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REVIEW

Role of RNA interference in plant improvement

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Abstract Research to alter crops for their better performance involving modern technology is underway in numerous plants, and achievements in transgenic plants are impacting crop improvements in unparalleled ways. Striking progress has been made using genetic engineering technology over the past two decades in manipulating genes from diverse and exotic sources, and inserting them into crop plants for inducing desirable characteristics. RNA interference (RNAi) has recently been identified as a natural mechanism for regulation of gene expression in all higher organisms from plants to humans and promises greater accuracy and precision to plant improvement. The expression of any gene can be down-regulated in a highly explicit manner exclusive of affecting the expression of any other gene by using RNAi technologies. Additional research in this field has been focused on a number of other areas including microRNAs, hairpin RNA, and promoter methylation. Manipulating new RNAi pathways, which generate small RNA molecules to amend gene expression in crops, can produce new quality traits and having better potentiality of protection against abiotic and biotic stresses. Nutritional improvement, change in morphology, or enhanced secondary metabolite synthesis are some of the other advantages of RNAi technology. In addition to its roles in regulating gene expression, RNAi is also used as a natural defense mechanism against molecular parasites such as jumping genes and viral genetic elements that affect genome stability. Even though much advancement has been made on the field of RNAi over the preceding few years, the full prospective of RNAi for crop improvement remains to be fully realized. The intricacy of RNAi pathway, the molecular machineries, and how it relates to plant development are still to be explained.

Keywords Co-suppression · Crop improvement · Gene silencing · RNA interference · Stress · Transgene

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Introduction

Human beings fully depend on plants directly or indirectly not only for their basic needs such as food, fodder, and shelter but also on other plant-derived products including gum, resin, timber, fiber, oil, dyes, pharmaceutically important secondary metabolites, drugs, and fossil fuels. In the face of global scarcity of arable land, water resources, abiotic, biotic stresses, and climate changes are the major limiting factors responsible for inferior plant growth, development, yield, and plant products. As world population increases rapidly, the demand for plants increases, which has led to the future food security, malnutrition, and famine (Brown and Funk 2008; Lobell et al. 2008; Godfray et al. 2010). To overcome these problems, new modern breeding, molecular genetics, recombinant DNA, and biotechnology studies consisting of genomics and proteomics will be needed to be augmented for the crop yield by developing better disease-resistant and environmental stress-tolerant, high-yielding crop varieties (Mittler and Blumwald 2010; Tester and Langridge 2010). The quality and quantity of plants have been improved by conventional plant breeding methods which are well known and still in the practice, but these are time consuming, laborious, and have several

other ecological, physiological, and biological constraints. However, the precision of biotechnological approaches, mainly genetic engineering, contributed rapid and significant changes in the crop improvement by offering a wide array of novel genes and traits which can be effectively inserted into elite crops to raise yield, nutritional value, and confer resistance to abiotic and biotic stresses (Sharma et al. 2002).

But this technology has public concerns and doubts related to their use in contemporary agriculture, biosafety guidelines, and impact of the genetically engineered crops on the environment particularly when genes derived from organisms other than plants are used (Wolfenbarger and Phifer 2000; Herdt 2006). The potentiality to transfer and express genes from other than plant sources into edible crops has raised apprehensions about the possible dangers to human beings and the environment. Transgene movement to other varieties and wild relatives leading to monster crops, erosion of genetic diversity, and ecological disturbances are major worries. Hence, before releasing transgenic crops for regular use, transgenic plants are subjected to intricate tests to understand the risks and to ensure safety. Development of transgenic crops thus needs additional time, cost, and expertise. Therefore, there is a need to develop new strategies and safe ways for crop improvement which could prove to be more acceptable to the general public. In this regard, RNA silencing or RNA interference (RNAi) technology has attracted the minds of researchers working in different areas of molecular biology throughout the world. RNAi is an inclusive term for the action of small interfering RNAs (siRNA) and microRNAs (miRNA) result in gene silencing through cleavage of mRNAs and blockage of protein synthesis. The discovery of RNAi has boosted our knowledge of gene regulation, gene function, and gene analysis and opened up novel avenues to develop fascinating technology that has an immense potential for application in genetic analysis, plant protection, and many other areas related to crop improvement. The capability of RNAi in influencing plant growth, development, morphogenesis, polarity, and other physiological process has been well established. Detection of RNAi in mechanism of hormone signal transduction, tolerance to environmental stress, and prevention of microbe invasion is a noteworthy process. Based on these findings, a concept of development of artificial RNAi was conceived which can inhibit action of targeted genes which in turn can silence a specific trait necessary to the improvement of a crop plant. Several in-depth reviews and other publications have been extensively focused on functions and role of RNAi (Rahman et al. 2008; Shukla et al. 2008; Tang et al. 2008; Belostotsky and Sieburth 2009). However, the intention of this review article is to summarize current scattered information regarding the unambiguous applications of RNAi in various aspects of crop improvement (Fig 1) for documenting, projecting, and understanding the role of RNAi.

History and basic mechanism of RNA interference

RNAi is one of the most exciting and enlivening phenomenon in which short double-stranded RNA (dsRNA) prevents the expression of specific genes by causing degradation of sequence of specific target mRNA in the cytoplasm. RNAi phenomenon was first discovered in transgenic plant *Petunia hybrida* L. (Napoli et al. 1990) by enhancing anthocyanin pigments in *Petunia* by the introduction of chalcone synthase gene (*CHS A*) encoding key enzymes in anthocyanin biosynthesis pathway. Unexpectedly, transgenic plants producing white or chimeric flowers were obtained instead of dark purple flowers due to the silencing of endogenous homologous gene and this phenomenon was termed as "co-suppression". RNAi occurrence is conserved among various organisms, also labeled as post-transcriptional gene silencing (PTGS) in plants, quelling in fungi (Romano and Macino 1992) and RNA interference in animals (Fire et al. 1998). Later on, Fire et al. (1998) elucidated the mechanism of RNAi in the nematode, *Caenorhabditis elegans*, and proposed the term RNA interference for the first time. The mechanism of RNA silencing works on at least three different levels in plants viz. cytoplasmic silencing by dsRNA results in cleavage of mRNA, endogenous mRNAs are silenced by

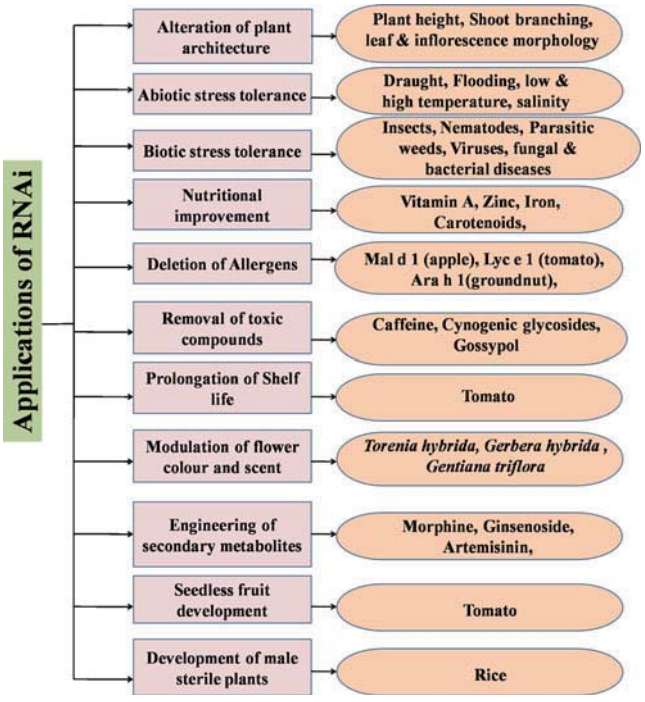

Fig. 1 Applications of RNAi in crop improvement

micro-RNAs (miRNAs), which negatively regulate gene expression by base-pairing to specific mRNAs, resulting in either RNA cleavage or blocking protein translation called post-transcriptional gene silencing (PTGS), and RNA silencing is associated with sequence-specific methylation of DNA and the consequent suppression of transcription [transcriptional gene silencing (TGS)] (Mansoor et al. 2006). The generalized procedure of RNA interference consists of cloning and insertion of targeted/interested gene into suitable plasmid to form recombinant plasmid. The recombinant plasmid is transformed to an appropriate vector like *Agrobacterium* which is suitable for plant transformation. There are several other methods like microprojectile bombardment, vacuum infiltration, syringing, and spraying infection methods available for plant transformation (Shao et al. 2008). The general process of RNA interference in plants is initiated by 21–24-nt-long, staggered cut small interfering RNAs (siRNAs) which are intracellularly generated from long endogenous or exogenous dsRNA molecules through the cleavage activity of a ribonuclease III-type enzyme called Dicer (Hamilton and Baulcombe 1999; Zamore et al. 2000). These siRNAs (21–24 nt) are then incorporated to RNA-induced Silencing Complex (RISC) which contains several proteins like AGO (Baumberger and Baulcombe 2005; Vaucheret 2008) besides siRNAs. The ATP-activated RISC unwinds the double-stranded siRNA. The antisense strand of siRNA molecule is incorporated into a nuclease containing RISC complex upon the loss of sense strand of the siRNA duplex by an RNA helicase activity (Kusaba 2004). RISC with antisense siRNA sequence then targets the homologous transcript by base-pairing interaction and cleaves the mRNA or blocks the translation leading to inhibition of protein synthesis (Bartel 2004) (Fig 2).

RNAi technology could be an alternative biotechnological approach having numerous advantages particularly more specific, dominant, sequence-based gene silencing. This tremendous potentiality of RNAi has been effectively exploited for inducing desirable traits as discussed here separately characteristic wise.

Applications of RNAi in plant improvement

Alteration of plant architecture

Alterations in plant architecture related to plant height, shoot branching, stem elongation, and leaf and inflorescence morphology have been of interest for centuries, as they control several important agronomic traits like yield, physiological and biochemical processes, lodging, and resistance to environmental stress (Khush 1999; Camp 2005; Wang and Li 2006). The negative impact of current global climate change and scarcity of water and nutrients on yield could be minimized by manipulating root system architecture towards a uniform distribution of roots in the soil that efficiently optimizes water and nutrient uptake (de Dorlodot et al. 2007). The recent studies carried out in model plants, *Arabidopsis* and *Petunia*, and crop plants including tomato, maize, and rice served as platforms for understanding the molecular basis of plant architecture (Wang and Li 2006, 2008). Recently, RNAi technology is used to improve crop productivity by modifying plant architecture. Adventitious root emergence and development were significantly inhibited in the *OsPIN1* RNA interference (RNAi) transgenic plants, which were similar to the phenotype of NPA (*N*-1-naphthylphalamic acid, an auxin transport inhibitor)-treated wild-type plants. α-Naphthylacetic acid (α-NAA) treatment was able to rescue the mutated phenotypes occurring in the RNAi plants. Over-expression or suppression of the *OsPIN1* expression through a transgenic approach resulted in changes of tiller numbers and shoot/root ratio suggesting that *OsPIN1* played an important role in auxin-dependent adventitious root emergence and tillering (Xu et al. 2005).

Endogenous plant hormones, including gibberellins (GAs) and brassinosteroides (BRs), are major plant regulators that control plant height. The genetic manipulations of genes related to GA biosynthesis are major targets to alter plant height (Coles et al. 1999; Hedden and Phillips 2000). Zhou et al. (2006) demonstrated the task of interfering RNA to suppress the expression of *OsGLU1* gene encoding a membrane-bound endo-1, 4-b-D-glucanase gene in rice (*Oryza sativa* L.) which affected plant internode elongation causing structural changes in cell walls altering cell wall composition and leading to develop a dwarf phenotype. RNAi was used to suppress the expression of the *OsGA20ox2* gene in rice (*O. sativa* L.), which encodes the regulatory enzyme GA 20-oxidase, for the syntheses of biologically active gibberellins (GAs) in plants. A phenotypic series of transgenic lines were recovered expressing reduced content of endogenous biologically active GA_1, which decreased plant height and resulted in semi-dwarf phenotype. The semi-dwarf lines exhibited shorter stem, lodging-resistant and better productivity as compared to the wild-type plants (Qiao et al. 2007). In another study, Hu et al. (2009) showed that the expression of rice histone deacetylases (*HDAC*) genes displays specific expression patterns and divergent developmental functions compared with closely related homologs in *Arabidopsis* and most of them are responsive to drought or salt stresses. Over-expression of several rice *HDACs* did not produce any visible phenotype, whereas down-regulation of a few *HDAC* genes affected different developmental aspects. Specifically, down-regulation of *HDA703* by miRNA reduced rice peduncle elongation and fertility, while inactivation of a closely related homolog *HDA710* by RNAi affected vegetative growth. *HDA704*

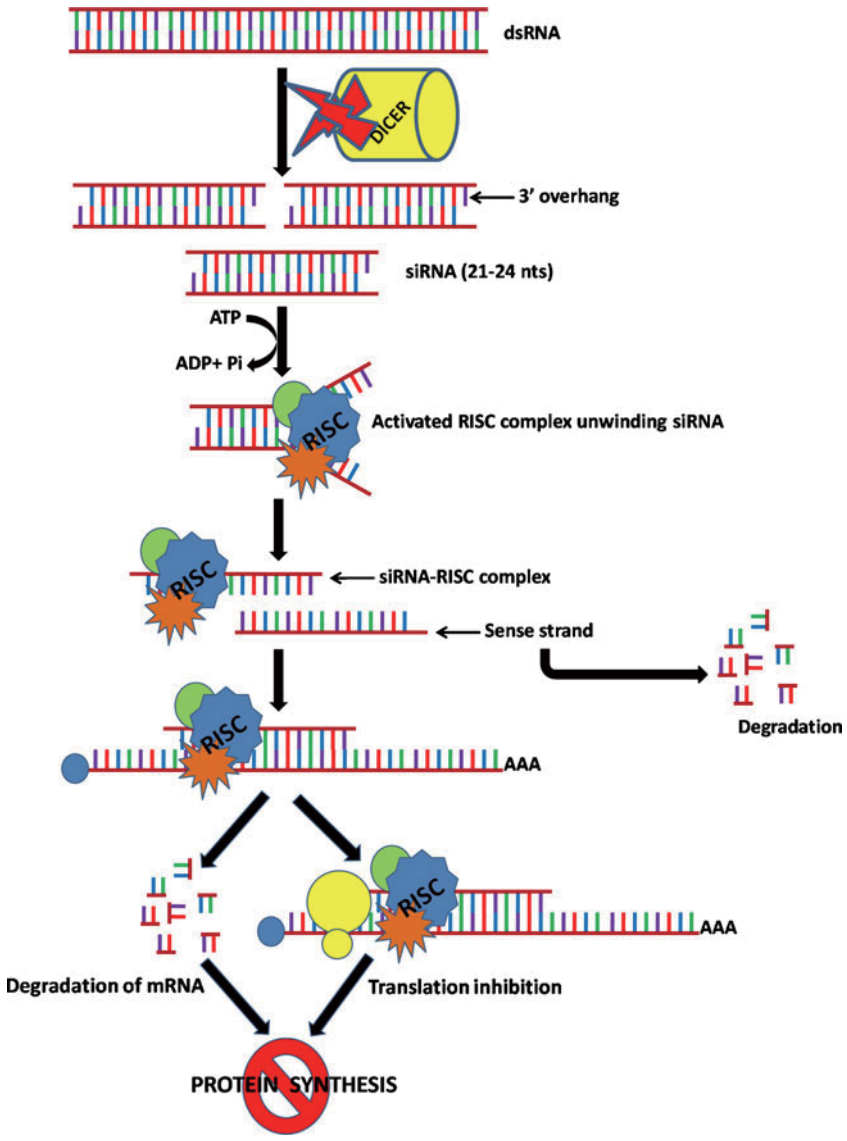

Fig. 2 The RNAi mechanism—dsRNA is processed by DICER RNase III into 21–24 nt siRNA duplexes. The siRNAs are then incorporated into RISC. The siRNA–RISC complex then targets a sequence, complementary to the siRNA, in a piece of mRNA. The protein synthesis is blocked either by degradation of mRNA or inhibition of translation

RNAi altered plant height and flag leaf morphology. Down-regulation of *HDT702* led to the production of narrowed leaves and stems. The feasibility of manipulation of plant architecture using RNAi technology has a wide utility in flowering, ornamental, plantation crops, and forest trees, for example, appropriate easy access for leaves plucking in tea or mulberry plants, harvesting of fruits or seeds from tall trees, and absence of thorns in roses.

Abiotic stress tolerance

Environmental abiotic factors such as drought, flooding, salinity, and fluctuations in temperatures reduce the plant productivity considerably. The discovery of novel genes, determination of their expression patterns in response to abiotic stress, and an improved understanding of their roles in stress adaptation (obtained by the use of functional genomics) will provide the basis of effective engineering strategies leading to greater stress tolerance (Cushman and Bohnert 2000; Pardo 2010). Recent evidence indicated that RNAi are involved in abiotic stress responses in plants. The role of miRNAs in response to abiotic stresses such as drought, cold, salinity, and oxidative stress was described by Sunkar and Zhu (2004) in *Arabidopsis* seedlings exposed to different abiotic stresses and showed that miR393 was strongly up-regulated by cold, dehydration, high salinity, and abscisic acid (ABA) treatments. Furthermore, miR319c, miR389a, miR397b, and miR402 were regulated by different abiotic stress treatments to varying degrees in *Arabidopsis*. The other reports have indicated that miR395 and miR399 are up-regulated when plants suffer from sulfur and phosphate starvation in *Arabidopsis* (Jones-Rhoades and Bartel 2004). The miR395 targets the ATP sulfurylases genes that catalyze the first step of

inorganic sulfate assimilation as well as *AST68* gene (encoding a sulfate transporter) (Takahashi et al. 2000; Jones-Rhoades and Bartel 2004). This result was unusual for miRNA-mediated gene regulation because miR395 appeared to regulate two different groups of genes that function in the same metabolic pathway. Subsequently, miR399 was shown to down-regulate ubiquitin-conjugating enzyme (*UBC24*) by targeting the 5′ untranslated region (UTR), and this regulation was demonstrated to be important for plant responses to Pi starvation by altering the expression of miR399 or UBC24 in *Arabidopsis* (Fujii et al. 2005; Chiou et al. 2006). The function of miR393 has also been shown to inhibit the expression of *TIR1* to down-regulate auxin signaling and seedling growth under abiotic stress conditions and also contributed to antibacterial resistance (Vierstra 2003; Jones-Rhoades and Bartel 2004; Navarro et al. 2006). Moreover, miR159 also responded to hormone signaling and dehydration responses in *Arabidopsis* (Achard et al. 2004; Reyes and Chua 2007). In addition, several stress-related miRNAs have been discovered in rice and only two miR393 and miR169g have been found to be related to abiotic stress; both were up-regulated by dehydration (Zhao et al. 2007). In another report, Jian et al. (2010) identified novel stress-related miRNAs from rice (*O. sativa* L. ssp. *Japonica* cv 9522) seedlings exposed to cold, dehydration, salinity, and abscisic acid (ABA) as well as wild-type seedlings. Recent studies have shown that the receptor for activated C-kinase 1 (*RACK1*) was a highly conserved scaffold protein with versatile functions and played important roles in the regulation of plant growth and development. Transgenic rice plants, in which the expression of *RACK1* gene was inhibited by RNAi, elucidated the possible functions of *RACK1* in response to drought stress in rice. The tolerance to drought stress of the transgenic rice plants was higher as compared with the non-transgenic rice plants. The various intrinsic antioxidants systems in plants that include a variety of enzymatic scavengers like superoxide dismutases (SODs) constitute the first line of defense against highly toxic superoxide radicals accumulated during stress conditions. Da-Hong et al. (2009) studied the superoxide dismutase activity and showed the superoxide dismutase activity in transgenic rice plants which was significantly higher than those in non-transgenic rice plants. It was suggested that *RACK1* negatively regulated the redox system-related tolerance to drought stress in rice plants (Da-Hong et al. 2009).

Biotic stress resistance

Biotic stress factors caused by insects, nematodes, parasitic weeds, and viral, bacterial, and fungal diseases create several constraints to crop productivity, which influence the total agricultural production. However, maximum loss of plant productivity is because of viral diseases. Viruses are difficult to control because they use a variety of strategies to multiply and to spread both through and between plants. Direct transmission of viruses from parent to progeny and indirect transmission involving insect vectors as well as widespread cultivation in monocultures are the major factors responsible for viral diseases. An important and often defining characteristic of plant virus epidemiology determining the rate and extent of disease development in economically important plant populations including fruit crops (banana, papaya, orange, grape, apple), cash crops (cotton, sugarcane, cassava), and food crops (potato, rice) leading to severe losses stands second only to the fungi. Therefore, there is an urgent need to develop eco-friendly, non-chemical, and publicly acceptable control measures for crop protection. The various "pathogen-derived resistance" (PDR) approaches (in which the resistance to a determined pathogen could be obtained from its own genetic material) have been used to develop disease-resistant plants. These approaches consist of (1) PDR through the expression of viral proteins viz. viral coat proteins (CP) and replication-associated proteins (Reps) and (2) PDR without protein expression which includes gene silencing, PTGS by antisense, and hpRNA mechanisms (Shepherd et al. 2009).

A number of plant viruses have RNA genomes and replicates via dsRNA intermediates, thereby serving as potent inducers of RNAi during early replication and as silencing targets in later infections. Because RNAi is an antiviral mechanism, it is not surprising that many plant viruses encode suppressors of RNAi, hence, was first used by Waterhouse et al. (1998) to develop potato virus-resistant plants harboring vectors for the simultaneous expression of both the sense and antisense transcripts of the viral helper-component proteinase (*HCPro*) gene showing complete immunity to potato virus Y (PVY). Commercial variety of potato (cv Spunta) was transformed with dsRNA derived from the 3′ terminal part of the coat protein gene of PVY. The selected transgenic lines were highly resistant to three strains of PVY and infection of transgenic plants with potato virus X (PVX) simultaneously or prior to the challenge with PVY did not interfere with PVY resistance (Missiou et al. 2004).

The fruit plant papaya, known for its edible fruits and as a major source of papain enzyme, is drastically affected by viral attacks reducing the yield considerably. Coat protein mediated transgenic plants were raised, and although the coat protein gene was detected in all transgenic lines only a single line showed a high degree of rearrangement of the inserted coat protein expression cassette. In the resistant lines, the amount of the truncated coat protein mRNA was significantly decreased confirming that the RNA-mediated mechanism of coat protein-mediated resistance in papaya is

probably based on post-transcriptional gene silencing (Kertbundit et al. 2007).

Nicotiana benthamiana plants expressing the coat protein gene of sweet potato feathery mottle virus were established by Nazmul-Haque et al. (2007). From these plants, two silenced and two non-silenced lines were selected to investigate the manifestation of transitive RNA silencing in which RNA-dependent RNA polymerase (RdRp) amplifies the RNAi signal throughout the plant, silencing genes in other plant tissues and organs, by graft experiments. Non-silenced scions carrying the entire transgene were grafted onto silencing inducer rootstocks. When non-silenced scions were grafted onto 5′ silencing inducer rootstocks, RNA silencing was induced in the non-silenced scions and spread toward the 3′ region of the transgene mRNA. Similarly, when non-silenced scions were grafted onto 3′ silencing inducer rootstocks, RNA silencing was induced in the non-silenced scions, but was restricted to the 3′ region of the transgene and did not spread to the 5′ region. Results from crossing experiments, involving non-silenced and 3′ silencing inducer plants, confirmed the findings (Nazmul-Haque et al. 2007).

However, Schwind et al. (2009) produced transgenic tomato plants provided with hairpin RNA that exhibited resistance against potato spindle viroid. Hairpin RNA in excess amount is the key factor responsible for resistance. Genome mapping carried out by the authors revealed an unequal distribution of RNAi along the PSTVd genome suggesting the opportunity to engineer plants for viroid resistance. In another report, the engineered transgenic cassava plants showed resistance to African cassava mosaic virus (ACMV), by expressing dsRNAs. Transgenic cassava lines with high levels of AC1-homologous small RNAs have ACMV replication associated with protein coding sequence imparting Rep/AC1-homologous hairpin double strain immunity (Vanderschuren et al. 2009).

However, attempts to obtain RNAi-mediated resistance against fungal disease, caused by *Phytophthora parasitica* var. *nicotianae* by targeting glutathione *S*-transferase (GST) enzyme, resulted in significant increase in resistance of *Nicotiana tabacum* to infection following gene silencing for glutathione *S*-transferase-silenced plants compared with control plants. GSTs are present in eukaryotes and in prokaryotes, where they catalyze a variety of reactions and accept endogenous and xenobiotic substrates. Silencing of glutathione *S*-transferase was achieved by cloning a glutathione *S*-transferase gene in sense and anti-sense orientation to an RNAi vector for the purpose of gene silencing to arrest the spread of black shank disease (Hernández et al. 2009). It was noticed that few defense genes were up-regulated in glutathione *S*-transferase-silenced plants during the interaction with the pathogen.

The RNAi has been, in addition, used effectively to protect plants against phytopathogenic bacteria, mainly *Agrobacterium tumefaciens*, which causes crown gall disease, by targeting two genes viz. *iaaM* and *ipt* responsible for inducing resistance to crown gall disease (Dunoyer et al. 2006).

Root knot nematodes (*Meloidogyne* spp.) are plant parasites that exist in the soil and cause severe damage to crops and drain plants nutrients. Several parasitism proteins encoded by parasitism genes expressed in esophageal gland cells mediate infection and parasitism of plants by root knot nematodes (RKN). Bioengineered crops expressing dsRNA that target RKN parasitism genes to disrupt the parasitic process represent a viable and flexible means of developing novel durable RKN-resistant crops and could provide crops with unprecedented broad resistance to RKN (Huang et al. 2006). Tobacco plants expressing dsRNA hairpin structures targeting a root knot nematode were obtained by Fairbairn et al. (2007). The authors concluded that RNA interference triggered (HD-RNAi) silencing of parasite genes provides a novel disease resistance strategy with wide biotechnological applications. The potential of HD-RNAi is not restricted to parasitic nematodes but could be adapted to control other plant feeding pests. Cyst nematodes utilize parasitism proteins to successfully parasitize plants which are necessary for parasitism leading to the establishment of specialized feeding cells required by the nematode for their nourishment. Inhibition of cyst nematode genes should disrupt the parasitic cycle and render the host plant resistant. RNAi data were studied for cyst nematodes related to four parasitism genes in transgenic *Arabidopsis thaliana*, which is a host for the sugar beet cyst nematode *Heterodera schachtii*. Targeted nematode genes were specifically reduced in nematodes feeding on plants expressing corresponding RNAi constructs (Sindhu et al. 2009) and RNAi of all four nematode parasitism genes decreased the number of mature nematode females. This could be an effective way to check problem of cyst nematodes (Sindhu et al. 2009).

RNAi technology is also used as a genetic tool for engineering host resistance against parasitic weeds (Yoder et al. 2009). Parasitic flowering plants comprising 19 families and about 4,100 species are known which cause huge economic losses in a variety of crops including trees. Dependence of weeds on their host is a very complex mechanism involving numerous biochemical and physiological processes. The use of RNAi as a genetic tool for engineering host resistance against parasitic weeds has been elegantly reviewed by Yoder et al. (2009). The strategy is to transform a host plant with a plasmid encoding a double-stranded hairpin RNA (hpRNA) targeted against one or more vital parasite genes but having no phenotypic effect on the host; however, this approach has a striking effect on the parasites that have taken up the parasite-specific RNAi

from the host through the haustorium (Yoder et al. 2009). In transgenic maize, to produce weed resistance, mainly *Striga*, hairpin constructs were made that specifically targeted five *Striga asiatica* genes; two required for fatty acid biosynthesis, one for the synthesis of aromatic amino acids, and another for the biosynthesis of adenosine monophosphate and a fifth gene controlling vacuole morphogenesis. Transformed maize plants were subsequently challenged with germinating seeds of *S. asiatica* (L.) Kuntze (Yoder et al. 2009) to confirm the results.

In another weed, transgenic plants of *Triphysaria* that were expressing the beta-glucuronidase reporter gene (GUS) in their roots to determine whether transcript levels in the parasite would be affected by expression of an hpGUS in the host. The visual assay for GUS activity provided a higher-resolution analysis of gene activity in the parasite than could be achieved using the RNAi targets (de Framond et al. 2007).

Transgenic parasite roots expressing GUS were made using an *Agrobacterium rhizogenes*-mediated root transformation system and *Triphysaria* roots keep their capability to develop haustoria in response to host factors and to invade host roots (Boisson-Dernier et al. 2001; Tomilov et al. 2007). GUS expressing *Triphysaria* roots were allowed to parasitize lettuce roots and silencing activity of the hpGUS in different transgenic lettuce lines was constitutively expressed in GUS gene into lettuce leaves expressing hpGUS (Wroblewski et al. 2007). Lettuce lines that most strongly silenced GUS in the transient assays were used as hosts for *Triphysaria*. Three weeks after placing GUS-expressing *Triphysaria* roots onto hpGUS-expressing lettuce roots, haustoria and attached root tissues were stained for GUS activity. Reduction in active GUS protein was only observed in root tissues made after the RNAi signal has been translocated across the haustorium confirming hpRNA constructs engineered into host plants which can silence the expression of transgenes in root parasitic plants (Tomilov et al. 2008).

The noticeable accomplishment of RNAi approaches in controlling root knot nematodes provides additional utility against parasitic plants. It may be desirable to include multiple hpRNA molecules on a single transformation vector so that multiple parasite genes, preferably in distinct pathways, are targeted at the same time. Silencing of multiple loci is technically feasible by making chimeric constructions (Dafny-Yelin et al. 2007), and there are many potential parasite genes to target (Torres et al. 2005). Genomic resources are being developed for representative species in order to facilitate RNAi and other biotechnological approaches to parasitic weed management (Yoder et al. 2009). The existing database sequences should prove a valuable source for identifying vital parasite genes to target using RNAi. There are several cases of hpRNA constructs made against a target gene being active in silencing in one species, which could be a homolog in the second. Phytopathogenic viruses have also been used to silence host genes called virus induced gene silencing (VIGS), where a recombinant vector carrying a sequence of host gene infects the plant, and upon transcription this sequence from vector triggers the host gene silencing (Shao et al. 2008). One of the most striking examples is the VIGS silencing of the *N. benthamiana* PDS by a sequence homologous to the monocot *Lilium longiflorum* PDS gene. It seems likely that parasite-associated genes will be sufficiently conserved in Orobanchaceae (a family of parasitic plants) to allow the same hpRNA construction to be used against multiple species.

The multiple applications of RNAi discussed here for biotic stress tolerance have been summarized in Table 1. The technology of Bt transgenics has been well established in cotton, legumes, cereals, and transgenic Bt plants that have been planted in several countries for commercial production. Transgenic Bt crops producing insecticidal crystalline proteins from *Bacillus thuringiensis*, known as *Cry* toxins, have proved effective in controlling insect pests. However, the future of Bt crops is threatened by the evolution of insect resistance. Therefore, RNAi technology could be a substitute biotechnological approach having numerous advantages than Bt transgenics to control resistant insects without the use of chemical insecticides (Bravo and Soberôn 2008). The RNAi-mediated insect-resistant plants possess several characters over transgenic Bt crops as it is specific, dominant, sequence-based, eco-friendly, and minimize off-target effects which occurred when sequence homology allows novel small RNAs to degrade mRNA for genes that were not the intended silencing targets. There are several examples where RNAi may be successfully applied to develop biotic stress tolerant crops and are given in Table 2, and in the future, RNAi technology would be preferred over the present Bt transgenic plants.

Deletion of allergens

Food allergy is defined as a hypersensitive response to normally harmless food components which are generally proteins that are mediated mainly as a result of an immunoglobulin E (IgE) mechanism (Johansson et al. 2004). Although generally most food allergies cause relatively mild and minor symptoms, some food allergies can cause severe reactions and could even be life-threatening. There is no cure for food allergies. Strict avoidance of food allergens and early recognition and management of allergic reactions to food are important measures to prevent serious health consequences. There are eight types of foods that account for 90% of all food allergic reactions, namely peanut, tree nuts (walnut, Brazil

Table 1 Use of RNAi in biotic stress tolerance of plants

Type of biotic stress	Target organism	Region targeted	Impact	System used	Reference
Viral diseases	Potato virus Y (PVY)	HC-pro	Immunity	Potato	Waterhouse et al. 1998
	Mungbean yellow mosaic India virus (MYMIV)	Bidirectional promoter	Recovery from infection	*Vigna mungo* (black gram)	Pooggin et al. 2003
	African cassava mosaic virus (ACMV)	Replication-associated protein gene	Reduced virus accumulation	Tobacco protoplast	Vanitharani et al. 2003
	Pepper mild mottle virus (PMMoV)	Arbitrary sequence	Block in viral infectivity	Tobacco	Tenllado et al. 2003
	Alfalfa mosaic virus (AMV)	Arbitrary sequence	Recovery from infection	Tobacco	Tenllado et al. 2003
	Tobacco etch virus (TEV)	Arbitrary sequence	Arbitrary sequence symptoms appeared	Tobacco	Tenllado et al. 2003
	Potato virus Y (PVY)	Coat protein gene	Resistant	Potato	Missiou et al. 2004
	Potato spindle tuber viroid (PSTVd)	PSTVd-specific siRNA	Recovery from infection	Tomato	Sano and Matsuura 2004
	Tomato yellow leaf curl Sardinia virus	Replication-associated protein gene	Poor resistance	Tomato	Noris et al. 2004
	Beet necrotic yellow vein virus (BNYVV)	Coat protein	Tolerance	Tobacco	Andika et al. 2005
	Tobacco mosaic virus (TMV)	Replication-associated protein	Inhibition of TMV replication	Tobacco	Zhao et al. 2006
	Tomato yellow leaf curl virus (TYLC)	Intron-hpRNA construct (involving TYLC replication associated protein gene (C1) and castor bean catalase intron)	Resistant	Tomato	Fuentes et al. 2006
	Papaya ringspot virus type W (PRSV-W)	Coat protein gene	Resistant	*Cucumis melo* L. var. *cantalupensis* cv. Sun Lady	Krubphachaya et al. 2007
	Plum pox virus (PPV)	Coat protein gene	Resistant	*Nicotiana benthamiana*, *Prunus domestica*	Hily et al. 2007
	Cucumber green mottle mosaic virus (CGMMV)	Coat protein gene	Resistant	*Nicotiana benthamiana*	Kamachi et al. 2007
	Rice tungro bacilliform virus (RTBV)	dsRNA having similar	Resistant	Rice	Tyagi et al. 2008
	African cassava mosaic virus (ACMV)	Replication-associated protein	Immunity	Cassava	Vanderschuren et al. 2009
	Potato spindle tuber viroid (PSTVd)	hpRNA construct derived from PSTVd	Resistant	Tomato	Schwind et al. 2009
	Citrus tristeza virus (CTV)	Transgene-derived siRNAs	Resistant	Mexican lime	López et al. 2010
Fungal diseases	*Blumeria graminis* f. sp. *tritici*	Knockdown of MLO	Resistant	*Triticum aestivum*	Riechen 2007
	Phytophthora parasitica var. *nicotianae*	Glutathione *S*-transferase (GST)	Resistant	*Nicotiana tabacum*	Hernández et al. 2009
Bacterial diseases	*Agrobacterium tumefaciens*	*iaaM* and *ipt* genes	Resistant	*Arabidopsis thaliana*	Dunoyer et al. 2006
	Crown gall			*Lycopersicon esculentum*	
Insects	Cotton bollworm (*Helicoverpa armigera*)	P450 monooxygenase gene *CYP6AE14*	Sensitive to gossypol	Tobacco *Arabidopsis thaliana*	Mao et al. 2007
	Corn rootworm (*Diabrotica virgifera virgifera* LeConte)	V-type ATPase gene	Reduce feeding damage by corn rootworm	Maize	Baum et al. 2007

Table 1 (continued)

Type of biotic stress	Target organism	Region targeted	Impact	System used	Reference
Nematodes	Root knot nematodes (RKN)	HD-RNAi	Resistant	Tobacco	Fairbairn et al. 2007
Parasitic weeds	*Striga asiatica* L.	hpRNA	Resistant	Maize	Yoder et al. 2009
		Target genes required for fatty acids, aromatic amino acid, and AMP biosynthesis also gene controlling vacuole morphogenesis			

nut, cashew, etc.), soybean, wheat, milk, egg, fish, and shellfish (Zuidmeer et al. 2008; Sicherer and Sampson 2010). The unique mechanism of RNAi has been exploited to delete allergens from food items in an effective manner.

Soybean is a major legume edible plant but the allergy is dominated by protein Gly m Bd 30 K, also referred to as P34. Herman et al. (2003) used transgene-induced gene silencing to prevent the accumulation of immunodominant soybean allergen Gly m Bd 30 K protein in transgenic soybean seeds without showing any compositional, developmental, structural, or ultrastructural phenotypic differences when compared with control plants.

Gilissen et al. (2005) used RNAi technology to inhibit the expression of a prominent apple (*Malus domestica*) allergen Mal d 1, which displays IgE antibodies cross-reactivity to the birch pollen allergen Bet v 1. Transgenic apple plantlets, consisting of a construct coding for an intron-spliced hairpin RNA containing a Mal d 1-specific inverted repeat sequence separated by a Mal d 1-specific intron sequence, showed up to a 10-fold reduction in Mal d 1 leaf expression without affecting phenotypic characteristics as compared with the wild type (Gilissen et al. 2005). In addition, further work is necessary to study the expression of Mal D 1 in the fruit and followed by feeding studies to assess nutritional impact.

In another report, Le et al. (2006b) used an RNAi strategy to silence the two allergens Lyc e 1 (having two isoforms Lyc e 1.01 and Lyc e 1.02) which corresponded to a ubiquitous eukaryotic protein profilin and Lyc e 3, a non-specific lipid transfer protein (nsLTP) of tomato. The transgenic lines showed 10-fold reductions in Lyc e 1 accumulation in fruits, reduction in height and yield as well as delayed ripening as compared to wild plants (Le et al. 2006a). On the other hand, level of Lyc e 3 in transgenic fruits was decreased to less than 0.5% of that in wild-type fruits producing phenotypically normal plants. The skin prick tests of transgenic tomato fruit extracts revealed highly reduced allergenicity. These results are promising for the production of hypoallergenic tomatoes (Le et al. 2006b).

Peanut (*Arachis hypogaea* L.) allergy is one of the most life-threatening food allergies causing severe or fatal food-associated anaphylaxis and is one of the serious challenges facing the peanut and food industries. The RNAi technology was used to alleviate peanut allergy by silencing Ara h 2, the most immune dominant allergen causing gene over 85% allergic reactions and with over 52.5-fold more potency than Ara h 1. Transgenic peanuts were produced by infecting peanut hypocotyls explants with *A. tumefaciens* EHA 105 harboring the pDK28 construct. The allergenicity of transgenic peanut seeds was expressed as IgE binding capacity which was evaluated by ELISA using sera of patients allergic to peanut. The results showed a significant decrease in the IgE binding capacity of selected transgenic seeds without affecting plant morphology, growth rate, and reproduction as compared to the wild type. Crude peanut extract from the transgenic plants showed up to 25% reduction in Ara h 2 content, hence, demonstrating the feasibility of alleviating peanut allergy using RNAi technology (Dodo et al. 2008). There are several plants where RNAi technology could be applied successfully to develop plants free from allergic substances to ensure its use in human diet as explained in Table 2.

Development of male sterile plants

Hybrid production systems rely on an efficient and effective mechanism for inducing male sterility in one of the parental lines so as to ensure purity of the resultant hybrid seed. Engineered male sterility is an alternative method for developing hybrids in cases where natural male sterility is not available. Nawaz-ul-Rehman et al. (2007) investigated the potential for producing male sterile lines by the specific down-regulation of the anther-specific gene *TA29* of tobacco (*N. tabacum* cv. Samsun) by RNAi. *TA29* is expressed exclusively in anthers at the time of microspore development. About 10 out of 13 tobacco lines transformed with a hairpin RNAi construct containing *TA29* sequences were male sterile. Transgenic plants were phenotypically indistinguishable from non-transgenic plants. At the anthesis stage, pollen grains from transgenic, male sterile plants were aborted and lysed in comparison to the round and

Table 2 Future opportunities for improvements in crop plants by the use of RNAi technology

1. Alteration in plant architecture—height, number of leaves, tillers, branches, leaf angle, number of flowers, flower morphology, fruit size, root structure, surface area, length etc. to increase yield, easy plucking, efficient nutrient uptake in crop plants, ornamental and fruit plants.
2. Abiotic stress tolerance—development of crop plants tolerant to cold, heat, flooding, salinity, drought, oxidative stress, heavy metal tolerance, changing environmental condition will be necessary to feed ever-increasing population.
3. Biotic stress resistance—development of crop plants resistant to various diseases, pest, and viruses will reduce the off-target effects of harmful chemicals like herbicides, pesticides, and insecticides. Some are listed as follows.

Biotic stress type	Host plant	Target organism
A. Viral diseases	Banana	Banana bunchy top virus (BBTV)
	Banana	Banana bract mosaic virus (BBrMV)
	Cotton	Cotton leaf curl virus (CLCuV)
	Sugarcane	Sugarcane mosaic virus (SCMV)
	Pepper	Pepper mottle virus (PepMV)
B. Fungal disease	Banana	Black Sigatoka (*Mycosphaerella fijiensis*)
	Banana	Panama disease (*Fusarium oxyporum* f. sp. *cubense*)
	Potato	Potato blight (*Phytopthora infestans*)
C. Insect pest	Cotton	Cotton bollworm (*Helicoverpa armigera*)
	Potato	Colorado beetle (*Leptinotarsa decemlineata*)
	Tomato	Tomato fruitworm (*Heliothis armigera*)
	Brinjal	Fruit and shoot borer (*Leucinodes orbonalis*)

4. Nutritional improvement—synthesis and optimization of various key nutrients iron, carotenoids, flavonoids, antioxidants, vitamins, fatty acid, and amino acid composition in cereals, fruits, etc. to develop biofortified crops
5. Deletion of allergens/toxic substances—to develop plants free from allergens/toxic substances to ensure its use in human diet.

Crop plant	Allergens/toxic chemical to be removed
Brassica oleracea	Glucosinolates
Capsicum spp.	Capsaicin
Glycine max	Protease/amylase inhibitors
Solanum lycopersicum	Tomatine
Solanum tuberosum	Solanine
Phaseolus lunatus	Cyanogenic glucosides
Lathyrus sativus	Oxalyl-diaminopropionic acid
Prunus dulcis	Cyanogenic glucosides
Ricinus communis	Ricin

6. Prolongation of shelf-life—increase in shelf-life of fruits and vegetable to minimize senescence and deterioration of fruit quality and post-harvest spoilage during transportation and storage, e.g., mango, banana, strawberry, and tomato
7. Modulation of flower color and scent—flower color and scent are important horticultural traits as it contributes to the aesthetic and economic value. Future plant scientists and biotechnologists will dream to create novel plants including blue rose, blue anthurium, white *Petunia*, *Gerbera*, and *Torrenia* as well as scented rose, chrysanthemum, tulip, and orchids.
8. Enhancement of secondary metabolites—the RNAi will be used to facilitate synthesis and production of commercially valuable plant-derived drugs, fragrances, pigments, flavors, and volatile oils.
9. Seedless fruit development—seedless fruits are appreciated by the consumers for fresh consumption as well as processed fruit products. To create seedless fruits in guava, watermelon, and custard apple have a great potential.
10. Development of male sterile lines—development of male sterile line gives great value in breeding systems to ensure purity of the resultant hybrid seed. To develop male sterility is an alternative method for developing hybrids in cases where natural male sterility is not available.

fully developed pollen in non-transgenic plants. Male sterile transgenic plants set seed normally when cross-pollinated with pollen from non-transgenic plants, confirming no adverse effect on the female parts of the flower.

Nucleases play critical roles in nucleic acid metabolism in all organisms and are involved in a variety of basic cellular and genetic processes. Moritoh et al. (2005) cloned a new member of the RAD2/XPG nuclease family, OsGEN-L (OsGEN-like), from rice (*O. sativa* L.). The OsGEN-L possesses two domains, the N- and I-regions that are conserved in the RAD2/XPG nuclease family. The RAD2/XPG nuclease functions in nucleotide excision repair (NER). To elucidate the function of OsGEN-L, Moritoh et al (2005) generated rice OsGEN-L-RNAi

transgenic plants in which OsGEN-L expression was silenced. Most of the OsGEN-L-RNAi plants generally displayed low fertility and were male sterile. OsGEN-L-RNAi plants lacked mature pollen resulting from a defect in early microspore development. To study the subcelluar localization of OsGEN-L protein, a 35S:OsGEN-L-GFP fusion construct was made and transiently introduced into onion epidermal cells by particle bombardment. Further, the authors noticed an OsGEN-L-green fluorescent (GFP) fusion protein which was localized in the nucleus, and the OsGENL promoter was specifically active in the anthers. A recombinant OsGEN-L protein possessed flap endonuclease activity and both single-stranded and double-stranded DNA-binding activities indicating that OsGEN-L played an essential role in DNA metabolism required for early microspore development in rice (Moritoh et al. 2005).

Cytoplasmic male sterility (CMS) is one of the most ideal phenomena known in higher plants to describe the incompatibilities between mitochondrial–nuclear genomic interactions. Causes of CMS in plants have been studied for two decades, and mitochondrial chimeric genes have been predicted to induce CMS. In a previous study, Fujii et al. (2007a) performed microarray analysis and showed that 140 genes was aberrantly regulated in anthers of CW-type CMS of rice (*O. sativa* L.). In another study, Fujii and Toriyama (2008) investigated down-regulated genes in CW–CMS encoding a protein phosphatase 2C (PP2C). *DCW11* mRNA was preferentially expressed in anthers, with the highest expression in mature pollen. The N-terminal sequence, *DCW11* signal peptide-green fluorescent protein (GFP) fusion protein, was localized in mitochondria. Removal of *DCW11* in wild-type rice by RNAi caused a major loss of seed-set fertility, without visible defect in pollen development and this down-regulation of *DCW11* is correlated with CW–CMS. Mitochondrial retrograde signaling controls the up-regulation of alternative oxidase 1a (*AOX1a*), which is known to be regulated by in *DCW11* knockdown lines. Down-regulation of *DCW11* and up-regulation of *AOX1a* were also observed in two other types of rice CMS. These results indicated that *DCW11* could play a role as a mitochondrial signal transduction mediator in pollen germination.

Engineering of secondary metabolites

Plant secondary metabolites are sources of drugs, fragrances, pigments, food additives, and pesticides. It is estimated that 70–80% of the people worldwide rely mainly on traditional, largely herbal, medicines to meet their primary healthcare needs (Canter et al. 2005). Presence of precursors and conversion to the desirable end products for the synthesis of secondary metabolite is a major prerequisite controlled by an array of genes. However, this process in some cases is blocked by synthesis of undesirable compounds which could be inhibited by antisense or RNAi. On the other hand, suppressing genes that up-regulate the pathway or by enhancing catabolism would yield similar results (Gomez-Galera et al. 2007; Borgio 2009). The versatility of RNAi for controlling multigenes responsible for metabolite production and across a number of tissues and developmental steps has been well recognized as an effective strategy (Borgio 2009). Allen et al. (2004) was able to knockdown the activity of codeinone reductase genes through DNA-directed RNAi in transgenic opium poppy (*Papaver somniferum* L.), which resulted in accumulation of precursor (s)-reticuline. Reticuline is a key compound in the biosynthetic pathway for isoquinoline alkaloids in plants, which include morphine, codeine, and berberine. Similarly, Fujii et al. (2007b) used RNAi technology to suppress berberine bridge enzyme (*BBE*) activity in California poppy (*Eschscholzia californica*) cells. In these transgenic cells, end-products of isoquinoline alkaloid biosynthesis, such as sanguinarine, were considerably reduced and reticuline was accumulated at a maximum level. These cells also produced a methylated derivative of reticuline and laudanine, which could scarcely be detected in control cells. The over-expression and suppression of the gene encoding the morphinan pathway enzyme salutaridinol 7-*O*-acetyltransferase (salAT) in opium poppy affected the levels of alkaloid products that accumulated (Allen et al. 2008). Kempe et al. (2009) developed transgenic *P. somniferum* plants in which *salAT* transcript has been reduced using RNAi technology. RNA interference of *salAT* led to accumulation of the intermediate compounds, salutarydine and salutaridinol, in a ratio ranging from 2:1 to 56:1. The post-transcriptional gene silencing of dammarenediol synthase (DDS) by RNAi strongly suppressed the expression of DDS mRNA and resulted in reduced ginsenoside accumulation (85.4%) in transgenic *Panax ginseng* roots Han et al. (2006). These results indicated that expression of DDS played a vital role in the biosynthesis of ginsenosides in *P. ginseng*. Artemisinin is an effective anti-malarial drug isolated from *Artemisia annua* L. The artemisinin content of *A. annua* was increased by suppressing the expression of SQS (squalene synthase), a key enzyme of sterol pathway, by means of a hairpin-RNA-mediated RNAi technique. The artemisinin content of some transgenic plants was significantly increased, by 3.14-fold as compared to untransformed control plants (Zhang et al. 2009). Potato (*Solanum tuberosum* L.) tubers have recently emerged as bioreactors for the production of human therapeutic glycoproteins, increasing the yield of recombinant proteins, targeting the produced proteins to specific cellular compartments, and diminishing expensive protein purification steps. The potato patatins were eliminated almost completely via RNAi technology to develop potato tubers as a more efficient protein expression system (Kim et al. 2008).

Modulation of flower color and scent

Modification of flower color and scent is one of the most important traits in floriculture and is a subject of intense interest in conventional breeding and plant biotechnology as it has a great economic and aesthetic value. A change in flower color pattern always fetches an enormous value in the market. Flower color is an important horticultural trait and is mainly produced by the synthesis of flavonoid pigments and anthocyanins. Primarily produced to attract pollinators, flavonoids also protect the plant and its reproductive organs from UV damage, pests, and pathogens (Gronquist et al. 2001). The recent RNAi technology could induce more efficient and stronger gene silencing than the conventional breeding and genetic transformation for alteration in flower pigments (Waterhouse et al. 1998). RNAi has been applied to suppress some structural genes in anthocyanin biosynthesis, causing the inhibition of anthocyanin accumulation resulting in a change in flower color in transgenic plants. The flower color of the garden plant *Torenia hybrida* cv. Summerwave Blue, a commercially important garden plant, was successfully modulated by RNAi against a gene of chalcone synthase (*CHS*), a key enzyme for anthocyanin and flavonoid biosynthesis. By using each of the coding region and the 3′-untranslated region of the *CHS* mRNA as an RNAi target, exhaustive and gene-specific gene silencing were successfully induced, and the original blue flower color was modulated to white and pale colors (Fukusaki et al. 2004). In another study, Nishihara et al. (2005) applied RNAi for the effective suppression of the genes coding for chalcone isomerase (*CHI*) in tobacco (*N. tabacum*). The transgenic tobacco plants showed *CHI* suppression by RNAi had reduced pigmentation and change of flavonoid components in flower petals. The plants also accumulated high levels of chalcone in pollen, showing a yellow coloration. This suggested that *CHI* plays a major part in the cyclization reaction from chalcone to flavanone.

Osteospermum hybrida is a popular ornamental plant having rose to lilac flower colors that are mainly based on delphinidin-derived anthocyanins. The predominant synthesis of delphinidin derivatives is referred to as a strong endogenous flavonoid 3′,5′-hydroxylase (F3′5′H) activity. Furthermore, since dihydroflavonol 4-reductase (DFR) of *Osteospermum* does not convert dihydrokaempferol (DHK) to leucopelargonidin (LPg), synthesis of pelargonidin-based anthocyanins is naturally not realized. In order to redirect anthocyanin biosynthesis in *Osteospermum* towards pelargonidin derivatives, Seitz et al. (2007) introduced cDNAs coding for DFRs which efficiently convert DHK to LPg. But neither the expression of *Gerbera hybrida* DFR nor of *Fragaria*×*Ananassa* DFR—the latter is characterized by an unusual high substrate preference for DHK—altered anthocyanin composition in flowers of transgenic plants. However, chemical inhibition of F3′5′H activity in ray florets of DFR transgenic plants resulted in the accumulation of pelargonidin derivatives. Accordingly, retransformation of a transgenic plant expressing *Gerbera* DFR with a construct for RNAi-mediated suppression of F3′5′H activity resulted in double transgenic plants accumulating predominantly pelargonidin derivatives in flowers (Seitz et al. 2007). Recently, Nakatsuka et al. (2010) used chimeric RNAi technology to produce transgenic gentian (*Gentiana triflora*×*Gentiana scabra* interspecific cultivar ‘Albireo’) plants with down-regulated anthocyanin 5,3′-aromatic acyltransferase (5/3′AT) and flavonoid 3′,5′-hydroxylase (F3′5′H) activities, which are both essential enzymes for gentiodelphin biosynthesis. Two lines off less color-modified plants were obtained from 15 transgenic gentian plants. Clone no. 1 exhibited a lilac flower color and clone no. 15 exhibited pale-blue flowers. RNA gel blot analysis confirmed that both transgenic lines had markedly suppressed 5/3′AT transcripts, whereas clone no. 15 had fewer F3′5′H transcripts than clone no.1 and untransformed control plants. HPLC analysis of anthocyanin compositions showed that down-regulation of the 5/3′AT gene led to increased accumulation of non-acylated anthocyanins, as expected. These results demonstrated that genetic engineering to reduce the accumulation of polyacylated anthocyanins could cause modulations of flower color.

Koseki et al. (2005) analyzed the mRNA levels of six genes involved in anthocyanin biosynthesis in flower colors and only the level of chalcone synthase (*CHS*) mRNA was depressed in the unpigmented flower sectors. Presence of short interfering RNAs of CHS in the unpigmented sectors was detected. These results confirmed sequence specific degradation of *CHS* RNA involved in the formation of white sectors in ‘Red Star’ flowers.

The scented flowers also constitute a commodity with strong aesthetic, emotional, and economic values. The floral scent is a mixture of volatile compounds belonging to the monoterpenoids or phenylpropanoid/benzenoid classes of compounds, and plays an important role in reproduction of many plants by attracting pollinators (Pichersky and Dudareva 2007). Underwood et al. (2005) used RNAi technology in petunia to modify phenylpropanoid/benzenoid floral scent profiles by the elimination of some volatile compounds from the scent bouquet. The RNAi-mediated silencing of the *PhBSMT* (petunia benzylalcohol/phenylethanol benzoyltransferase) gene resulted in transgenic petunia plants that lack the major scent component methylbenzoate, with minimal changes in the emission of other volatiles. RNAi silencing of the *Petunia* phenylacetaldehyde synthase gene (*PhPAAS*) led to the complete elimination of the emission of phenylacetaldehyde and 2-phenylethanol (Kaminaga et al. 2006). The silencing of benzylalcohol/phenylethanol benzoyltransferase (*PhBPBT*) by

RNAi resulted in *Petunia* plants whose flowers did not emit benzylbenzoate or phenylethylbenzoate, although emission of all other volatiles remained unchanged while plants with fully suppressed *PhBPBT* expression also had clear morphological differences, such as bigger flowers and larger leaves (Orlova et al. 2006). The silencing of coniferyl alcohol acyltransferase (CFAT), the enzyme that catalyzes the formation of coniferyl acetate led to almost complete elimination of isoeugenol emission in *Petunia* flowers, with little effect on the emission of other phenylpropanoid/benzenoid volatiles (Koeduka et al. 2006; Dexter et al. 2007).

Nutritional improvement

Plants can provide most of the nutrients required in the human diet; however, the major staple crops are often deficient in some of these nutrients. Thus, malnutrition, with respect to micronutrients and vitamins affected >40% of the world's population (Tucker 2003). The use of conventional methods have been proved to be time consuming and have several constraints of limited genetic resources, loss of gene pools occurring during the domestication, and inherent breeding problems of crop plants. Natural traits having tissue-specific expression (such as in seeds and fruits) may be beneficial while it may be harmful when expressed in other plant tissues such as vegetative tissues (Tang et al. 2007). Advances in molecular biology, modern breeding, genetics, and biotechnology studies are being exploited to produce crops having enhanced key nutrients. Other nutritional targets include the modification of fatty acid composition and the enhancement of antioxidant levels, particularly carotenoids, such as lycopene, and flavonoids (Tucker 2003). The use of this genetic modification approach, however, has been met with considerable consumer resistance amid concerns for its safety; stringent controls, and therefore the provision of more public information, is necessary to assure and encourage public acceptance (Hirschi 2008). RNAi technology provides a publicly acceptable alternative for the development of biofortified foods. To change fatty acid composition of cottonseed oil, hpRNA-mediated gene silencing was adopted to down-regulate the seed expression of two key fatty acid desaturase genes. The resulting down-regulation of gene substantially elevated stearic acid level from 2% to 3% up to as high as 40% and silencing of the other gene enhanced oleic acid content, up to 77% compared with about 15% in seeds of untransformed plants (Liu et al. 2002). This seed-specific RNAi strategy has been successfully used to generate dominant high lysine corn by suppressing the expression of 22-kDa maize zein storage proteins (Segal et al. 2003).

RNAi technology was used to enhance β-carotene content in potato by silencing the β-carotene hydroxylase gene (*BCH*), which converts β-carotene to zeaxanthin. *A. tumefaciens*-mediated transformation was employed to introduce two RNAi constructs having the tuber-specific granule bound starch synthase (GBSS) promoter, and the other contained the strong constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter into potato lines. The transformants derived from the GBSS construct contained more β-carotene than CaMV 35S transformants. These results demonstrated that silencing *BCH* has the potential to increase the content of two health-promoting carotenoids, β-carotene and lutein, in potato which will provide a new agriculturally based tool for mitigating the incidence of vitamin A deficiency in populations (Eck et al. 2007). Nunes et al. (2006) demonstrated that silencing myo-inositol-1-phosphate (*GmMIPS1*) gene expression in soybean was an effective strategy to greatly reduce phytate content (up to 94.5%) and improved phosphorus availability in soybean seeds. This technology is a foundation for production of low phytate varieties, resulting in improved nutrient availability for animal feed and reduced environmental impact of livestock production. Nevertheless, experiments have been carried out to evaluate utilization of the *GmMIPS1* silencing strategy to inhibit seed development in fructiferous plants with the potential to develop seedless fruits. An enhanced synthesis of amylose type starch by RNAi reduction has been reported in four lines of wheat (Regina et al. 2006); similarly, Barley lines with low expression of SBE IIa or SBE IIb, and with the low expression of both isoforms were generated through RNA-mediated silencing technology (Regina et al. 2010).

Strawberry (*Fragaria*×*Ananassa*) fruit contains several anthocyanins that give the ripe fruits their attractive red color. The enzyme that catalyzes the formation of the first stable intermediate in the anthocyanin pathway is anthocyanidin-3-*O*-glucosyltransferase. In a report, a putative glycosyltransferase sequence (*FaGT1*) was cloned from a strawberry fruit cDNA library and the recombinant *FaGT1* transferred UDP-glucose to anthocyanidins and, to a lesser extent, flavonols, generating the respective 3-*O*-glucosides. Quantitative polymerase chain reaction revealed that transcripts of *FaGT1* were almost undetectable in green fruits, but gene expression increased dramatically in both turning and ripe red fruit, corresponding closely to the accumulation of anthocyanins during fruit ripening. The expression of *FaGT1* is fruit associated and negatively regulated by auxin. The in planta function of *FaGT1* revealed that significant down-regulation of *FaGT1* transcript levels corresponded to reduced concentrations of anthocyanin pigments in ripe strawberry fruits. In contrast, significant levels of epiafzelechin—formed by anthocyanidin reductase (ANR) from pelargonidin—were identified in *FaGT1*-silenced fruits, indicating competition of *FaGT1* and *FaANR* for the common anthocyanidin substrate. Thus, *FaGT1* represents an important branching-point enzyme

because it is channeling the flavonoid pathway to anthocyanins. These results demonstrated a method to redirect the anthocyanin biosynthesis into flavan-3-ol production to increase the levels of bioactive natural products or modify pigments in plant tissues (Griesser et al. 2008).

The levels of sinapate esters in transgenic *Brassica napus* were reduced by 76% in seeds of the T3 generation by inhibiting UDP-Glc:sinapateglucosyltransferase gene activity using RNAi (Hüsken et al. 2005). In another report, Wei et al. (2009) suppressed the expression of regulatory gene *DE-ETIOLATED1* (*DET1*) in *B. napus* (canola) which was expressed constitutively in a seed using a CaMV 35S promoter and the napin promoter, respectively. Constitutive silencing of *DET1* resulted in transgenic seeds with substantially elevated levels of lutein, β-carotene, and zeaxanthin relative to non-transgenic seeds. Levels of these carotenoids were also enhanced but to a lesser extent in seeds of transgenic plants with seed-specific silencing of *DET1*. The levels of 1,2-di-*O*-sinapoylglucose in seeds in both sets of transgenic plants were lower compared to non-transgenic seeds. The results revealed that *DET1* suppression in *B. napus* can increase the levels of carotenoids and reduce the levels of sinapate esters simultaneously in the seeds which are responsible for bitter taste, poor meal palatability, and unpleasant flavor to the meat and milk of animals fed on a *B. napus* seed meal diet, thus favorably changing their overall nutritional value.

Prolongation of fruit shelf-life

Fruit ripening has received considerable attention because of the dramatic changes in a wide range of metabolic processes that occur before and after this event, as well as due to its commercial importance. Fruits are an important source of supplementary diet. The quality of fruit is determined by a wide range of desirable characteristics such as nutritional value, flavor, processing qualities, and shelf-life. The massive losses accrue during transportation and post-harvest handling of the fruit which run into billions of dollars worldwide. Ethylene evokes several responses during ripening through a signaling cascade and thousands of genes participate which not only sets in ripening but also responsible for its spoilage (Bapat et al. 2010). Genetic engineering appears to be the most promising and cost-effective means to prevent these losses but was not acceptable by general people. Xiong et al. (2005) used RNAi technology to increase shelf-life in tomato. The dsRNA of tomato ACC oxidase expression unit was successfully introduced into tomato cultivar Hezuo 906 under the cauliflower mosaic virus 35S promoter by *A. tumefaciens*-mediated transformation. Plants which were produced had fruits having traces of ethylene and had a prolonged shelf-life of more than 120 days with similar levels of total soluble sugar, titratable acid, amino acids, and total soluble solids as the control plants. The RNA and protein analyses indicated that there was non-RNA interference, semi-RNA interference, and full-RNA interference of ACC oxidase in the population of transgenic tomato plants. Vrebalov et al. (2009), through RNAi repression, demonstrated that tomato *AGAMOUS-LIKE1* (*TAGL1*) played an important role in regulating both fleshy fruit expansion and the ripening process that together were necessary to promote seed dispersal of fleshy fruits.

Removal of toxic compounds

Both cultivated and wild plants are known to contain toxins of various types which often hinder the process of extraction of pure desirable products, and removal of unwanted compounds from plants is a cumbersome and a costly process which needs synergy of engineering and chemistry. RNAi would be a powerful technology to make the plants toxin-free. Ogita et al. (2003; 2004) used RNAi technology for simultaneous down-regulation of three distinct methylation steps of the caffeine biosynthetic pathway. In order to regulate caffeine biosynthesis in planta, they suppressed expression of *CaMXMT1* (7-*N*-methylxanthine methyltransferase or theobromine synthase) by the double-stranded RNAi method. Specific sequences in the 3′ untranslated region (UTR) of *CaMXMT1* messenger RNA were selected for construction of RNAi short and long fragments. The RNAi transgenic lines of embryogenic tissues derived from *Coffea arabica* and transgenic plantlets of *Coffea canephora* demonstrated a clear reduction in transcripts for *CaMXMT1* in comparison with the control plant. The caffeine content of transgenic plants was reduced by up to 70%, indicating that it is possible to produce decaffeinated coffee beans.

Cassava is a major staple food in tropical countries but contains unnecessary glucosides. The cassava plant gives the highest yield of food energy per cultivated area per day among crop plants and plays a particularly important role in farming in developing countries. It also offers flexibility to resource-poor farmers because it serves as either subsistence or a cash crop. Jørgensen et al. (2005) used RNAi to prevent production of the cytochrome P450 enzyme that makes the first committed step in the biosynthesis of linamarin and lotaustralin, and generated transgenic cassava (*Manihot esculenta*) plants with elimination of cyanogenic glucosides in the leaves (<1% of non-transgenic amounts) and a 92% reduction of cyanogenic glucoside amounts in tubers.

Cotton is a major cash crop produce fibers and oil. The oil is mainly used in cooking and in other industrial processes. Sunilkumar et al. (2006) reduced the toxic terpenoid gossypol in cotton seeds and cotton oil by engineering small RNAs for the cadinene synthase gene

in the gossypol biosynthesis pathway. A seed-specific promoter ensured that the gene was silenced in cotton seed, while allowing the leaves to synthesize normal terpenoid levels for protection against insects.

Cadmium (Cd), one of the most toxic heavy metals, causes several health problems even at trace levels. Li et al. (2007) carried out studies to reduce the accumulation of Cd in rice (*O. sativa* L. ssp. *japonica*) seeds by inhibiting the expression of phytochelatin synthase (PCS) gene *OsPCS1* by RNAi. A hairpin construct of a *PCS* fragment was designed in the pRNAi-*OsPCS1* under the control of ZMM1, a seed-specific promoter from maize. The construct was introduced into rice through *A. tumefaciens*. The RNAi rice plantlets were selected and cultivated in pots exposed to 10 mg/kg Cd. The transcriptional level of *OsPCS1* declined in seeds of some RNAi rice compared to the wild type. As a result, Cd accumulation was reduced by about half in the seeds of RNAi rice. As expected, no apparent difference of growth appeared between RNAi and wild-type plants. This is a noteworthy result which suggests that this new approach can be used to control heavy metal accumulation in crops.

A number of compounds including tobacco-specific nitrosamines (TSNAs) present in tobacco (*N. tabacum* L.) products have been reported to contribute to adverse health effects, including cancer. TSNAs are a class of compounds generated through the nitrosation of pyridine alkaloids during the curing and processing of tobacco. *N*′-nitrosonornicotine (NNN), a carcinogenic compound, is formed via the nitrosation of nornicotine, a secondary alkaloid produced through enzymatic *N*-demethylation of nicotine. Nornicotine is produced via the oxidative *N*-demethylation of nicotine by a nicotine *N*-demethylase enzyme during senescence and curing. Lewis et al. (2008) developed transgenic lines of burley tobacco (line DH98-325-5) carrying an RNAi construct designed to inhibit the expression of nicotine demethylase gene. The transgenic lines exhibited a 6-fold decrease in nornicotine content relative to untransformed controls. Analysis of cured leaves revealed a commensurate decrease in NNN and total TSNAs. The inhibition of nicotine demethylase activity is an effective means of decreasing significantly the level of a key defined animal carcinogen present in tobacco products. The result demonstrated the utility of RNAi technology for reducing the level of an undesirable metabolite to a value that has not yet been achieved using conventional methods. The rice mutant line LGC-1 (Low Glutelin Content-1) was the first commercially useful cultivar produced by RNAi (Kusaba et al. 2003). This dominant mutation produces hpRNA from an inverted repeat for glutelin, the gene for the major storage protein glutelin, leading to a lower glutelin content in the rice through RNAi. The gluten is a complex mixture of components with the monomeric gliadins accounting for about 50% of the gluten proteins present in wheat grain, which is largely responsible for the functional properties of dough. Gliadins contribute mainly to the extensibility and viscosity of gluten and dough, with the polymeric glutenins being responsible for elasticity. Gliadins are also associated with the development of celiac disease, a food-sensitive enteropathy caused by the ingestion of gluten proteins. Gil-Humanes et al. (2008) used RNAi technology to silence the expression of specific γ-gliadins by RNA interference and to demonstrate the feasibility of systematically silencing specific groups of gluten proteins. Two lines of bread wheat were transformed by particle bombardment. Seven transgenic lines were obtained and all of them showed reduced levels of γ-gliadins without affecting fertility, grain morphology, and seed weight as comparable to the control lines. The proportion of γ-gliadins was reduced by about 55–80% in the bread wheat cultivar 'Bobwhite' BW208 lines and by about 33–43% in the BW2003 lines (Gil-Humanes et al. 2008).

Seedless fruit development

Absence of seeds is a character appreciated by consumers both in fruits for fresh consumption (e.g., grape, citrus, and banana) as well as in conserved or processed fruit products (e.g., frozen eggplant, tomato sauce) and can contribute to increase the quality of the fruits when seeds are hard or have a bad taste. RNAi technology is used to suppress the function of *ARF7* in tomato. The transgenic plants resulted in the production of seedless fruits (De Jong et al. 2009). Recently, a new gene family involved in fruit set regulation was identified. The two members of the *AUCSIA* family coding for 53-amino-acid-long peptides are expressed in the ovary and are drastically down-regulated after pollination. The *AUCSIA* genes were functionally suppressed in tomato by RNAi (Molesini et al. 2009). *AUCSIA*-silenced plants generally displayed facultative parthenocarpy; these plants produced seedless fruits after flower emasculation. Obligatory parthenocarpy was observed in one *AUCSIA*-silenced line; in this line, the fruits were always seedless. Seedless fruits have been obtained by down-regulating chalcone synthase (*CHS*), the first gene in the flavonoid biosynthetic pathway (Schijlen et al. 2007). Interestingly, loss of CHS activity in *A. thaliana* caused an increase in polar auxin transport. It is possible that in *CHS*-silenced tomato, parthenocarpy results from an altered distribution of auxin caused by the reduced level of flavonoids.

Conclusion

Modern progress in molecular biology has generated elevated prospects for the potential role of RNA-mediated

trait for plant improvement and RNAi has become the technology of preference for scientists investigating gene function and manipulating plants to create novel characteristics. If thoughtfully used, this know-how may go an extensive way to narrow the gap through production of disease-, insect-, and virus-resistant, nutritionally rich, and toxic-free crops. Mainly, RNAi research has been carried out in model plants and has to be extended to the entire economically important crop for desirable traits. Facts on the constancy of traits achieved through RNAi are not obtainable but mutants that impersonate the RNAi effect have been shown to be steady for 20 generations (Kusaba et al. 2003). To facilitate silence gene expression, time-specific and inducible promoters active in the target tissues which could, when required, minimize "off-target" effects. Conventional transgenic technologies generally need the expression of whole genes, which are in contrast to comparatively small size of the RNAi transgene required for silencing, permitting multiple genes to be targeted in a single construct. For changing stages in a particular metabolic pathway or resisting multiple pathogen attack, this would assist to lessen the amount of manipulation and time required to accomplish the desired traits.

Regulatory agencies and risk analysts have to become familiar with the science of RNAi and its relevance to plant biotechnology. Biotechnologists need to raise precise problems concerning potential hazards and exposures to ensure that applicable data are collected and characterize uncertainty in menace assessments (Auer and Frederick 2009). Logical questions will require to be answered about off-target effects, non-target effects, and the impact of genetic mutations and polymorphisms (Auer and Frederick 2009). For example, silencing of unwanted or pathogen genes merely in the root stock of grafted crops, and using the silencing signal to influence the scion would avoid a number of the problems associated with gaining the public's acceptance of the use of the established transgenic technologies (Mansoor et al. 2006). It is necessary to acquire novel diagnostic instruments for the detection and quantification of small RNAs for an assortment of purposes, mainly crop identity preservation, monitoring, and segregation. Low detection limit and a high degree of specificity for each RNAi crop, while being relatively low cost, practical under field conditions, and operable by individuals with diverse backgrounds and training are the basic requirements (Auer and Frederick 2009).

The RNAi mechanism is intricate having high specificity and sensitivity posing difficulties during operating procedures and theoretical developments. In future opportunities, RNAi may even hold guarantee for development of gene-specific therapeutics or a complete understanding of genomics. With the methodical research in RNAi mechanisms and understanding the entire development of RNAi technology, it would be feasible to create a new biological science offering massive economic and social spin-offs.

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