

RNA interference in designing transgenic crops

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Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double stranded RNA; RISC, RNA-induced silencing complex

RNA interference (RNAi) is a sequence-specific gene silencing mechanism, triggered by the introduction of dsRNA leading to mRNA degradation. It helps in switching the targeted gene on and off, which might have a significant impact in developmental biology. Discovery of RNAi represents one of the most promising and rapidly advancing frontiers in plant functional genomics and in crop improvement by plant metabolic engineering and also plays an important role in reduction of allergenicity by silencing specific plant allergens. In plants the RNAi technology has been employed successfully in improvement of several plant species- by increasing their nutritional value, overall quality and by conferring resistance against pathogens and diseases. The review gives an insight to the perspective use of the technology in designing crops with innovation, to bring improvement to crop productivity and quality.

Introduction

RNA interference (RNAi) is a gene-silencing phenomenon, whereby sequence specific RNA degradation takes place. The RNA degradation process is triggered by introduction of double stranded RNAs (dsRNA) via transgenes, which are further cleaved by the enzyme Dicer to form duplexes of 21-nucleotides (nt) with symmetric 2 nt 3' overhangs. These duplexes are commonly referred to as small interfering RNAs (siRNAs), which mediates the degradation of mRNA, thus leading to suppression or alteration of the gene expression.

RNAi is a scientific breakthrough with its most promising and rapidly advancing frontiers in genetic improvement of crops. In plants the RNAi technology has been employed successfully in improvement of several plant species- by increasing their nutritional value, overall quality and by conferring resistance against pathogens and diseases. In this review, the main focus is on the mechanism and application of RNAi as an efficient tool in plant gene function analysis, plant metabolic engineering and reduction of specific allergens and toxic products in plants.

Discovery of RNAi

The discovery of RNAi, however, very accidental was unfolded by a surprising observation made in petunias. In 1990, Jorgensen et al.¹ were trying to deepen the color of petunia flowers by upregulation of the gene coding for chalcone synthase (pigment producing gene) under the control of constitutive 35s promoter. To their surprise, instead of the expected deep purple color petunias the flower appeared variegated or even white. Since the expression of both the transgene and the homologous endogenous gene was suppressed, the phenomenon was termed cosuppression. Later Guo and Kemphues,² while investigating the function of par-1 gene in the nematode worm *Caenorhabditis elegans* showed that injecting either sense or antisense RNA for the par-1 gene resulted in its suppression. Further Andrew Fire³ and Craig Mello⁴ observed that injection of the double stranded RNA (dsRNA) mixture i.e., both sense and antisense strand together resulted in more efficient silencing of the target gene, than either strand alone. This turned out to be the defining moment in RNAi research and the effect came to be known as "RNA interference". Since then, work in this area has been booming and researchers in this field believe that RNAi has greater potential over other posttranscriptional regulators like antisense technology. As known already, the action of RNAi relies upon an antisense mechanism, since ultimately a single-stranded RNA molecule binds to the targeted RNA and recruits a ribonuclease to degrade the targeted RNA. However, in spite of the compelling similarity between antisense and RNAi, there are several important differences. RNAi is a naturally occurring phenomenon found exclusively in eukaryotes as the oldest and most ubiquitous antiviral system, while the majority of antisense RNAs are found in prokaryotes. This might imply that by nature RNAi is more suitable than antisense RNAs for silencing genes in eukaryotic cells. Further, self-amplification and a "cell to cell" spreading of RNAi results in a long-lasting suppression of the targeted gene in plants and worms, while antisense RNA represents a rather transient suppression of the targeted gene in prokaryotes.⁵

RNAi Machinery and Mechanisms

Components of the RNAi pathway. The RNAi pathway involves two ribonuclease machines- firstly the ribonuclease III enzyme Dicer that leads to cleavage of dsRNA into active siRNAs, thus initiating the RNAi pathway. The second one is the

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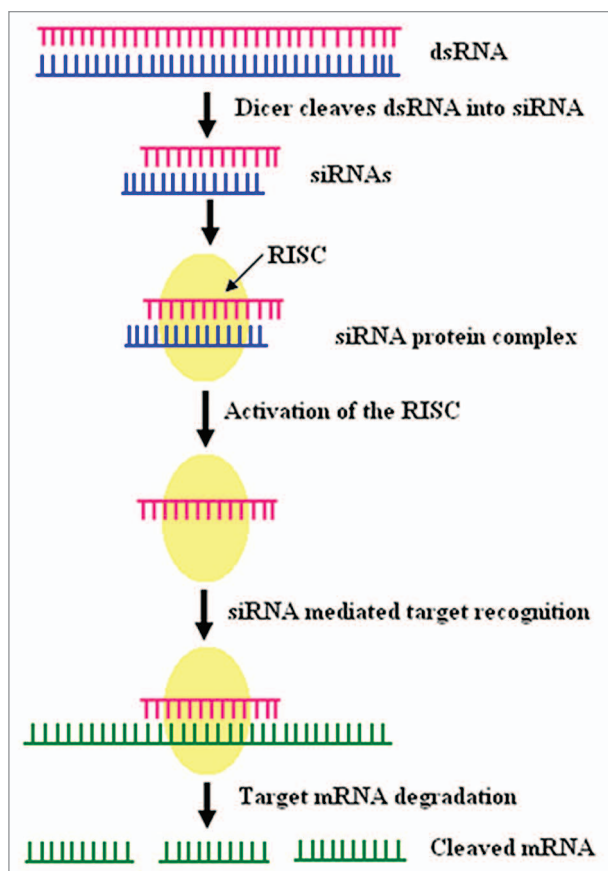


Figure 1. Mechanism of the RNAi pathway.

RNA-induced silencing complex (RISC),⁶ which brings about the silencing effect together with its RNase H core enzyme Argonaute.⁷⁻⁹ The RNase III family members shows specificity for dsRNAs¹⁰ and ultimately cleaves them with 2 nt overhangs at 3' and 5'-phosphate and 3'-hydroxyl termini.¹¹ The Dicer¹² is ATP dependent and contains four characteristic domains: an amino terminal helicase domain, a PAZ (Piwi/Argonaute/Zwille) domain, dual RNase III domains and a dsRNA binding domain.^{13,14} The model for Dicer catalysis as described by Zhang et al.¹⁵ suggests that the two RNase III domains in the Dicer are associated in an intramolecular pseudo-dimer, thus creating an active site. It has been shown that each domain cuts a single strand of the duplex and generates one new terminus.¹⁵

The final step of the RNAi pathway is the nucleolytic destruction of the target mRNA, which is achieved by the multicomponent protein complex RISC. Integral to this complex is a member of the Argonaute family, which has the characteristic nuclease activity responsible for mRNA target cleavage.⁷ Earlier, it has been already reported that the RISC has a sequence specific nuclease activity which has siRNA as an integral component.¹⁶ The siRNAs associated to nuclease, actually served as guides to target specific messages based upon sequence recognition. Among the various Argonaute family members, Argonaute 2 (AGO2)¹⁷ has been identified as an important protein component of RISC. AGO2 is a 130 KDa protein

containing polyglutamine residues, PAZ and PIWI domains characteristic of the Argonaute gene family members.¹⁸

Mechanism of action. RNA interference can be divided into four stages: (1) Double stranded RNA cleavage by the Dicer, (2) silencing complex (RISC) formation, (3) silencing complex activation and (4) mRNA degradation.¹⁴ The initial step of RNAi is connected with the delivery of dsRNA in the cell, which is perfectly homologous, in sequence, to the target gene.¹⁹⁻²¹ The Dicer enzyme, recognizes the ds RNA,²²⁻²⁴ and further processes dsRNA in an ATP dependent reaction into double stranded siRNA 21–25 nucleotides in length, depending upon the species.^{11,25} In the second step, the siRNAs produced by Dicer are incorporated into a multicomponent nuclease complex- the RNA-induced silencing complex (RISC), which is inactive in this form to conduct RNAi.²⁶ The third step involves unwinding of the siRNA duplex in an ATP dependent process by a helicase and further remodeling of the complex to create an active form of the RISC.²⁷ The final step includes the recognition and cleavage of mRNA complementary to the siRNA strand present in RISC. The target mRNA is cleaved into fragments of about 22 nucleotides long (Fig. 1). After the cleavage is complete, the RISC departs and the siRNA can be used in a new cycle of mRNA recognition and cleavage.²⁸ One interesting feature encountered in RNAi is its apparent catalytic nature. Though the cleavage of dsRNA into small siRNAs results in some degree of amplification, it is not sufficient to bring about continuous mRNA degradation. Studies made by Lipardi et al.²⁹ and Sijen et al.³⁰ provides very convincing genetic and biochemical evidence that RNA-dependent RNA polymerase (RdRP) plays a very important role in amplifying the RNAi effects. The enzyme RdRP uses siRNAs as primers to generate new dsRNAs that can be further cleaved into new siRNAs.³¹

RNAi as a Tool for Plant Functional Genomics

Functional genomics is an important aspect of genomics, where the specific functions of genes and their vitality to an organism, determining the function of all genes in a plant genome is very challenging. For this purpose, insertional mutagenesis has been a very efficient tool and has been extensively applied to the functional characterization of plant genes. However, this approach has limitations, arising from the randomness of mutagenesis, which further restricts high-throughput reverse genetic analysis of plants.³² Hence, silencing effect of RNAi is being exploited for plant functional genomics, utilizing its ability to specifically target the chosen gene. Further, the degree of gene silencing can be varied in different transgenic lines by using the same ihpRNA constructs.³³ Moreover, the expression of ihpRNAs from inducible promoters can control the extent and timing of gene silencing.³⁴ All these characters make RNAi the technology of choice for efficient plant gene function analysis.

The potential of RNAi in plant functional genomics has been utilized in various plants. For example, Travella et al.³⁵ introduced dsRNA expressing constructs of two genes- phytoene desaturase (PDS) and Ethylene Insensitive 2 (EIN2) into hexaploid wheat (*Triticum aestivum*). The PDS is an enzyme involved

in the carotenoid biosynthetic pathway and its reduction will lead to the inhibition of carotenoid biosynthesis.³⁶ The other gene EIN2 encodes a transmembrane protein of ethylene signaling pathway.³⁷ Travella et al.³⁵ successfully delivered the RNAi constructs expressing these two genes and obtained a highly specific and heritable phenotypic series, in the transformants, that may be a useful feature for gene discovery and functional genomics. They showed that RNAi mediated gene silencing of both genes resulted in reduction of transcripts by up to 93% for PDS and 99% for EIN2, in fact the endogenous target mRNA levels of homologous genes were decreased in RNAi transgenic lines, which displayed a strong correlation between decreased mRNA levels and increased severity of phenotypes. This clearly shows the potentiality of dsRNAi in being a useful and efficient tool for functional genomics in hexaploid wheat and provides means to manipulate gene expression experimentally.

RNAi has been also utilized for genomic scale analysis of gene function in maize (*Zea mays*). The functional genomics approach was pursued by McGinnis et al.³⁸ to study the role of 130 chromatin related maize genes in controlling a range of epigenetic phenomenon. They used an RNAi based approach for this study as it offered two advantages- first, RNAi induced mutations are dominant and secondly RNAi has the potential to reduce expression of multiple closely related genes with single transgene locus.

In 2005, Li et al.³⁹ while investigating the role of actin cytoskeleton during fiber development characterized 15 *Gossypium hirsutum* ACTIN (GhACT) cDNA clones. Of the 15 GhACT genes, GhACT1 was studied in detail using RNAi technology. The RNA interference of GhACT1 caused severe reduction in its mRNA and protein levels and lead to disruption of the actin cytoskeleton network in fibers, which ultimately, inhibited fiber elongation. The expression study of GhACT1 gene using RNAi clearly predicts its role in cotton fiber development.³⁹

Moreover, the rice (*Oryza sativa*) genome has been sequenced^{40,41} and hence the functional identification of rice genes has become a priority. So, to help identify the functions of genes in rice, Miki and Shimamoto⁴² developed a Gateway vector, pANDA, for RNA interference of rice genes. The destination vector which they developed contains the maize ubiquitin promoter with an intron, and also a 920 bp fragment of the coding region for the *Escherichia coli* *gus* gene which acts as a linker between two inverted repeats of the gene sequence derived from target genes. The vector so developed is very efficient in suppression of mRNA expression in transgenic rice plants and thus presents itself as a useful RNAi tool for functional genomics of rice. Another novel PCR based RNAi vector pTCK303 was constructed by Wang et al.⁴³ for efficient knock-down of gene expression in rice. Based on the vector pUN1301, a modified pCAMBIA1301,⁴⁴ the RNAi vector pTCK303 was developed, which has two specific multiple cloning sites separated by a rice intron of 478 bp.⁴³ In both pANDA and pTCK303, the presence of a maize ubiquitin promoter makes the vector appropriate for monocot gene silencing, as compared to pHANNIBAL and pHELLSGATE,³³ both of which are driven by a cauliflower mosaic virus 35S promoter, which limits them to dicotyledons only. More recently, a set of modular

pSAT RNAi vectors have been constructed, which are suitable for hpRNA expression under various constitutive promoters. The pSAT RNAi plasmids consists of a Chs A intron sequence that is flanked by two unique MCS suitable for insertion of target gene in reverse orientations. Since the plasmids can accommodate several hpRNA expression cassettes into a single Agrobacterium binary vector, it facilitates the analysis of multiple gene function in plant cells.⁴⁵

RNAi and its Role in Crop Improvement

RNAi mediated plant metabolic engineering. Since plants are the principal natural resource and provide food, for human and livestock consumption and also supply our pharmaceutical demand, many efforts have been focused on improving plant nutritional value and also increasing amounts of secondary metabolites. In this respect, plant metabolic engineering utilizing RNAi mechanisms provides a very promising approach towards realizing these efforts. In recent years, RNAi technology has been used in metabolic engineering of plants with respect to different traits and targets.

RNAi has shown promise in development of tomato (*Lycopersicon esculentum*) fruit with enhanced carotenoid and flavonoid content, both of which are highly beneficial for human health. Davuluri et al.⁴⁶ used fruit specific promoter combined with RNAi to suppress an endogenous photomorphogenesis regulatory gene DET1 in tomato, which represses several signaling pathways controlled by light. In contrast to control wild type (non transgenic) tomato (*Lycopersicon esculentum*), the transgenic tomatoes so obtained showed specific degradation of Det1, along with an increase in the carotenoid and flavonoid content.⁴⁶ Similarly, the carotenoid content of rapeseed (*Brassica napus*) were also enhanced by utilizing RNAi to downregulate the expression of lycopene epsilon cyclase (ϵ -CYC). The transgenic Brassica seeds thus obtained showed increased levels of β -carotene, zeaxanthin, violaxanthin and lutein.⁴⁷

In several other plants RNAi has been used to improve their nutritional value. For example, amylose content in wheat has been markedly increased with RNAi approach, by suppressing simultaneously the expression of SBEIIa and SBEIIb. The suppression yielded >70% amylose in wheat.⁴⁸ Similar RNAi approach has been made in case of sweet potato (*Ipomoea batatas*), yielding potato with higher amylose content.⁴⁹ RNAi was further applied to engineer decaffeinated coffee (*Coffea canephora*) plants by using constructs containing CaMXMT1 sequence that encodes the theobromine synthase gene involved in the caffeine biosynthetic pathway. The RNAi mediated suppression of theobromine synthase thus led to reduction of caffeine content by up to 70% in comparison to controls.⁵⁰

RNAi technology has also been used successfully in genetically modifying the fatty acid composition of cotton seed oil. Liu et al.⁵¹ utilized a hairpin RNA (hpRNA) mediated RNAi method to downregulate two key fatty acid desaturase genes encoding stearoyl-acyl-carrier protein $\Delta 9$ -desaturase and oleoyl-phosphatidylcholine $\omega 6$ -desaturase. Downregulation of these two genes

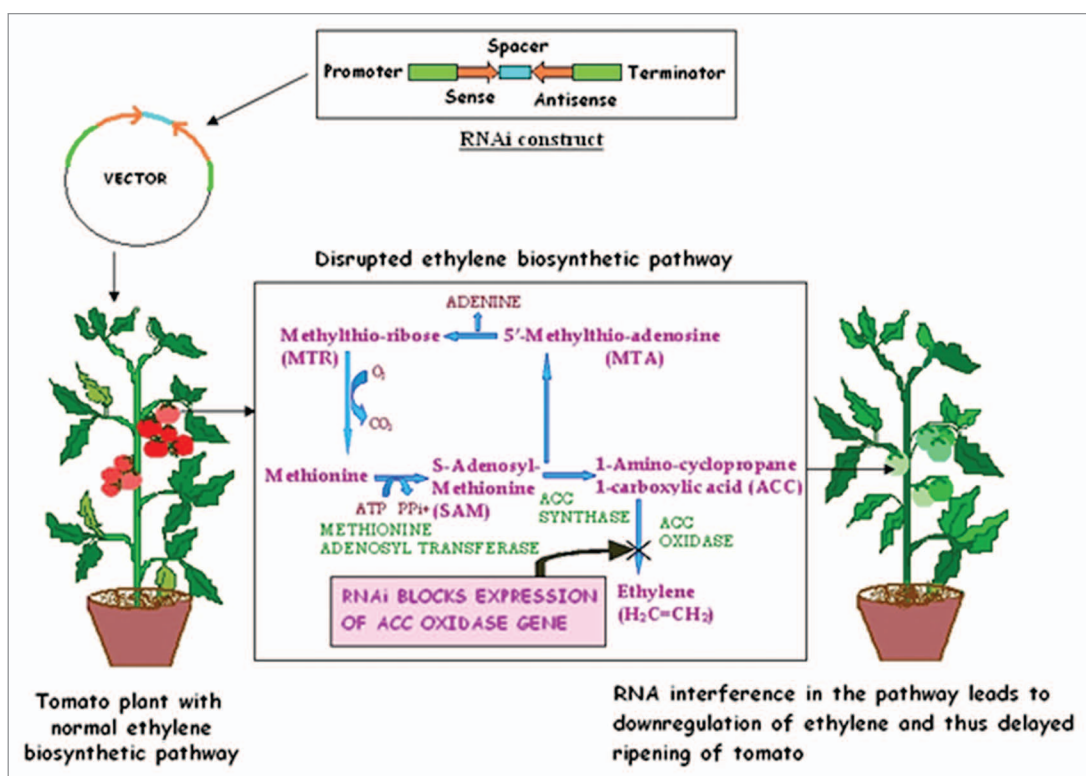


Figure 2. Schematic illustration of RNAi mediated downregulation of ethylene biosynthetic pathway in tomato.

in cotton resulted in nutritionally improved high stearic and high oleic cotton seed oils which are essential fatty acids for better health of the human heart.

In 2003, Kusaba et al.⁵² reported hpRNA mediated silencing of Low Glutelin Content 1 (Lgc1) gene in rice. Glutelin is a major seed storage protein, which accounts for about 60% of total endosperm protein in rice. LGC1 is a dominant mutation that suppresses the glutelin multigene family by a tail to tail inverted repeat of two highly similar glutelin genes, which results in dsRNA, thus inducing gene silencing. In mutant line LGC1, the glutelin content is reduced so that kidney patients, who need to restrict their protein intake might benefit from this.⁵²

RNAi has even been used to increase the shelf life of tomato by delaying its ripening. Tomato being a climacteric fruit, has a burst of autocatalytic ethylene during ripening process. Xiong et al.⁵³ introduced a unit of ACC oxidase dsRNA in tomato and thus blocked the expression of its gene. In fruits of transgenic plants, the ethylene production rate of ripened fruits and leaves was significantly inhibited (Fig. 2), which ensured a prolonged shelf life.

In a more recent work, Meli et al.⁵⁴ have identified and targeted two ripening-specific N-glycoprotein modifying enzymes, α -mannosidase (α -Man) and β -D-N-acetylhexosaminidase (β -Hex). They have further shown that the suppression of these genes enhances shelf life of tomato, owing to the reduced rate of softening.

As already known that lysine is one of the most important essential amino acids and due to its presence in limiting levels in major food crops various efforts have been focused on enhancing

its levels. For instance, RNAi constructs were successfully used to obtain a dominant opaque phenotype with stable and heritable expressions of recombinant sequences in maize that produce a specific reduction in 22 KD α -zein proteins. This further, leads to a shift in amino acid balance, due to which the transgenic opaque seeds of maize had higher levels of lysine and reduced levels of leucine, alanine and glutamine.⁵⁵ More recently, in corn (*Zea mays*), production of high lysine was reported by RNAi mediated suppression of the lysine catabolic enzyme lysine-ketoglutarate reductase/saccharopine dehydrogenase (ZLKR/SDH) in endosperm.⁵⁶ Lysine ketoglutarate reductase is the first enzyme in the α -amino adipic acid pathway, which catabolizes lysine in glutamate, α -amino adipic acid and acetyl CoA.^{57,58} Since lysine is an important essential amino acid, increasing its content in major cereal crops like rice and wheat through such RNAi approaches will prove to be fruitful.

A very interesting example of metabolic engineering was the RNAi mediated replacement of morphine with non-narcotic alkaloid reticuline in opium poppy (*Papaver somniferum*). In 2004, Allen et al.⁵⁹ were the first to report gene silencing in transgenic opium poppy using RNAi. It was also the first time when RNAi was used to interfere with multiple steps of a complex biochemical pathway. An hpRNA construct was designed to silence all members of the multigene Codeine reductase (COR) family. After the gene silencing through RNAi, the transgenic plants accumulated (S)-reticuline, a precursor non-narcotic alkaloid which occurs seven enzymatic steps upstream of codeinone in the pathway. The (S)-reticuline accumulation in transgenic opium poppy occurred at the expense of morphine, codeine, opium

and the baine.⁵⁹ In another study, RNAi was successfully used to silence the N-demethylase gene, designated as CYP82E4 and its close homologues in an effective means for suppressing nicotine to nornicotine conversion in tobacco (*Nicotiana tabacum*).⁶⁰ Nor-nicotine is the precursor of N'-nitrosonornicotine (NNN), which is a tobacco specific nitrosamine (TSNA) having carcinogenic properties.⁶¹ Further, Lewis et al.⁶² tested the efficacy of RNAi-mediated suppression of nicotine demethylase activity for reducing NNN and total TSNAs in commercial air-cured tobacco curing situations, by generating an array of transgenic lines of tobacco expressing an RNAi construct targeted towards CYP82E4, and this material was evaluated in a multi-environment test for nicotine conversion and TSNA formation. Hence, RNAi provides an effective strategy for reducing nornicotine and subsequent NNN levels in tobacco.

RNAi technology has also been used in soybean (*Glycine max*) in order to silence the myo-inositol-1-phosphate (GmMIPS1) gene, which resulted in a drastic reduction (up to 94.5%) of phytate content in the developed transgenic lines. By means of this silencing, it was further observed that the reduction in the phytate content of soybean lines showed poor seed development, demonstrating an important correlation between GmMIPS1 gene expression and seed development.⁶³ Much work has been done to produce low phytic acid crops in order to increase the bioavailability of minerals and micronutrients and thereby improve their nutritional quality. Various low phytic acid mutants have been produced in case of barley (*Hordeum vulgare*) and maize^{64,65} by inducing mutations in genes encoding specific enzymes involved in phytate biosynthesis. The phytate biosynthesis has also been reduced recently in rice by transformation with Ins (3)P1 synthase (MIPS) gene (RINO1) in antisense orientation.⁶⁶ Further, in the rice study, it has been reported that the considerable reduction of phytate-P content is associated with a similar increase in the level of inorganic phosphorus (Pi).⁶⁷ Thus, here is a possibility where RNAi could be well targeted to downregulate specific genes involved in phytate biosynthesis and hence develop crops with lower phytic acid levels and improved micronutrient (iron and zinc) absorption.

RNAi mediated reduction of allergenicity and toxicity in plants. Food allergies, though a rare phenomenon, are a cause of concern, in today's world. People allergic to a particular food or food items often avoid that which causes the allergy, which results in deprivation of the diet of a wide range of common plant foods that have important nutritional value. As a matter of fact, the allergic reactions are not elicited by the whole food item, but only by certain components called allergens, which are antigenic and so capable of sensitizing the body in such a way that unusual response occurs in hypersensitive individuals. Apart from allergies, people are also compelled to avoid highly beneficial and nutritious plant products due to presence of certain toxic metabolites. Hence, engineering plants with reduced allergenicity and toxicity is very important and required. In this context, RNAi gives us a feasible opportunity to face this challenge successfully.

RNAi being sequence-specific is highly efficient in silencing specific allergens and toxic metabolites, to the extent that it limits only their allergic potential without hampering the essential

cellular functions, which these allergens and metabolites may perform.⁶⁸ Le et al.⁶³ have demonstrated a very promising design of tomato fruits with reduced allergenicity. They reported the molecular characterization of Lyc e3, a new tomato allergen and the efficient downregulation of its expression in transgenic tomato plants. The Lyc e 3 encodes a non-specific lipid transfer protein (ns-LTP), which is hydrophilic and enhances specific intermembrane lipid transfer. Specific dsRNAi constructs of LTPG1 and LTPG2 were utilized for the suppression of Lyc e 3 accumulations. Further, tests were conducted for the allergenic potential of transgenic tomato plants by measuring the histamine release from sensitized human basophils as against parental lines.⁶⁹ In another study, an allergen designated as Mal d 1 in apple (*Malus domestica*), leads to cross-reactive IgE antibody responses, which causes adverse reactions in allergic patients. In order to inhibit the expression of this allergen RNA interference approach was used. An intron containing Mal d 1 gene was isolated from the cultivar Gala, to build the RNAi construct for successful gene silencing of Mal d 1. Skin prick testing and immunoblotting using apple leaf plantlets then monitored the effect of silencing on the expression of Mal d 1. The results suggested about 10-fold reduction in the expression of Mal d 1.⁷⁰

A very interesting study was made in cotton as its high quality protein content which makes cottonseed a nutrient rich resource for food cannot be utilized due to the presence of toxic gossypol within the seed tissue. Gossypol is a cardio and hepatotoxic terpenoid, which makes it unsafe for human and monogastric animal consumption.⁷¹ Hence any means, which could yield gossypol-free cottonseed, would significantly contribute to human nutrition and health. In 2006, Sunilkumar et al.⁷² reported successful use of RNAi to disrupt gossypol biosynthesis in cottonseed tissue by interfering with the expression of the δ -cadinene synthase gene during seed development. A tissue specific promoter for this RNAi approach was used, so that the gossypol content was reduced only in the seeds of cotton, without affecting its levels in any other part of the plant. In spite of being toxic to humans' gossypol is required by the plant to protect itself against insects and pathogens. The transgenic cottonseeds obtained showed 99% reduction of gossypol as compared to non transgenic wild types. However, no such reduction was observed in the other parts of transgenic plants. This approach in cottonseed has been further followed by the development of similar approaches in other potential food sources like *Lathyrus sativus*, cassava and fava beans that can be hampered by their toxicity.⁷³

Conclusion

Many gene silencing techniques have been discovered, but RNA interference technology is the first of its kind, with its potentially powerful, sequence specific mechanism, which shows higher gene silencing efficiency and potency. Since its discovery (1998), the field of RNAi has progressed at an amazing rate and within a short period of time it has become the technology of choice for plant scientists. RNAi has been further recognized as an attractive tool for plant gene function analysis and also for manipulation of both desirable and undesirable genes to generate plants with

improved and novel traits. In recent years, the studies focused on RNAi have revealed a much clearer picture about its mechanisms and applications, but still there is more, which needs to be known and understood. Moreover, one very important aspect of RNAi that needs to be highlighted is the ability to predict the effects of off-target silencing, which in turn would make RNAi technology

more specific and effective in its action. As far as the future prospects of this technology are concerned, it looks promising and seems to be advancing at a very rapid pace. Finally it could be stated that it is just a beginning of unfolding many unknown gene functioning and the exciting journey of plant science will move ahead.

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