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REVIEW

RNA interference: concept to reality in crop improvement

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Abstract The phenomenon of RNA interference (RNAi) is involved in sequence-specific gene regulation driven by the introduction of dsRNA resulting in inhibition of translation or transcriptional repression. Since the discovery of RNAi and its regulatory potentials, it has become evident that RNAi has immense potential in opening a new vista for crop improvement. RNAi technology is precise, efficient, stable and better than antisense technology. It has been employed successfully to alter the gene expression in plants for better quality traits. The impact of RNAi to improve the crop plants has proved to be a novel approach in combating the biotic and abiotic stresses and the nutritional improvement in terms of bio-fortification and bio-elimination. It has been employed successfully to bring about modifications of several desired traits in different plants. These modifications include nutritional improvements, reduced content of food allergens and toxic compounds, enhanced defence against biotic and abiotic stresses, alteration in morphology, crafting male sterility, enhanced secondary metabolite synthesis and seedless plant varieties. However, crop plants developed by RNAi strategy may create biosafety risks. So, there is a need for risk assessment of GM crops in order to make RNAi a better tool to develop crops with biosafety measures. This article is an attempt to review the RNAi, its biochemistry, and the achievements attributed to the application of RNAi in crop improvement.

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Introduction

RNAi is an evolutionarily conserved defence mechanism occurring naturally against double-stranded RNA (dsRNA) that can target cellular and viral mRNAs. In this biological process small RNA interferes with the translation of target mRNA transcript eventually suppressing the gene expression. The small non-coding RNAs are the cleavage product of dsRNA called microRNA (miRNA) and small interfering RNA (siRNA). The cleavage is carried out by a ribonuclease called DICER or Dicer-like enzyme (Pare and Hobman 2007). The small non-coding RNAs in association with RNA-induced silencing complex (RISC) (Redfern et al. 2013; Wilson and Doudna 2013), Argonaute (AGO) (Riley et al. 2012; Ender and Meister 2010) and other effector proteins lead to the phenomenon called RNAi illustrated in Fig. 1. The discovery of this phenomenon has transformed it into a powerful tool of genetic engineering and functional genomics.

The improvement of crop plants by alteration of traits using traditional plant breeding programme is time consuming and labour intensive. Since last two decades the researchers are switching towards biotechnological approaches for crop improvement. The manipulations in gene expression for quality traits in crop can now easily be achieved by RNAi. It can be employed by identifying the target gene(s) developing vectors as an RNAi construct, transforming plant and finally screening and evaluating the traits (Table 1). The abbreviation of RNAi-relevant terms used in this review are summarized in Table 2. The

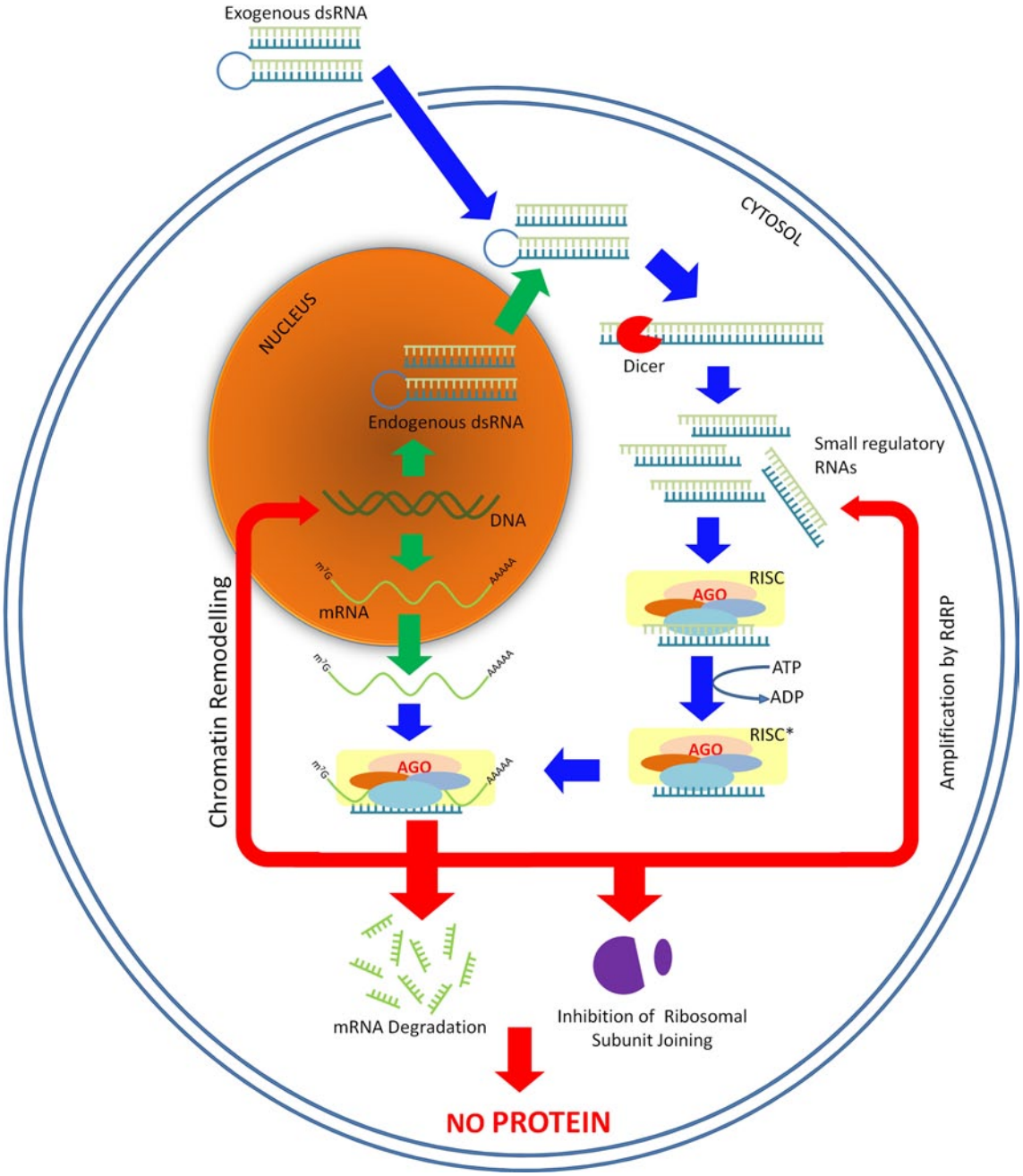

Fig. 1 The schematic representations of pathways involved in biogenesis of small regulatory RNAs (siRNAs, miRNAs) and their role in mechanisms for RNA interference. *Green arrows* denote natural pathway of molecular biology. *Blue arrows* denote the pathway involved in biogenesis of activated small regulatory RNAs from double stranded RNA (dsRNA). *Red arrows* denote the RNA interference-mediated pathways leading to gene silencing. *RISC* RNA induced silencing complex, *RISC** activated RNA induced silencing complex, *AGO* argonaute proteins, *RdRP* RNA-dependent RNA polymerase

useful links as tabulated in Table 3 may assist in performing genetic engineering with RNAi. The RNAi construct is designed to express self-complementary sequence homologous to the target gene in form of hairpin RNA (hpRNA). The insertion of a spacer sequence (usually an intron) between the two complementary sequences, expressing intron-hairpin RNA (ihpRNA), enhances the efficiency (Wesley et al. 2001). The tailoring of construct for various strengths and patterns of gene silencing is of most important for genetic engineering (Fig. 2). For specific plants and applications, the use of appropriate vector, promoter, marker, and transformation method are needed for enhanced efficiency of RNAi. The potential of RNAi as the most efficient tool for genetic engineering to

Table 1 A roadway to perform genetic engineering with RNA interference

STEP I	Identification of target gene and its related pathway • Genome sequencing • Applying bioinformatic tools • Analysis of transcriptome, proteome and metabolome
STEP II	Vector development to prepare RNAi constructs and screening for RNAi constructs • Selection of suitable vector • Selection of suitable promoter • Screening by selectable markers
STEP III	Transforming and screening transgenic plant • Delivery of RNAi • Tissue culture of transgenic line(s) • Screening and selection of transformed plants
STEP IV	Evaluation of transgenic lines for improved quality • Morphological evaluation • Transcriptome evaluation • Biochemical evaluation

knockdown the gene expression is summarized as its benefits in Table 4.

From the beginning of twenty-first century, role played by small non-coding RNAs in regulatory processes of post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) continues to be investigated one after another. The investigations revealed the outcome of gene silencing leading to the understanding of mechanisms involved. Till date several classes of small non-coding regulatory RNAs have been discovered in different model organisms. These include miRNA, siRNA, piRNA (PIWI-interacting RNA), qiRNA (QDE-2-interacting RNA), svRNA (small vault RNA), etc., having different biochemical approaches for their biogenesis (Aalto and Pasquinelli 2012).

In brief, the process for the biogenesis of miRNA and siRNA initially differs to form their respective dsRNA precursors. Later on, both siRNAs and miRNAs are produced by the cleavage of dsRNA precursors by Dicer or Dicer-like enzyme, a member of the RNase III family of dsRNA-specific endonucleases (Bernstein et al. 2001; Hutvagner et al. 2001). Finally, the small non-coding RNAs (miRNAs and siRNAs) in association with RNA-induced silencing complex (RISC), Argonaute (AGO) and other effector proteins lead to gene silencing.

MicroRNAs (miRNAs)

The miRNA was first described as regulator of the juvenile-to-adult transition in *Caenorhabditis elegans* (Lee et al. 1993; Reinhart et al. 2000). In past few years, it was found to regulate several developmental transitions in plants also (Wu and Poethig 2006; Wu et al. 2009) like *Arabidopsis thaliana* (Aukerman and Sakai 2003; Sieber et al. 2007), *Zea mays* (Chuck et al. 2007a, b), *Antirrhinum majus* and *Petunia hybrida* (Cartolano et al. 2007).

The miRNAs are endogenous ~23 nt RNAs transcribed by RNA Polymerase II (Lee et al. 2004), emerging as the most abundant and important class of small regulatory RNAs that mediate important gene-regulatory events by pairing to the mRNAs of protein-coding genes to direct their repression. Till date miRNAs are reported in plants, animals, unicellular algae and even viruses. In plants, the processing of both pri-miRNA to pre-miRNA and pre-miRNA to mature miRNA involves a homolog of dicer, DCL1.

After generation of mature miRNAs, the miRNA-induced silencing complex (miRISC) having Argonaute and other effector proteins, is recruited. The miRISC can cause miRNA-mediated gene silencing by target mRNAs through antisense base-pairing with specific miRNAs. The silencing mechanisms can differ depending upon cellular condition, cell type, developmental stage, target site, etc.

Huntzinger and Izaurralde (2011) proposed that the gene expression can be down-regulated by (1) the formation of miRNA complex which inhibits translational initiation or ribosome subunit joining, induce premature degradation of the nascent polypeptide chain and increase ribosome drop off; or (2) inducing deadenylation and destabilization of target mRNA.

Short-interfering RNAs (siRNAs)

Gene silencing by RNAi can be initiated by long dsRNA or short-hairpin RNA (shRNA) precursors, which are homologous in sequence to the gene to be silenced (Fire 1999; Tuschl 2001). The entry of long dsRNA such as an introduced transgene, a rogue genetic element (like transposable elements or repetitive elements) or a viral intruder inside the cytosol triggers the RNAi pathway of cells by recruiting the enzyme dicer (Bernstein et al. 2001). The dicer cleaves the dsRNA into short, 21–25 bp fragments known as siRNA (Hamilton and Baulcombe 1999).

The siRNAs are short, 5′-phosphorylated dsRNAs with two nucleotide overhangs at the 3′ end, generated by dicer from longer dsRNAs (Bernstein et al. 2001; Elbashir et al. 2001). The siRNA-induced silencing complex (siRISC) is recruited to distinguish between the two siRNA strands as either sense or antisense and resulting in the degradation of sense strands (with exactly the same sequence as the target gene). The siRISC is then incorporated into the antisense strand of siRNA with the target messenger RNAs (mRNA) in a sequence-specific manner. The target mRNA is cleaved by RISC having Argonaute (AGO) and other effector proteins, inhibiting the process of translation. The activated RISC can repeatedly participate in mRNA degradation and

Table 2 Abbreviations

RISC	RNA-induced silencing complex
AGO	Argonaute
ihpRNA	Intron-hairpin RNA
PTGS	Post-transcriptional gene silencing
piRNA	PIWI-interacting RNA
qiRNA	QDE-2-interacting RNA
svRNA	Small vault RNA
CMT3	Cytosine methyltransferase
ARF	Auxin response factor
GA	Gibberellic acid
β-Hex	β-D-*N*-Acetylhexosaminidase
Msh1	MutS HOMOLOG 1
GWD	Glucan, water dikinase
SEX4	Phosphoglucan phosphatase
LGC-1	Low glutenin content 1
SPS	Sucrose-phosphate synthase
Suc6P	Sucrose-6-phosphate
SPP	Sucrose phosphatase
VI	Vacuolar invertase
2-PRENCSO	*S*-2-propenyl-L-cysteine sulfoxide
1-PRENCSO	*trans*-*S*-1-Propenyl-L-cysteine sulfoxide
BOAA	Beta-*N*-oxalyl-ami-noalanine-L-alanine
ULGCS	Ultra-low gossypol cottonseed
TSNA	Tobacco-specific nitrosamine
NNN	*N*'-Nitrosonornicotine
BoHc	45 kDa C-terminal half of the heavy chain of botulinum type A neurotoxin
LDLRAP1	Low-density lipoprotein receptor adapter protein 1
CETS	CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING
PEBP	Phosphatidylethanolamine binding protein
FT	FLOWERING LOCUS T
TFL1	TERMINAL FLOWER I
Cg1	Corngrass1
CHI	Chalcone isomerase
TP	Tyrosine phosphatase
MSP	Mitochondrial stress-70 protein precursor
PDR	Pathogen-derived resistance
CP	Coat protein
BNYVV	Beet Necrotic Yellow Vein Virus
PVY	Potato Virus Y
PRSV-W	Papaya Ring Spot Virus type W
PPV	Plum Pox virus
CGMMV	Cucumber Green Mottle Mosaic Virus
TSV	Tobacco Streak Virus
ACMV	African Cassava Mosaic Virus
CBSD	Cassava Brown Streak Disease
CBSV	Cassava Brown Streak Virus
CBSUV	Cassava Brown Streak Uganda Virus
MYMIV	Mungbean Yellow Mosaic India Virus
TYLC	Tomato Yellow Leaf Curl Virus
RTBV	Rice Tungro Bacilliform Virus
CTV	Citrus Tristeza Virus

Table 2 continued

PSTVd	Potato Spindle Tuber Viroid
ABA	Abscisic acid
FTA or FTB	Farnesyl transferase genes
C4H	Cinnamate 4-hydroxylase
C3H	Coumarate 3-hydroxylase
HCT	Hydroxycinnamoyl CoA-shikimate/quinate transferase
CCoAOMT	Caffeoyl-CoA-3-*O*-methyltransferase
CCR	Cinnamoyl CoA reductase
F5H	Ferulate 5-hydroxylase
COMT	Caffeic acid-3-*O*-methyltransferase
CAD	Cinnamyl alcohol dehydrogenase
PPO	Polyphenol oxidase
BBrMV	Banana Bract Mosaic Virus

Table 3 Useful links that assist to perform genetic engineering with RNAi

a	Resources on RNAi
	http://www.ambion.com/techlib/resources/RNAi/
	http://srna-tools.cmp.uea.ac.uk/plant/
b	Computation model to predict gene function
	http://www.sciencedaily.com/releases/2010/01/100131142436.html
c	Target finder
	http://www.ambion.com/techlib/misc/siRNA_finder.html
	http://bioinfo3.noble.org/psRNATarget/
d	For careful selection of an insert gene sequence
	http://bioinfo2.noble.org/RNAiScan/RNAiScan.html
e	RNAi design tool
	http://www.ccts.uky.edu/Research/microRNA/miRNAResearchHome.aspx
	https://rnaidesigner.invitrogen.com/sirna/
	http://biotools.idtdna.com/rnai/
	http://wmd.weigelworld.org/
	http://www.protocol-online.org/prot/Molecular_Biology/RNA/RNA_Interference/siRNA_Design_Rules/
f	siRNA selection
	http://hydra1.wistar.upenn.edu/Projects/siRNA/siRNAindex.htm
	http://jura.wi.mit.edu/siRNAext/register.php
g	Find restriction sites in given gene sequence, NEB cutter V2.0 (New England Biolabs)
	http://tools.neb.com/NEBcutter2/
h	miRNA database
	http://www.mirbase.org/
	http://bioinformatics.cau.edu.cn/PMRD/
	http://www.misolrna.org./
	http://ted.bti.cornell.edu/cgibin/TFGD/sRNA/home.cgi
i	*M. truncatula* RNAi database
	https://mtrnai.msi.umn.edu/
j	Arabidopsis Genomic RNAi Knock-out Line Analysis (AGRIKOLA) project
	http://www.agrikola.org/index.php?o=/agrikola/html/index
k	Probabilistic functional gene network of *Arabidopsis thaliana*
	http://www.functionalnet.org/aranet/

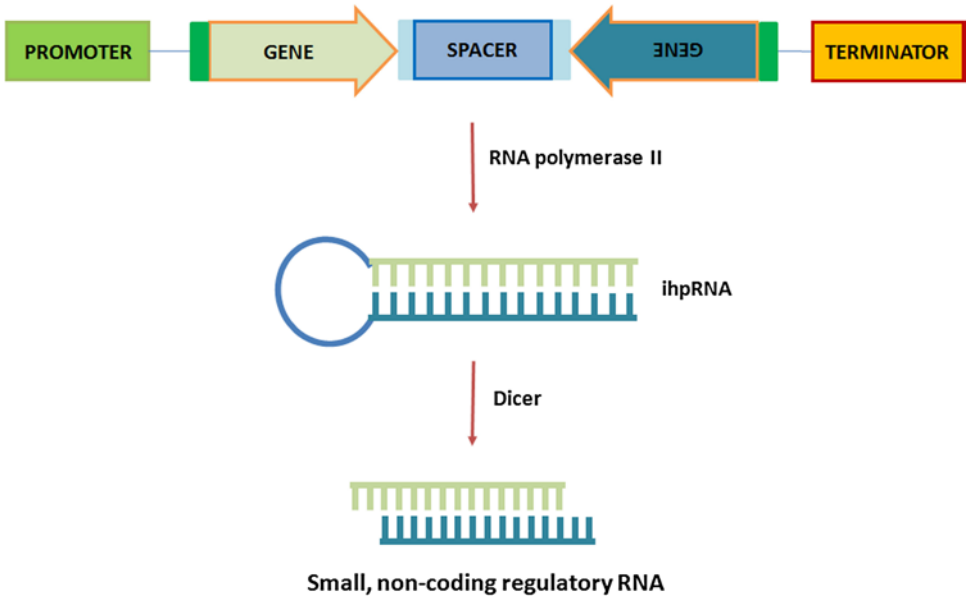

Fig. 2 Tailoring of vectors for more efficient, precise and stable RNA interference. In transformed plants, the expression of gene (in sense and antisense orientations) along with the spacer sequence forms ihpRNA. Initially, ligase-based vectors such as pHANNIBAL and pKANNIBAL were used for RNAi constructs. Now, the vectors used for RNAi construct are based upon Gateway Vectors like pHELLSGATE, pSTARGATE and pWATERGATE. In many RNAi constructs constitutive promoters like 35s CaMV, plant pathogen promoters, Rice actin promoter, Plant ubiquitin promoter (Ubi), Maize alcohol dehydrogenase 1 promoter (Adh-1) can efficiently drive foreign gene expression in plant cells

Table 4 Benefits of RNAi for gene silencing in plants

a	Precise	No off-target effects reported. But may affect the feedback loops
b	Efficient	CSIRO Plant Industry has reported that 70–100 % of transformed plants show the silencing phenotype
c	User friendly	By employing several user-friendly tools available to perform RNAi in plants
d	High-throughput	High-throughput vectors are designed to make an hpRNAi construct (Wesley et al. 2001, Xu et al. 2010, Yan et al. 2012)
e	Stable	hpRNAi has been shown to be stably inherited over several generations (Kusaba et al. 2003)
f	Flexible	Individual or multiple genes can be silenced with single construct. Allen et al. (2004) have shown effective silencing of two genes from one hpRNAi construct
g	Better than antisense	The highest silencing obtained with an antisense construct was only as good as the least silenced plant with hpRNAi (Wesley et al. 2001). In comparing the ability of sense, antisense, dsRNA at generating RNA-mediated virus resistance via PTGS in tobacco and silencing of an endogenous GUS reporter gene in rice; Waterhouse et al. 1998, has shown that in both cases duplex RNA was more effective than either sense or antisense RNA at silencing the target gene

inhibiting protein synthesis results into post-transcriptional gene silencing (PTGS).

Not only this, siRNAs also have a role as co-transcriptional silencers of gene expression by chromatin regulation (Burkhart et al. 2011; Fagegaltier et al. 2009). Ossowski et al. (2008) reported that siRNA recruits several DNA- and histone-modifying proteins including the cytosine methyltransferase, CHROMOMETHYLASE3 (CMT3), which together mediate the formation of a silent chromatin state with minimal transcriptional activity; leading to transcriptional gene silencing (TGS).

Applications in crop improvement

The demand for plants increases with the rapid increase in population and at the same time population is facing the problems of food security, malnutrition, and famine (Godfray et al. 2010). To overcome these problems, the interplay of genetic engineering with plant physiology, proteomics and genomics will be needed (Mittler and Blumwald 2010; Tester and Langridge 2010). This has been effectively demonstrated in terms of high-yielding varieties with improved traits (Sharma et al. 2002). The RNAi approach and its

contribution in achieving desired traits by manipulating the genetic expression have proven its potential in crop improvement.

Seedless fruit development

It is well known that the phytohormone plays a key role in regulation of transition between flowering, fertilization and fruiting. Parthenocarpy is potentially useful for producing vegetables and fruits when pollination or fertilization is affected due to extreme temperatures, as in winter (Tomes 1997) or, more generally, to ensure yield stability in case of unfavourable pollination conditions. Recent studies have shown that seedlessness can increase the texture and shelf life of fruits, for instance in the case of watermelon and eggplant (Pandolfini 2009). In watermelon, it was observed that seeds are the origin of fruit deterioration. Thus, replacing seeds and seed cavities with edible fruit tissue is desirable (Varoquaux et al. 2000) and can be of great value for consumers, the processing industry and breeding companies. In addition, it has been shown that seed development in fruits restricts the yield in cucumber (Tiedjens 1928; Denna 1973) and tomato (Falavigna and Soressi 1987).

Two members of ARF family, *ARF8* of *A. thaliana* and *ARF7* of tomato show high level of expression in non-pollinated flowers and are down-regulated after pollination. De Jong et al. (2009) indicated that *SlARF7* acts as a modifier of both auxin and gibberellin responses during tomato fruit set and development. Normally, *SlARF7* transcript levels are reduced after pollination and fertilization (Vriezen et al. 2008; De Jong et al. 2009). Reduction of *SlARF7* transcript levels by an RNAi approach may release the repression of the auxin and GA signalling pathways that are imposed by *SlARF7* independently of pollination and fertilization, resulting in the partial activation of these pathways and thus in parthenocarpic fruit growth in tomato (*Solanum lycopersicum*). Hence, the fertilization-dependent step of the auxin signalling transduction pathway may be bypassed, which might be necessary to initiate cell division activity and stimulate GA biosynthesis.

Enhanced shelf life

Fruits and vegetables are more prone to spoilage than cereals due to their nature and composition, and this spoilage results in inedible waste. In spite of being one of the largest producer of fruits and vegetables, India loses about 30 % of total fruits and vegetables produced due to spoilage (Agricultural Research Data Book 2004). So, there is a necessity for increase in shelf life of vegetables and fruits as another essential agronomic trait which may minimize the deterioration and spoilage of vegetables and fruits, thus minimizing the horticultural loss.

The shelf life in tomato has been increased by silencing of genes associated with either ethylene production or ripening. Xiong et al. (2005) used RNAi technology to increase shelf life in tomato. They introduced a unit of dsRNA and blocked the expression of ACC oxidase gene in tomato. The ethylene production rate in ripened fruits and leaves of transgenic plants was found to be significantly inhibited, ensuring a prolonged shelf life of tomato. On the other hand, Meli et al. (2010) have suppressed two ripening-specific N-glycoprotein modifying enzymes, α-mannosidase (α-Man) and β-D-*N*-acetylhexosaminidase (β-Hex) after their identification in tomato. They have shown that RNAi suppression of these genes enhances shelf life of tomato, through reduced rate of softening.

Male sterility and fertility

The development of male sterility is one of the most important traits chosen to ensure purity in order to construct hybrid plant for hybrid seed production. Several methods based on conventional as well as genetic engineering are used by plant biologists for the abortion of pollens from various crop species. Today, genetic engineering is used to produce male-sterile plant varieties like tobacco and tomato through RNA interference. Not only this, RNAi can also be employed to restore the fertility of male-sterile plants (Nizampatnam and Kumar 2011).

Recently, Zhu and Deng (2012) have highlighted the relationship of small RNA (osa-smR5864) with photoperiod- and temperature-regulated male sterility.

In plants, MutS HOMOLOG 1 (*Msh1*) is a nuclear gene product that maintains genome stability in both mitochondria and plastids. Under abiotic stresses, the suppression of *Msh1* triggers a plastidial response that involves non-genetic inheritance and alters numerous plant metabolic pathways (Xu et al. 2012). Sandhu et al. (2007) also used RNAi technology to disrupt the expression of *Msh1* in tobacco and tomato which resulted in rearrangements in the mitochondrial DNA associated with naturally occurring cytoplasmic male sterility. The breeding through these RNAi lines may have few drawbacks like the hybrid may carry the construct in F1 generation leading to severe effects.

Nutritional improvement

Biofortification

The diet of over two-thirds of the world's population lacks one or more essential mineral elements (White and Broadley 2009). RNAi confers the biofortification of plants such as tomatoes with dietary antioxidants (Niggeweg et al. 2004) and essential elements like Zn, Mg, Cu, Se, Ca, Fe, I, S, P, etc., (Table 6).

The production of potential pharmaceutical compounds using genetic engineering and transient expression systems such as agro-infiltration, virus infection and magnification (Obembe et al. 2011) is known as molecular pharming or bio-pharming. It focuses mainly on the biosynthesis of proteins and secondary metabolites which are very useful for humans, but are expensive in the market. Various therapeutics and nutraceuticals are being produced using molecular pharming (Obembe et al. 2011). The production potential of RNAi for these products has been reported in many papers, some of which are discussed in this article.

In 2005, Hüsken et al. had used RNAi to reduce the levels of sinapate esters by 76 % in transgenic canola seeds of the T3 generation by inhibiting UDP-Glc:sinapateglucosyltransferase gene activity. Removal of sinapate esters enhances the flavour of canola seeds exhibiting the utility of RNAi in over production/over-expression of certain gene to get the desired trait.

Plants can be improved by RNAi technology to increase the starch content in the leaves. It has been shown that starch phosphorylation and dephosphorylation are critical components of leaf starch degradation, where glucan, water dikinase (GWD) adds phosphate to starch, and phosphoglucan phosphatase (SEX4) removes these phosphates. In maize, the pathway of leaf starch degradation is less well characterized (Weise et al. 2011). Weise et al. (2012) had manipulated phosphate metabolism in *Zea mays* (maize) and *A. thaliana* using RNAi constructs to increase the starch content.

Kusaba et al. 2003 were able to reduce the level of glutenin employing RNAi-mediated gene silencing by GluB hairpin RNA and produced a rice variety called *LGC-1* (low glutenin content 1), a relief to the kidney patients unable to digest glutenin.

The consumption of alpha-linolenic acid (18:3) was found to be unhealthy for human as well as animals. The reduction of alpha-linolenic acid (18:3) is good to improve soybean oil flavour and stability with reduced need for its hydrogenation. The linoleic acid (18:2) is converted into alpha-linolenic acid (18:3) in presence of enzyme omega-3 fatty acid desaturase. Flores et al. (2008) have constructed Hairpin RNA for the down regulation of omega-3 fatty acid desaturase (*Gm*FAD3A, *Gm*FAD3B and *Gm*FAD3C), using glycinin promoter for seed-specific silencing. Transgenic soybean seed has been reported to have 1–3 % of alpha-linoleic acid in comparison with 7–10 % in non-transgenic soybean seed.

Sucrose biosynthesis involves two enzymatic steps: sucrose-phosphate synthase (SPS) catalyses the synthesis of sucrose-6-phosphate (Suc6P), which is further, hydrolysed by sucrose phosphatase (SPP) to yield sucrose and inorganic phosphate (Pi). Storage of potato tubers at low temperature (4 °C) leads to the accumulation of glucose and fructose in a process called 'cold sweetening'. Chen et al. (2008) used CaMV 35S promoter-driven hairpin RNAi construct containing part of the coding region of the tobacco *NtSPP2* gene to reduce SPP expression in transgenic potato tubers. They reported that Suc6P accumulates in RNAi-silenced sucrose phosphatase (SPP) potato tubers upon cold storage at 4 °C. They have revealed from northern analysis that cold-induced expression of vacuolar invertase (VI) was blocked in SPP-silenced tubers explaining a reduced sucrose-to-hexose conversion. Suc6P levels were found to be negatively correlated with VI expression.

Gil-Humanes et al. (2008) used RNAi technology to silence the expression of specific γ-gliadins and reported the reduced level of γ-gliadins by about 55–80 % in the bread wheat cultivar 'Bobwhite' lines (BW208) and by about 33–43 % in the 'Bobwhite' lines (BW2003). Further, Gil-Humanes et al. (2012) reported that down-regulation of gamma-gliadins by RNAi in wheat lines produced a compensatory effect in the rest of the gluten proteins, with no statistically significant changes in the total content of gliadins, whereas the glutenin content was increased. As a consequence, the total protein content was slightly increased in most of the transgenic lines.

Allergen and toxin elimination

Food allergy is an exaggerated immune response of our body triggered by allergens present in food such as peanuts, apple, mango or some other specific food. So, there is a need to reduce or eliminate the content of allergens from our food. Not only this, there is also a need to develop plants free from toxic substances as the natural toxins are present in a wide variety of plants commonly consumed as food. These toxic substances when ingested in significant amount or when they are not processed appropriately can be potentially harmful to human health causing food poisoning. The elimination of allergens and toxic substances can be achieved by employing RNA interference that may alter the biosynthesis of allergens by altering its biochemical pathway to improve the food quality by minimizing the risk of food allergy and toxicity.

RNAi was employed to inhibit the expression of major apple allergen, Mal d1 that belongs to a group of pathogenesis-related protein PR10. The Mal d1 expression has been reduced successfully by Gilisen et al. (2005) through RNAi-based gene silencing. In 2008, Dodo et al. demonstrated about 25 % reduction of Ara h 2 content in crude peanut extract by down-regulating its expression through RNAi using its hpRNA construct. In peanut, the Ara h 2 is one of the most allergic protein present out of 7 allergenic proteins. To remove Lyc e3 from tomato, Le et al. (2006) have demonstrated an efficient silencing of Lyc e3, a tomato allergen that encodes a nonspecific lipid transfer protein in the RNAi-silenced tomato plants.

Siritungam and Sayre (2003) have reduced the linamarin content, a cyanogenic substance in cassava plants. Jørgensen et al. (2005) used RNAi to suppress the cytochrome P450 enzyme production inhibiting the biosynthesis of linamarin and lotaustralin, and generated transgenic cassava (*Manihot esculenta*) plants with elimination of less than 1 % of cyanogenic glucosides from leaves and 92 % reduction of cyanogenic glucosides from tubers.

Allium species synthesize a unique set of cysteine-derived secondary sulphur metabolites such as *S*-alk (en) yl-L-cys sulfoxides, which include *S*-2-propenyl-L-cysteine sulfoxide (alliin or 2-PRENCSO) and *trans*-*S*-1-propenyl-L-cysteine sulfoxide (isoalliin or 1-PRENCSO). On disruption of tissues, these cysteine derivatives are cleaved by alliinase (EC 4.4.1.4) into sulfenic acids and volatile sulphur compounds giving characteristic flavour and bioactivity. The conversion of 1-propenyl sulfenic acid to propanthial *S*-oxide (tear-inducing, lachrymatory factor, LF) is mediated by an enzyme, named lachrymatory factor synthase (LFS). Eady et al. (2008) suppressed lfs gene by hpRNAi using 35S CaMV promoter reducing LFS activity in wounded onion resulting in the production of tearless onion.

People of countries like India, Bangladesh and Ethiopia, consume grass pea (*Lathyrus sativus*). Grass pea or chickling pea contains a neurotoxin, beta-*N*-oxalyl-ami-noalanine-L-alanine (BOAA), which can cause paralytic disease called lathyrism (Spencer et al. 1986). BOAA is also known to impart immunity to plant under extreme stress conditions, but the use of RNAi technology in bringing down the level of BOAA to an appropriate concentration can render the crop safe for its consumption (Angaji et al. 2010).

Cotton seeds are rich in dietary protein but unsuitable for human consumption because of their toxic terpenoid content, gossypol. Gossypol is a cardio and hepatotoxic terpenoid, which makes it unsafe for human and monogastric animal consumption (Risco and Chase 1997). Gossypol is also important in plant defence mechanism as it provides protection against insects and pathogens. So utilizing seed-specific promoter does not affect terpenoid level in leaves and other parts of cotton plant. RNAi has been used to produce cotton stocks with seeds containing lower levels of delta-cadinene synthase, a key enzyme in gossypol biosynthesis pathway, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pests. It has been reported that the transgenic cotton seeds have about 99 % reduced gossypol content as compared to wild types (Sunilkumar et al. 2006). Recently, Rathore et al. (2012) reported that RNAi-knockdown of delta-cadinene synthase gene was used to engineer cotton plants that produced ultra-low gossypol cottonseed (ULGCS). They also showed that RNAi-mediated ULGCS trait exhibited multi-generational stability.

As nor-nicotine is the precursor of a carcinogenic, tobacco-specific nitrosamine (TSNA), *N*′-nitrosonornicotine (NNN) (Hecht 1998) and the conversion of nicotine to nor-nicotine is mediated by an enzyme nicotine demethylase. The inhibition of nicotine demethylase activity is an effective mean of decreasing significantly the level of defined carcinogen present in tobacco products. Gavilano et al. (2006) used RNAi for silencing the *N*-demethylase (CYP82E4) gene for suppressing the conversion of nicotine to nor-nicotine in tobacco (*Nicotiana tabacum*). Further, Lewis et al. (2008) developed RNAi-silenced nicotine demethylase transgenic lines of burley tobacco line (DH98-325-5) exhibiting sixfold decrease in nor-nicotine content.

Therapeutics

Recent finding has proved the role of RNAi in therapeutics. Yuki et al. (2012) demonstrated utilization of efficient advance MucoRice system for generating highly immunogenic mucosal vaccines. They used RNAi suppression of a combination of major rice endogenous storage proteins (13 kDa prolamin and glutelin A), and could express highly a vaccine comprising the 45 kDa C-terminal half of the heavy chain of botulinum type A neurotoxin (BoHc), at an average of 100 μg per seed (MucoRice-BoHc). MucoRice-BoHc could induce high levels of mucosal immune response when nasally administered together with mucosal adjuvant. Mice were immunized with nasal vaccine composed of 25 μg of MucoRice-BoHc and mucosal adjuvant. Results suggested that MucoRice-BoHc with nontoxic chimera adjuvant CTA/LTB has the potential to be used as a promising nasal vaccine against botulism.

Vaucheret and Chupeau (2012) have mentioned exogenous plant miRNAs are present in the sera and tissues of various animals and that these exogenous plant miRNAs are primarily acquired orally, through food intake directly influence gene expression in animals after migration through the plasma/sera and delivery to specific organs. Zhang et al. (2012a) have reported a surprising finding that plant miRNAs acquired orally through food are present in the sera and tissues of various animals and can regulate the expression of target genes in mammals. In the sera of healthy Chinese men and women, MIR168a of rice is found highly enriched plant miRNA. In vitro and in vivo studies have demonstrated that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in liver decreasing LDL removal from mouse plasma leading to increase in LDL content. This phenomenon shows the therapeutic potential of exogenous supply of plant-derived miRNA to regulate the expression of specific gene in cross-kingdom such as human beings.

Altered phenotype

Since antiquity, ornamental plants and flowers are improved by modifying their various traits such as colour, shape and size of flowers and architecture of plants. The alteration in architecture of crop plants may also lead to increase crop yield, e.g. Dwarf Rice. So, there is a need to improve the phenotype of plants and flowers.

Altered architecture

Plant architecture is a basic agronomic trait shaped by indeterminate and determinate meristems, and their activities subsequently guide different patterns of plant growth (Sussex and Kerk 2001) such as, plant height and canopy, leaf size and number, branches, number of flowers, fruit size and shape, root size and structure, etc., to increase yield in ornamental, fruit and crop plants.

Recently, McGarry and Ayre (2012) reviewed the roles of the genes belonging to the CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING (CETS) family which share homology to phosphatidylethanolamine binding protein (PEBP) genes. FLOWERING LOCUS T (FT) and TERMINAL FLOWER I (TFL1) of Arabidopsis and orthologs from other species are best-characterized in controlling the balance and distribution of indeterminate and determinate growth. These genes can be manipulated to enhance the crop yield or improve plant/flower morphology. The RNAi had shown its potential by its employment in plants to alter their morphology as per requirement (such as height, inflorescence, branching and size).

In poplar, Rubinelli et al. (2013) over-expressed a unique miRNA gene from maize belonging to the MIR156 family called Corngrass1 (Cg1) under the control of the cauliflower mosaic virus 35S promoter. Transgenics had significantly greater axillary meristem outgrowth, shorter internode length, and up to a 30 % reduction in stem lignin content compared to stem lignin of the wild-type.

Recently, McGarry and Ayre (2012) reported that the HSPp::FT1/FT2-RNAi lines also formed inflorescences, suggesting that FT1 signalling is sufficient for reproductive onset.

Altered colour/scent of flowers

Nowadays, the demand of flowers has increased for the purpose of decoration and scent. There is a need to develop flowers like lotus, rose, tulip, petunia, orchid and poppy in different colours and scents, as per their demand in market. Many researches are being performed to improve the flowers through gene silencing (Table 5).

Seitz et al. (2007) and Nakatsuka et al. (2010) suggested that reduced accumulation of polyacylated anthocyanins by RNAi could cause modulations of flower colour. In 2005, Nishihara et al. had applied RNAi-mediated silencing of chalcone isomerase (CHI) in tobacco and reported reduced pigmentation and change of flavonoid components in flower petals. In pollen, it showed a yellow colouration which was indicative of accumulation of high levels of chalcone suggesting that CHI plays a major role in the cyclization reaction from chalcone to flavanone.

Defence improvement

The evaluation of RNAi from greenhouse studies to field trials provides critical information for successful anti-resistance strategies. These strategies could be employed to control the biotic stresses by improving defence mechanism

Table 5 RNAi-mediated improvised defence

S. no.	Defence improvement	Resistance against	Targeted gene	Plant used	References
1	Insect resistance	*Helicoverpa armigera*	*CYPAE14*	Cotton	Mao et al. (2007)
		Corn rootworm	V-ATPase A	Maize	Baum et al. (2007)
2	Virus resistance	Rice Dwarf Virus (RDV)	PNS12	Rice	Shimizu et al. (2009)
		Bean golden mosaic virus (BGMV)	AC1 gene	Bean	Bonfim et al. (2007)
		BYDV (Barley Yellow Dwarf Virus)	BYDV-PAV	Barley	Wang et al. (2000)
3	Nematode resistance	*Meloidogyne incognita*	splicing factor and integrase	Tobacco	Yadav et al. (2006)
		Meloidogyne	16D10	Arabidopsis	Huang et al. (2006)
		Meloidogyne javanica	Tis11	Tobacco	Fairbairn et al. (2007)
4	Bacteria resistance	*Xanthomonas citri* subsp. *citri* (Xcc)	PDS and CalS1	Lemon	Enrique et al. (2011)
5	Fungus resistance	*Phytophthora infestans*	SYR1	Potato	Eschen-Lippold et al. (2012)
		Phytophthora parasitica var. *nicotianae*	GST	Tobacco	Hernández et al. (2009)
		Blumeria graminis f. sp. *tritici*	MLO	Wheat	Riechen (2007)

Table 6 The gene targeted for RNAi-mediated crop improvement

S. no.	Crop improvement	Traits improved	Gene targeted	Plant used	References
1	Biofortification	β-Carotene and lycopene	NCED1	Tomato	Sun et al. (2012)
		Carotenoid	ε-CYC	*Brassica napus*	Yu et al. (2007)
		Carotenoid and flavonoid	DET 1	Tomato	Davuluri et al. (2005)
		Carotenoid with decreased sinapate esters	DET1	*Brassica napus*	Wei et al. (2009)
		β-Carotene and lutein	BCH	Potato	Eck et al. (2007)
		Starch	AtGWD	Maize	Weise et al. (2012)
			AtGWD and AtSEX4	Arabidopsis	Weise et al. (2012)
		Lysine	ZLKR and SDH	Maize	Houmard et al. (2007)
			Maize zein storage protein	Maize	Segal et al. (2003)
		Amylose	SBE IIa and SBE IIb	Wheat	Regina et al. (2006)
			SBE IIa and SBE IIb	Barley	Regina et al. (2010)
		Stearic- and oleic- fatty acids	Stearoyl-acyl-carrier protein Δ9-desaturase and oleoyl-phosphatidylcholine ω6-desaturase	Cotton	Liu et al. (2002)
		Vitamin C	APX	Tomato	Zhang et al. (2011)
2	Bio-elimination	Caffeine	CaMXMT 1	*Coffea canephora*	Ogita et al. (2003)
		Cadmium	PCS	Rice	Li et al. (2007)
		Morphine	Codeine Reductase (COR)	*Papaver somniferum (Opium poppy)*	Allen et al. (2004)
3	Altered phenotype	Flower colour: blue to white	CHS	Torenia hybrida cv. Summerwave Blue	Fukusaki et al. (2004)
		Scent profile modification	PhBSMT	Petunia	Underwood et al. (2005)
		Parthenocarpy	AUCSIA	Tomato	Molesini et al. (2009)
			CHS	Tomato	Schijlen et al. (2007)
		Male sterility	TA29	Tobacco	Nawaz-ul-Rehman et al. (2007)
			GEN-L	Rice	Moritoh et al. (2005)
			BCP1	*Arabidopsis thaliana*	Tehseen et al. (2010)
		Fertility restored	orfH522	Tobacco (male sterile)	Nizampatnam and Kumar (2011)

in crop plants (Hollomon 2012). However, further research is needed to explore the novel strategies amounting to efficient and durable gene silencing. Numerous papers suggest the efficacy of RNAi for better control over the parasitic weeds, virus, bacteria, fungi, nematodes and insects (Table 6).

Parasitic weeds resistance

Plant parasitic weeds are widely distributed in and around crop fields of many countries, leading to enormous loss of agricultural yield. There are many conventional methods for its control but with several limitations, so there is a need to develop a biotechnological tool to control the parasitic weeds.

Recently, some researchers have reported the application of RNAi technology to develop weed-resistant plant varieties. Aly et al. (2009) have produced transgenic tomato plants bearing M6PR dsRNA-expression cassette. They found that the level of endogenous M6PR mRNA in the tubercles and underground shoots of *Orobanche aegyptiaca* grown on transgenic tomato plants was reduced by 60–80 % with a significant decrease in mannitol level and a significant increase in the percentage of dead *O. aegyptiaca* tubercles. A parasitic weed-resistant variety of maize was developed using hpRNA-mediated RNAi resistant to *Striga asiatica* L. (de Framond et al. 2007; Yoder et al. 2009).

Insect and nematode resistance

Insect pests cost billions of dollars in the form of crop losses and insecticides. Still farmers face an ever-present threat of insecticide resistance, fuelling a continual search for alternative pest-control strategies (Ferry et al. 2006; Gordon and Waterhouse 2007).

Annually crop loss of worth about US$125 billion was reported by unmanageable phytoparasitic nematodes. Gheysen and Vanholme (2007) demonstrated that dsRNA expression in a host plant against housekeeping or parasitism genes in the root-knot nematode results in nematode-resistant plants.

The differentiation of syncytium (plant root organ) is induced by plant-parasitic cyst nematodes for source of nourishment. Syncytium is formed through re-differentiation and fusion of root cells in large number. Hewezi et al. (2012) reported that miR396 has a role in phase transition in Arabidopsis. Strong down-regulation of miR396 in cells giving rise to the syncytium coincides with the initiation of syncytial formation phase and up-regulation of miR396 in the developed syncytium marks the beginning of maintenance phase, when no new cells are incorporated into the syncytium. Expression modulations of miR396 and its growth-regulating factor (GRF) target genes resulted in reduced syncytium size and arrested nematode development. This showed miR396 as a key regulator in reprogramming of root cells representing a powerful molecular target for parasitic animal to modulate plant cells into a novel developmental pathway.

Huang et al. (2006) were first to demonstrate resistance to more than one nematode species, targeting a gene involved in parasitism rather than a nematode housekeeping gene. It is likely that recent sequencing of the *Meloidogyne hapla* genome will reveal new targets for HD-RNAi (Oppermana et al. 2008).

Through host-induced RNAi, Sindhu et al. (2009) targeted all four nematode parasitism genes of sugar beet cyst nematode (*Heterodera schachtii*), 3B05, 4G06, 8H07 and 10A06, having host *A. thaliana*. They reported that no complete resistance was observed, but it led to 23–64 % reduction in the number of mature nematode females in different RNAi lines.

Ibrahim et al. (2011) targeted four different genes for RNAi constructs. The genes have high similarity with *Heterodera glycines* (essential soybean cyst nematode) and *C. elegans* to determine their efficacy to reduce galls formed by *Meloidogyne incognita* in soybean roots. Of the four, two constructs targeting the genes encoding tyrosine phosphatase (TP) and mitochondrial stress-70 protein precursor (MSP) were able to reduce gall formation by 92 and 94.7 %, respectively.

Virus resistance

Among different strategies to combat virus infections in plants, pathogen-derived resistance (PDR) is the most powerful approach. The application of the PDR concept has helped to engineer virus-resistant plants (Simón-Mateo and García 2011). There is one more strategy that targets multiple regions of a viral gene showing a broad-spectrum resistance against tospoviruses in tomato plants (Bucher et al. 2006). This strategy is most effective and is based upon the use of a miRNA construct that expresses multiple artificial miRNAs (amiRNAs) targeting multiple regions of a viral gene.

Impact of RNAi, targeting the coat protein (CP) gene of viruses is found to be quite effective in inducing resistance to the plant against viruses. There are many viral coat protein targeting RNAi-modified virus-resistant plants such as Beet Necrotic Yellow Vein Virus (BNYVV)-resistant tobacco (Andika et al. 2005), Potato Virus Y (PVY)-resistant potato (Missiou et al. 2004), Papaya Ring Spot Virus type W (PRSV-W)-resistant *Cucumis melo* L. var. *cantalupensis* cv. Sun Lady (Krubphachaya et al. 2007), Plum Pox virus (PPV)-resistant *Nicotiana benthamiana* and *Prunus domestica* (Hily et al. 2007) and Cucumber Green Mottle Mosaic Virus (CGMMV)-resistant *N. benthamiana* (Kamachi et al. 2007).

Pradeep et al. (2012) reported that the introduction of inverted repeats of the CP gene of Tobacco Streak Virus (TSV) may be an effective and reliable strategy for developing economically important crops with resistance to TSV. Zhou et al. (2012) have created an RNAi construct containing CP gene and disease-specific protein gene sequences from Rice Stripe Virus. Two susceptible japonica varieties, Suyunuo and Guanglingxiangjing, were transformed by RNAi construct to develop resistance against Rice Stripe Disease. It was found that the homozygous progeny of rice plants in the T5 and T7 generations containing RNAi constructs, after self-fertilization were strongly resistant to viral infection without any morphological and developmental differences.

RNAi-mediated silencing of African cassava mosaic virus (ACMV) resulted in a 99 % reduction in Rep transcripts and a 66 % reduction in viral DNA (Vanitharani et al. 2003). Only closely related strain of ACMV can be silenced by siRNA approach. In plant viruses, more than 40 viral suppressors have been identified (Ruiz-Ferrer and Voinnet 2007). Patil et al. (2011) first demonstrated RNAi-mediated resistance to Cassava Brown Streak Disease (CBSD) in cassava (*M. esculenta*) and protection against very distant isolates of causative organism (more than 25 % in nucleotide sequence) belonging to two different species: Cassava Brown Streak Virus (CBSV) and Cassava Brown Streak Uganda Virus (CBSUV). Today, CBSD is considered as the leading risk to cassava cultivation in East Africa.

Using black gram (*Vigna mungo*) as a study system, Pooggin et al. (2003) have discovered that the DNA of a replicating virus can also be a target of RNAi. They have observed recovery of *Vigna mungo* from MYMIV (Mungbean Yellow Mosaic India Virus) infection by silencing the gene associated with bidirectional promoter through RNAi approach.

Bonfim et al. (2007) have generated geminivirus-resistant BGMV-resistant common bean using RNAi method. Aragão and Faria (2009) have also presented data on the generation of two transgenic common bean (*Phaseolus vulgaris*) lines.

There are many other transgenic plants with enhanced defence by RNAi-mediated gene silencing, such as Tomato Yellow Leaf Curl Virus (TYLC)-resistant tomato (Fuentes et al. 2006), Rice Tungro Bacilliform Virus (RTBV)-resistant rice (Tyagi et al. 2008) and Citrus Tristeza Virus (CTV)-resistant mexican lime (López et al. 2010). Schwind et al. (2009) have even shown that using hpRNA construct in *Solanum lycopersicum* (tomato) against Potato Spindle Tuber Viroid (PSTVd) produce PSTVd-resistant tomato plant varieties.

Bacterial resistance

Bacterial diseases are one of the biggest challenges in crop field such as tomato, soybean and banana. The bacterial diseases spread very fast and become difficult to control, hence prevention is the only way to avoid bacterial infections. Employing RNAi for enhancing bacterial resistance on experimental plant *A. thaliana* has shown good results.

Escobar et al. (2001) showed that silencing of two bacterial genes (iaaM and ipt) could decrease the production of crown gall tumours (*Agrobacterium tumefaciens*) to nearly zero in Arabidopsis (Dunoyer et al. 2006), suggesting that resistance to crown gall disease could be engineered in trees and ornamental plants.

Fungal resistance

Research findings show the enhanced defence against fungi in genetically engineered crop plants based on RNAi. In 2010, Xin et al. reported that 24 miRNAs were responsive to powdery mildew infection caused by the obligate biotrophic fungus *Blumeria graminis* f. sp. *tritici*. and further predicted 149 target genes which were potentially regulated by the novel wheat miRNA. More examples are summarized in Table 5.

Abiotic stress tolerance

The application of transgenesis in crop plants was initially aimed to provide protection against pests and tolerance to herbicides (Shewry et al. 2008). Many plant miRNAs play critical roles in nutrient homeostasis, developmental processes, abiotic stresses and pathogen responses (Xin et al. 2010). The miRNA plays important regulatory roles in development and stress response in plants by negatively affecting post-transcriptional gene expression. Identification of miRNAs at the global genome-level by high-throughput sequencing is essential to functionally characterize miRNAs in plants (Wang et al. 2011b). Extensive studies of miRNAs have been performed in model plants such as rice, *A. thaliana* and other plants. Wang et al. (2011a) investigated about miRNAs involved in the very early stage during seed germination and reported that miRNA-mediated regulation of gene expression is present in maize imbibed seed.

Chen et al. (2012) provided valuable information of miRNAs in Al^{3+} toxicity and tolerance. They found that 18 miRNAs were providing response in 4 h of Al^{3+} treatment, 4 miRNAs belonging to 4 families were found to show response at both 4 h and 24 h of Al^{3+} treatment. The miR390 was down-regulated in response to 24 h Al^{3+} treatment and considered as the late responsive miRNAs. Only miR390 is known to regulate auxin response factor which is responsible for the lateral root development (Marin et al. 2010; Yoon et al. 2010).

Wang et al. (2011b) identified drought-responsive miRNAs in a legume model plant, *Medicago truncatula*. They reported that 22 members of 4 miRNA families were up-regulated and 10 members of 6 miRNA families were down-regulated in response to drought stress. Out of 29 new miRNAs/new members of known miRNA families, 8 miRNAs were responsive to drought stress with both 4 miRNAs being up-regulated and down-regulated, respectively. The known and predicted targets of the drought-responsive miRNAs were found to be involved in diverse cellular processes in plants, including development, transcription, protein degradation, detoxification, nutrient status and cross adaptation.

Sunkar and Zhu (2004) described the role of miRNAs in response to abiotic stresses. The Arabidopsis seedlings were exposed to different abiotic stresses such as drought, cold, salinity, and oxidative stress. It was observed that miR393 was strongly up-regulated by cold, dehydration, high salinity and abscisic acid (ABA) treatments. In addition, by exposing Arabidopsis to different abiotic stress of varying degrees, miR319c, miR389a, miR397b and miR402 were also found to be regulated. In 2010, Xin et al. reported that 12 miRNAs in wheat (*Triticum aestivum* L.) were responsive to heat stress. In another report, stress-related miRNAs has been identified in rice (*O. sativa* L. ssp. *Japonica* cv 9522) seedlings exposed to abiotic stresses (cold, dehydration, salinity and abscisic acid) as well as wild type (Jian et al. 2010). 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).

Under stress conditions, miR393 is known for its contribution to the anti-bacterial resistance and down regulation of auxin signalling and seedling growth by inhibiting the expression of TIR1 (Vierstra 2003; Jones-Rhoades

and Bartel 2004; Navarro et al. 2006). Moreover, it was reported that miR159 responds to hormone signalling and dehydration responses in Arabidopsis (Achard et al. 2004; Reyes and Chua 2007).

Recently, Zhang et al. (2012b) found that miR156, miR157, miR166 and miR172 have different expression levels between the juvenile and adult phases of precocious trifoliate orange (*Poncirus trifoliata* L. Raf.). They also reported that miR156 declined from juvenile to adult stage, whereas miR172 increased during this same period. Whereas the over-expression of miR172 accelerates flowering in Arabidopsis (Chen 2004) and exhibits a similar temporal expression pattern in maize where it targets Glossy15, a gene responsible for expression of juvenile epidermal traits (Lauter et al. 2005), the over expression of miR156 delays flowering and prolongs the expression of juvenile vegetative traits in Arabidopsis and maize (Wu and Poethig 2006; Chuck et al. 2007a, b). Thus the expression pattern of miR156 and miR172 is found to be inversely related.

Hu et al. (2009) showed that the expression of rice histone deacetylases genes display 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.

Hwang et al. (2011) identified drought stress-responsive miR171 family members (named miR171a, miR171b and miR171c) in potato plants, *Solanum tuberosum*. In water-deficit condition, Trindade et al. (2010) identified several conserved miRNAs having differential expression in *M. truncatula* plants as miR169 is down-regulated in roots, whereas miR398a/b and miR408 are strongly up-regulated in both shoots and roots. The RNAi-suppression of farnesyl transferase genes FTA or FTB, in canola leads to decreased stomatal conductance and thereby transpiration resulting in higher yields in a 3-year field trial (Wang 2005, 2009).

In response to the drought stress, 22 members in 4 miRNA families (miR399, miR2089, miR2111 and miR2118) were up-regulated whereas, 10 members from 6 miRNA families (miR164, miR169, miR171, miR396, miR398 and miR1510) were down-regulated (Wang et al. 2011b). miR399 and miR2111 have also been reported to be up-regulated by abiotic stresses such as phosphate starvation (Bari et al. 2006; Pant et al. 2009). PvPHR1 is supposed to be positive regulator of genes implicated in transport, mobilization and homeostasis of phosphorus. Valdés-López et al. (2008) demonstrated that miRNA399 of *P. vulgaris* (PvmiR399) is an essential component of the PvPHR1 signalling pathway in common bean (*P. vulgaris* L.). Jones-Rhoades and Bartel (2004) have indicated that miR395 and miR399 are up-regulated when plants suffer from sulphur and phosphate starvation in Arabidopsis.

Leguminous plants are characterized by their ability to develop nitrogen-fixing nodules via an interaction with symbiotic bacteria. Li et al. (2008b) found that silencing of *Asnodf32* delayed root nodule and bacteroid senescence with enlarged nodules and extended period for active nitrogen fixation. *Asnodf32*, which encodes a nodule-specific cysteine proteinase in *Astragalus sinicus*, is silenced by *Agrobacterium rhizogenes*-mediated RNAi. Further, Laporte et al. (2010) noted that MtSNARP2-silenced transgenic roots of *M. truncatula* showed aberrant early senescent nodules where differentiated bacteroids degenerate rapidly. Hence, a functional symbiotic interaction may be regulated through secreted RNA-binding peptides.

Future perspective

The global demand for food is likely to increase with the continuing population growth and consumption (Godfray et al. 2010). In the twenty-first century, the challenge for agriculture will be the improvement of crop production in a sustainable manner (Molesini et al. 2012). According to Godfray et al. (2010), the world is capable of producing more food and can ensure that it is used more efficiently by just employing genetic engineering and metabolic engineering to develop high-yielding crop varieties.

The biomolecules such as carbohydrate, lignin and lipid are well known for their potential to produce bioactive molecules. Carbohydrates and lignin can lead to chemical intermediates such as levulinic acid, vanillin and many other aromatic molecules for the synthesis of pharmaceutical products (Cherubini 2010); while lipids can be used to produce beneficial polyunsaturated fatty acids such as omega-3 (Octave and Thomas 2009). Over the years, several biotechnological techniques have been adopted for enhancing the bioactive molecules in medicinal plants (Khan et al. 2011a, b) like *Liquidambar styraciflua* tree, which produces shikimic acid, a key intermediate for the antiviral drug Tamiflu (Martin et al. 2010). Lim and Bowles (2012) reviewed the use of plant system to produce small bioactive molecules for health and nutrition; sweetener, flavour and fragrance; and pesticide and insect repellent. Taxol (a produce from *Taxus brevifolia*) is a mitotic inhibitor, used in the treatment of breast cancer, lung cancer and ovarian cancer. Similarly, diosgenine (*Dioscorea deltoidea* and *Trigonella foenum graecum*) inhibits migration and invasion of human prostate cancer PC-3 cells by reducing matrix metalloproteinases expression (Chen et al. 2011). It is also used for the commercial synthesis of cortisone, pregnenolone, progesterone, and other steroid products.

Metabolic engineering can be useful to facilitate synthesis and production of commercially valuable plant products such as drugs, pigments, fragrances, volatile oils and flavours (Table 7) on large scale. RNAi can be utilized with novelty for lowering the enzyme activities that will change the

Table 7 RNAi could be employed in future as a tool of metabolic engineering for synthesis and production of commercially valuable plant products

RNAi may enhance	Products	Source plant
Production of important natural pharmaceutical compounds	Codeine (Alkaloid)	*Papaver sominifera*
	Quinine (Alkaloid)	*Cinchona ledgeriana*
	Vincristine (Alkaloid)	*Catharanthus roseus*
	Scopolamine (Alkaloid)	*Dhatura stramonium*
	Taxol	*Taxus brevifolia*
	Diosgenine (steroid sapogenen)	*Dioscorea deltoidea* and *Trigonella foenum graecum*
Biosynthesis of scents in flowers	Rose	*Rosa species*
	Jasmine	*Tabernaemontana divaricata*
	Lavender	*Lavandula Angustifolia*
Biosynthesis of flavouring agents	Vanillin	*Vanilla planifolia, V. pompon* and *V. tahitiensis*
	Strawberry	*Fragaria* × *ananassa*
	Menthol, peppermint or mint	*Mentha arvensis*, *M. canadensis* and *M. piperita*
Increased number of floral parts	Stigma (Saffron)	*Crocus sativus*
	Petals	*Rosa species* and *Calendula officinalis*

biochemical reactions leading to formation of desired products instead of unwanted/toxic compounds. RNAi may help to improve the nutraceutical potential of a plant and/its products by enhancing the content of useful amino acids, fatty acids, fibre or by getting rid of allergenic/toxic compounds (Small 2007; Vaucheret 2006; Martino-Catt and Sachs 2008; Newell-McGloughlin 2008). Khan et al. (2012) reported that RNAi is a potential technique being used for the production of medicinal plants with potential traits for a particular marker compound which is needed for eradication of diseases.

The potential digestion of cell wall material by rumen bacteria has been hindered by cell wall lignifications. Total dry matter digestibility has been increased by the traditional breeders rather than cell wall digestibility, resulting in minimal reduction in cell wall lignification. Transgenic approaches down-regulating the genes for enzymes like cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl CoA-shikimate/quinate transferase (HCT), coumarate 3-hydroxylase (C3H), caffeoyl-CoA-3-*O*-methyltransferase (CCoAOMT), cinnamoyl CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid-3-*O*-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD) involved in monolignol synthesis have produced plants with reduced lignin content and improved cell wall digestibility. If the targeted gene is a housekeeper, it should be partially silenced by RNAi with gene-specific expression to maintain the cell wall strength.

Not only this, soon RNAi will prove its potential for inhibition of photorespiration to enhance the productivity of C_3 plants. This knockdown technology may be useful in inducing early flowering, delayed ripening, delayed senescence, breaking dormancy, stress-free plants, overcoming self-sterility, etc.

Recently there are artificial restriction enzymes designed to effectively alter any gene in an organism. These are alternative to gene silencing and have enough future scope. Curtin et al. (2012) has mentioned three sequence-specific nuclease systems used for crop plants. These are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and LAGLIDADG homing endonucleases (LHEs), also termed "meganucleases". Generally ZFNs and TALENs cause programmable genetic modifications by inducing breaks in the double strands of DNA. Recently, Gaj et al. (2013) has showed the therapeutic potential of ZFNs and TALENs including the emergence of clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases. *Xanthomonas* infects plant cells and delivers a cassette of proteins called transcriptional activator-like effectors (TALEs), which modifies the host's transcriptome by binding to specific DNA sequences. TALENs are the fusion of TALEs (programmable DNA binding domain) and FokI (cleavage domain) enabling the programmable, efficient and precise site-specific RNA-guided DNA nuclease to introduce double-strand breaks (DSBs) for targeted genome modification. TALEN have been used in plants like *A. thaliana* (Cermak et al. 2011), tobacco (Mahfouz et al. 2011) and rice (Li et al. 2012).

Conclusion

Malnutrition is a major problem especially in developing countries (WHO 2000). Chronic food deficits affect about 792 million people in the world (FAO 2000) including 20 % of the population in developing countries. To ensure

a healthy diet for healthy world, there is a necessity to develop bio-fortified cereals, fruits and vegetables through enrichment with nutritionally essential elements and compounds such as essential minerals, antioxidants, vitamins, fatty acids and amino acids.

One of the most important agronomical traits that should be developed in crop plants is the stress (biotic and abiotic) tolerance. So, there is a need to develop plant varieties that will tolerate phytopathogens and pests, along with changing environmental conditions like extreme temperatures, flood, and drought, oxidative stress and changes in soil compositions (heavy metal accumulation, salinity, decreasing fertility, etc.). These plant varieties will be necessary for food security to feed the increasing population. RNAi-based researches have proved its potential in crop improvement to overcome the problem of food security, malnutrition and famine.

Rigorous researches have been designed for finding small non-coding regulatory RNAs and reveal their biogenesis and effect on gene suppression; still some facts regarding complexity of RNAi pathway and its components has to be explained. However, the applications of research related with small non-coding RNAs is creating milestone for agricultural improvement, thereby improving the way of life.

RNAi has developed many novel crops such as nicotine-free tobacco, decaffeinated coffee, nutrient fortified and hypoallergenic crops. The genetically engineered Arctic apples are near close to receive US approval. The apples were produced by RNAi suppression of PPO (polyphenol oxidase) gene making apple varieties that will not undergo browning after being sliced. PPO-silenced apples are unable to convert chlorogenic acid into quinone product.

Now, RNAi can be used in production of blue rose by suppressing cyanidin genes; generating low lignin content jute varieties for high-quality paper; healthier oil production by suppressing the enzyme that converts oleic acid into a different fatty acid; regulating flowering time in crops. It can be a boon for production of BBrMV-resistant banana varieties against Banana Bract Mosaic Virus (BBrMV), a challenge for banana population in Southeast Asia and India (Rodoni and Dale 1999).

One of the major concerns about selection of transformed plant is the use of antibiotic resistance markers. The antibiotic resistance gene may evoke environmental concern as it may lead to antibiotic resistant microbes by horizontal gene transfer. Alternative ways such as the use of *pmi* (phosphomannose isomerase) gene, *wbc19* (ABC transporter) gene, *ak* (aspartate kinase) gene and *dhps* (dihydrodipicolinate synthase) gene leads to positive selection (Jaiwal et al. 2002). However, the NptII gene has earned "generally regarded as safe" status from the USFDA (1993) as it can be used in vectors in genetic engineering for crop development producing safe food. NptII protein imparts kanamycin tolerance and used as a tool to select transformed plants.

The phenomenon of RNAi cause transcriptional gene silencing by chromatin modification leading to heterochromatin formation. These modifications might become hereditary and may cause potential adverse effects leading to biosafety risks. The crop plants developed through RNAi should undergo risk assessment related to food safety and environment protection. So, there is a need to tailor customize vectors according to the necessity of crop improvement.

Nevertheless, RNAi can be highly effective for functional genomics and biotechnology of perennial plants (Li et al. 2008a). To meet the global demand for food, fibre and fuel the production of improved crops are needed (Chapotin and Wolt 2007), which will be provided with the advances in RNAi technology. There are several opportunities for the applications of RNAi in crop science for its improvement such as stress tolerance and enhanced nutritional level. Thus, there is huge potential of RNAi to improve the agricultural yield significantly.

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