between polyamine and ethylene metabolic pathways. Other than elucidating the steps of metabolic pathways, antisense RNA inhibition has found its use in identification of gene function as in case of a ripening gene (pTOM5), which was found to be a part of carotenoid pathway[45]. Another ripening-related gene of tomato (pTOM13) was found to be involved in ethylene synthesis and thus may be a part of ACC-oxidase system involved in conversion of ACC to ethylene[46]. *Arabidopsis* expressing antisense RNA against ankyrin repeat (AKR) containing gene indicated the involvement of AKR gene in regulation of chloroplast differentiation[47]. Antisense repression of nuclear encoded NADH-binding subunit of mitochondrial respiratory chain complex-I in potato plants led to normal vegetative growth but reduced male fertility which might be due to insufficient mitochondrial respiratory chain[48]. Similarly, the function of cytosolic phosphorylase was investigated in transgenic potato plants expressing antisense cytosolic phosphorylase, the tuber produced more shoots and transgenics flowered earlier than the wild plants[49].

Transgenic *Flaveria bidentis* plants with antisense rubisco gene were used to study the relationship between CO_2 assimilation and rubisco content in C_4 plants and it was observed that the inhibition of rubisco led to increase in CO_2 concentration and its leakage in bundlesheath[50]. The importance of peptide transport gene AtPTR2-B from *Arabidopsis* was evaluated by producing transgenic with antisense AtPTR2-B gene, the transgenics had altered phenotype, delayed flowering and no seed set, suggesting a major role of the gene in growth and development[51]. Other examples of the antisense RNA technology being utilized for elucidating gene functions include the transgenic tobacco-expressing antisense ascorbate peroxidase (APX) gene leading to increased susceptibility of transgenics to ozone injury, suggesting the major role of APX in oxidative stress tolerance[52]. Antisense inhibition of biotin carboxylase gene in tobacco led to severe retardation of growth, reinforcing the importance of biotin for plant growth[53].

The antisense RNA technology has also been used for crop improvement, besides being used to gain knowledge in the basics of plant development. The technology has been used in modifying seed oil composition of *Brassica* seed oil[54]. The desaturase enzyme gene was inhibited, leading to production of seeds with high-stearate oil content without there being any decrease in the seed lipid content.

Altering of lignin composition in tobacco by inhibiting lignin biosynthetic enzymes led to a significant decrease in syringyl and appearance of 5-hydroxyguaiacyl units, thus opening new avenues in lignin manipulation and its potential benefits[55]. Antisense RNA inhibition of

chitinase gene expression has resulted in increased fungal disease susceptibility in *Arabidopsis* plants, elucidating some role of chitinases in plant protection[56]. Polygalacturonase (PG) plays a major role in tomato fruit development as it is involved in cell wall degradation and fruit softening, and transgenic tomato plants constitutively expressing antisense PG gene were produced[17,57] and were found to have a longer shelf life compared to normal tomatoes though the red pigment (lycopene) accumulation and morphology of the transformed fruits was not disturbed[17]. In fact, these transgenic tomatoes (marketed as 'Flavr Slavr' by Calgene, USA) were the first of the transgenics commercialized in USA. Ethylene plays a major role in ripening of fruits. Transgenic cantaloupe melon plants expressing antisense ACC oxidase gene showed blockage of fruit ripening both on and off the vine, though the antisense effect could be reversed by exogenous ethylene treatment[58].

Antisense RNA has also been used to confer resistance to viral plant infections. Transgenic potato plants expressing antisense RNA to potato leaf roll luteovirus (PLRV) coat protein were resistant to the infection[59]. Stanley *et al.*[60] produced transgenic cassava-resistant to cassava latent virus (CLV) by introducing a tandem repeat of subgenomic DNA B of CLV. Transgenic *Nicotiana benthaniana* plants expressing antisense C1 gene (which encodes for Rep protein) of tomato yellow leaf curl virus (TYLCV) were found to be resistant to TYLCV infection[61], thus tomato transgenics may be produced which would be resistant to TYLCV, a major disease in tomato.

The antisense RNA technology has recently been reported to generate Hsp70 mutant in *A. thaliana* and this brought forward the protective role of Hsp70 in thermotolerance and a regulatory effect on heat shock transcription factors leading to autoregulation of the heat shock response[62].

The role of phenylalanine ammonia-lyase (PAL), which is the first enzyme in the phenyl propanoid biosynthetic pathway in microspore development, has been studied using antisense PAL cDNA under the control of rice tapetum specific promoter in tobacco, and it has been demonstrated that PAL activity in anthers is essential for microspore development[63]. The antisense gene strategy has been applied to inhibit the expression of an allergen gene during seed maturation in rice, and inhibition persisted in the progeny of the transformed plants[64].

Antisense oligonucleotides have tremendous applications especially in animal systems. As in the case of plants, antisense oligonucleotides have been used in the study of gene function in animal systems, e.g. interrelationship between morphin analgesia and G-protein was elucidated by using G-protein α-subunit antisense oligonucleotides[65]. Weisinger *et al.*[66] used antisense inhibition of a neuro-peptide gene to elucidate its role

Table 1. Various potential applications of antisense RNA technology

– Production of mutants with leaky mutations in various systems
– Study of promoter specificity, transcriptional and translational control
– Elucidation of various steps in metabolic pathways
– Study of interactions between various metabolic pathways
– Production of fruits with delayed ripening
– Production of flowers with altered pigmentation
– Alteration of components of seed oils
– Control of viral and fungal infections in various systems
– Inhibition of tumour growth in animal systems

in cell growth in other systems, besides nervous system. Antisense oligonucleotides have been extensively utilized in control of viral infections. Anazodo *et al.*[67] used antisense oligonucleotides complementary to 5-LTR of viral genome for the protection of cells against HIV-1 infections. Besides viral infections, antisense oligonucleotides have been used, both *in vitro* and *in vivo*, in murine systems to inhibit oncogene expression[68]. Antisense RNA has also been used to study murine cell differentiation regulated by *p53* gene[69].

Besides viral infection control and cancer therapy, oligonucleotides are expected to prove useful in the treatment of autoimmune, endocrinological, neurological diseases and bacterial infections[70]. Though most of such studies were performed *in vitro*, preliminary toxicity studies have been carried out in mice and rats. Phosphorothioate oligonucleotides were found in all organs even after 48 h of administration, pharmacokinetics of oligonucleotides is related to phosphate backbone and can be modulated by using carriers such as liposomes. Though dosage studies on animal systems are on, the long-term toxicity of oligonucleotides, including immune reactions, mutagenicity and possible incorporation into genome has to be evaluated before their use in therapeutics[70].

The antisense RNA technology has certain basic lacunae which need to be overcome before its full potential is exploited. Firstly, there seems to be no single universal mechanism of antisense action, each system has to be studied independently for its mechanistic aspects of inhibition. The degree of inhibition is quite variable even when single copy of antisense gene is present, this aspect needs to be studied further in correlation with copy number and positional effect on the antisense gene expression. Besides these, the long-term effects of expression of antisense gene or dosage of antisense oligonucleotides, their degradation and effects of non-specific binding need to be investigated.

In conclusion, the antisense RNA technology holds promise both for the plant systems as well as the animal systems but before its extensive use, the basics of the technology have to be elucidated and the technology accordingly modulated so that it may be exploited to its full potential.

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