RNAi induced gene silencing in crop improvement

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Abstract The RNA silencing is one of the innovative and efficient molecular biology tools to harness the down-regulation of expression of gene(s) specifically. To accomplish such selective modification of gene expression of a particular trait, homology dependent gene silencing uses a stunning variety of gene silencing viz. co-suppression, post-transcriptional gene silencing, virus-induced gene silencing etc. This family of diverse molecular phenomena has a common exciting feature of gene silencing which is collectively called RNA interference abbreviated to as RNAi. This molecular phenomenon has become a focal point of plant biology and medical research throughout the world. As a result, this technology has turned out to be a powerful tool in understanding the function of individual gene and has ultimately led to the tremendous use in crop improvement. This review article illustrates the application of RNAi in a broad area of crop improvement where this technology has been successfully used. It also provides historical perspective of RNAi discovery and its contemporary phenomena, mechanism of RNAi pathway.

Keywords RNAi · Crop improvement · Gene silencing · RNAi vector

Introduction

Once considered as a mere intermediate in gene expression, RNAs; key machinery in transmitting information from

DNA to protein, RNAs together are now found to be a major player in gene expression. In the quest of appealing products, a new paradigm of RNA has emerged. The real persuader behind this gene regulation is a group of 21–25 nucleotides long RNA molecules referred to as “Small RNAs”—a term that represents trans-acting small interfering RNA (tasiRNA), repeat associated small interfering RNAs (rasiRNAs), natural-antisense small interfering RNA (natsiRNA) and microRNA (miRNA). These small RNAs are consequence of RNA silencing pathway. RNA silencing in gene regulatory pathway limits the expression of target genes by suppressing transcription (Transcriptional gene silencing [TGS]), inducing sequence specific RNA degradation process (post-transcriptional gene silencing [PTGS]/RNA interference) or inhibiting the translation of their mRNA into protein. Owing to its high specificity and efficacy, RNAi has been widely used as an efficient tool to analyze gene function and also for improving plant traits. Hence in this review, I shall make an attempt to assess the potential of RNAi for improving crop traits as well as focus on the current understanding of the endogenous RNAi pathway and prevalent “RNAi vector technology”.

RNAi discovery: a brief history

PTGS/RNAi was first observed in plants but later on RNAi related phenomena were observed in almost all eukaryotic organisms, including protozoa, invertebrates, vertebrates, fungi and algae. In Plants, RNAi was first discovered serendipitously in 1990 when R. Jorgensen’s laboratory tried to get petunia flowers with darker purple hue by introducing numerous copies of a gene that code for deep purple flower i.e. Chalcone Synthase (*Chs A*), but surprisingly some of the final plants yielded white or patchy

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flowers. Somehow the transgene silenced the expression of both homologous endogenous and introduced loci (Napoli et al. 1990) and the phenomenon was then termed as co-suppression. But it was Fire and Mello's work that made those puzzling observations readily understandable. They observed the presence of dsRNA in *C. elegans* that triggered a marked silencing of genes containing sequence homology to the dsRNA (Montgomery and Fire 1998; Tabara et al. 1998). They termed this unusual phenomenon of gene silencing as "RNA interference". Later on it was observed that both plant co-suppression as well as plant gene silencing mediated by "Antisense technology" led to the production of cellular dsRNAs. In parallel, several reports showed that plants respond to RNA viruses by targeting their RNAs for destruction using the same gene silencing phenomenon as RNA viruses replicate through dsRNA intermediate. Several such contemporary phenomena of RNAi exist as a result of plant-virus interactions which are discussed in subsequent subsection.

Mechanism of RNA interference: unraveling the puzzle

The process of RNAi has been dissected biochemically in both plants and animals, and found to be conserved among diverse eukaryotes. It is the dsRNA which triggers the RNA silencing pathway. dsRNAs are produced by bidirectional transcription, transcription of an inverted repeat or hairpin sequence, exogenously introduced dsRNA, replication of RNA virus or unintended transcription of nuclear gene by neighboring promoter. In case of plants, transgene which transcribe sense or antisense RNA can also generate dsRNA and silence gene expression. The long dsRNAs thus produced are cleaved endonucleolytically into 21–25 nt long double stranded fragments termed as small interfering RNAs (siRNA) in ATP dependent manner (Zamore et al. 2000). siRNAs are produced by the enzyme Dicer, member of the RNase III family of dsRNA-specific endonuclease (Bernstein et al. 2001; Xie et al. 2004). These 21–25 nt long double stranded RNAs have a characteristic structure that reflects their production by the multidomain ribonuclease III (RNase III) enzyme, Dicer. Typically Dicer has an N-terminal RNA helicase domain followed by a PIWI/Argonaute/Zwille (PAZ) domain, tandem RNase III motif and c-terminal double stranded RNA binding domain (Bernstein et al. 2001). In contrast to animals, higher plants generate array of small RNAs (sRNA) with specialized functions. For instance, *A. thaliana* encodes four different types of dicer like (DCL) proteins (DCL1 to DCL4), 10 AGO (Argonaute protein, component of RISC catalytic core), six RDRs (RNA dependent RNA polymerase) and five DRBs (dsRNA binding proteins) which participate in at least five different endogenous small RNA pathways.

While miRNAs are produced from their corresponding imperfectly base paired fold-back precursor by DCL1, the other three DCL proteins generate different types of endogenous siRNA from perfectly double stranded RNAs, such as stress related 24-nt natural-antisense transcript siRNA (natsiRNA) by DCL2 (Borsani et al. 2005), 24-nt repeat-associated siRNA (rasiRNA) that guide chromatin remodeling by DCL3 (Xie et al. 2004), and DCL4 generates 21-nt trans-acting siRNA (tasiRNA) that has role in plant developments (Gascioli et al. 2005; Xie et al. 2005). These DCL proteins, however, reported to have exhibited some redundant function in *A. thaliana*. For instance, DCL1 can produce 21-nt tasiRNA in the *dcl2*, *dcl3*, *dcl4* triple mutant (Bouche et al. 2006) and DCL2 can generate 22-nt tasiRNA when the DCL4 activity is compromised (Gascioli et al. 2005; Xie et al. 2005). siRNA thus generated by Dicer activity is then incorporated into a different multicomponent ribonuclease called *RNA-induced silencing complex* (RISC). In plants, short siRNAs plays role in local RNA silencing whereas the long siRNAs are involved in systemic signaling and also in *RNA directed DNA methylation* (RdDM) (Zilberman et al. 2003). The longer one i.e. 24-nt long, is originated from repeat sequences and are known as repeat associated siRNA (rasiRNA). rasiRNAs have been associated with direct DNA methylation of repetitive DNA sequence in the plant genome (Chan et al. 2005). Methylated DNA is believed to act as a template for the transcription of aberrant RNA by a protein with RNA polymerase activity, either RNA polymerase II, RDR2, or the plant specific PolIVa. Aberrant RNA is then converted to dsRNA by RDR2 or PolIVa, or alternatively, PolIVa uses RDR2-transcribed dsRNA to produce additional aberrant RNA molecules (Vaucheret 2005). Then DCL3 processes this dsRNA into 24-nt siRNA that are methylated by HEN1 (HUA ENHANCER1) and used by AGO4 to direct the actual sequence specific DNA methylation step of RdDM, mediated by the combined action of the SNF2-like chromatin remodeling protein, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 (DRD1), the alternative form of the plant-specific PolIVb, and the primary de novo DNA methyltransferase, DOMAINS REARRANGED METHYLASE2 (DRM2; Cao et al. 2003; Kanno et al. 2004, 2005). The DNA methyltransferase, METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE3 (CMT3) are then primarily responsible for the maintenance of DNA methylation based on the sequence context of the methylated cytosine residue (Eamens et al. 2008).

It is the siRNA structure and its sequence which determines which one of the strand enters RISC as the guide strand and which is excluded from RISC, becoming passenger strand. It seems siRNA strand whose 5' end is more weakly bound to the complementary strand is more

readily retained in RISC, and the other strand is degraded. Several informatics and biochemical studies also corroborated this assumption (Khvorova et al. 2003; Schwarz et al. 2003). RNA and DNA viruses also produce virus associated siRNAs upon infection in plants. For example, siRNA of RNA viruses such as *Turnip crinkle virus* (TCV), *Cucumber mosaic virus* (CMV), and *Tobacco rattle virus* (TRV) are generated by combined action of DCL2,3,4, RDR1,2,6 and DRB4; and their slicing function requires at least AGO1 and AGO7 (Qu et al. 2008; Bouche et al. 2006; Diaz-Pendon et al. 2006; Donaire et al. 2008), whereas in DNA viruses, in addition to components mentioned above, viral siRNAs biogenesis needs DCL1 and AGO4 and Pol IV/V for their antiviral function. Another class of 30–40 nt long siRNA known as long-siRNA (lsiRNA) are reported to be associated with *P. syringae* infection. For instance, AtlsiRNA-1, contributes to plant bacterial resistance by silencing AtRAP, a negative regulator of plant defense (Katiyar-Agarwal et al. 2007).

As we have seen it is a dsRNA which induces RNA silencing pathway, then how does the sense or antisense RNA silence their corresponding target gene? Screening of genes required for gene silencing in such cases demonstrates a family of protein whose sequences suggest they are RNA-directed RNA polymerases (RDR) (Dalmay et al. 2000b; Sijen et al. 2001) that can synthesize complementary RNA (cRNA) and induce spreading of silencing signal. Moreover, the discovery of such RDR protein also provides a possible explanation for the remarkable efficacy of dsRNA in gene silencing. In natsiRNA pathway, dsRNA molecules are formed by endogenous cis-antisense gene transcribed from different DNA strands. The dsRNA molecules thus produced served as a substrate for DCL2 cleavage and generate 24-nt natsiRNA. The single 24-nt natsiRNA subsequently targets one of the cis-antisense gene pair transcripts for cleavage, and the cleaved RNA molecule is converted to dsRNA by RDR6 and SGS3, the coiled-coil protein, SUPPRESSOR OF GENE SILENCING3 (SGS3). dsRNA, in turn produced, is subjected to DCL1 mediated processing into phased 21-nt natsiRNA and subsequently used as a guide to direct sequence-specific silencing of homologous mRNA (Eamens et al. 2008). Kennerdell and Carthew 1998; observed in *Drosophila* embryos that only few molecules of dsRNA per cell or embryo were capable of silencing target mRNA thought to be present in more than two copies per cell. An RDR protein plays a central role in the amplification of the dsRNA or siRNA itself (Lipardi et al. 2001). The 'random degradative PCR model' proposes that the RDR utilizes the guide siRNA strand as a primer to synthesize new RNA using the target RNA as a template and thereby converting it into dsRNA that can then be destroyed by Dicer (Lipardi et al. 2001). Readers are suggested to go through the article

authored by Lipardi et al. (2001) who proposed the mechanism of the interaction of siRNA with target mRNA which led to its conversion into dsRNA. Ultimately, the nascent dsRNA greater than 39 bp in length is then cleaved by RNase III-related enzymes and this step generates new siRNAs to amplify mRNA degradation.

A phenomenon known as *transitivity* increases the initial pool of siRNA, by producing new siRNAs corresponding to sequences located outside the primer targeted regions of a primary RNA target site (Sijen et al. 2001; Vaistij et al. 2002). The direction in which transitive RNA silencing proceed, depends on the organism in which it occurs. In case of plants, transitive RNA silencing is dependent on RNA-dependent RNA polymerase activity and is bidirectional i.e. it can travel both in 5' → 3' and 3' → 5' direction (Voinnet et al. 1998; Vaistij et al. 2002). It is possible that RDR initiates synthesis of cRNA at the 3' end of the aberrant target mRNA with or without the aid of primer. 5' spreading of the signal is thought to be mediated by the primary siRNA, generated by the action of the Dicer in response to the trigger. The primary siRNA binds to 3' end of one of the two target mRNA fragments generated by Dicer and primes the synthesis of cRNA by RDR. The other target mRNA cleaved fragment (3' fragment) is probably sensed as an aberrant RNA by RDR and has been used as template for unprimed synthesis of dsRNA and thereby 3' silencing signal spreads. It is also speculated that primary siRNAs bring about the cleavage of transgene transcripts, giving rise to TAS-like (tasiRNA transcript) RNA fragments that are converted to double stranded RNA by RDR6 to produce secondary siRNAs that direct the spread of silencing into surrounding nontargeted sequences (Eamens et al. 2008).

Contemporary phenomena of RNAi

Gene silencing mediated by viruses can occur with both types of viruses viz. RNA as well as DNA viruses, which replicate in the cytoplasm and the nucleus respectively. Viruses may carry exogenous gene sequence in the specific location of its genome, and retain the infectivity of the RNA transcript. When these transcripts are used to infect plants, the foreign sequences also induce and become the target of the RNAi response of the host plant. *Virus induced gene silencing* (VIGS) is a technology which take advantage of the ability of viruses to carry and induce RNAi against foreign sequences (Baulcombe 1999). Several RNA or DNA viruses have been developed as potential VIGS. VIGS is particularly useful in plants species that are recalcitrant for transformation, provided there is VIGS system available for the species. VIGS may also be utilized in analyzing gene function as the infectious transcripts can

be applied to mature plants (Waterhouse and Helliwell 2003). Among VIGS vectors, the TRV-based are more promising because these are capable of inducing meristem gene silencing which has not been possible to achieve with other RNA virus based vectors. Tomato golden mosaic geminivirus (TGMV) vectors system has also been reported to be used as meristem gene silencing (Peele et al. 2001). One major limitation with VIGS is the host range that is most viruses used for VIGS have a limited number of hosts and thus virus host combination become a crucial factor in determining the efficacy of silencing. Moreover, some of the viruses used in VIGS can cause symptoms that may confuse the phenotype caused by the silencing of the target gene. Another viral silencing system, using SATELLITE RNAs, has also been developed. In this *satellite virus-induced silencing system* (SVISS; Gossele et al. 2002), the target sequence is inserted into the satellite RNA, which is then co-inoculated with the associated virus. Another RNAi related technology is amplicon mediated RNA silencing first described by Angell and Baulcombe (1997) for potato virus X (PVX). An amplicon is a transgene that comprises target gene sequence and viral genome but not necessarily that of all the genes of the native virus. Plants transfected with amplicon do not show symptom of being infected with virus. Amplicon can also overcome the host specificities which is the major limitation of VIGS. A PVX-GFP amplicon transgene induces GFP-specific RNAi in *Arabidopsis* (Dalmay et al. 2000a) even though *Arabidopsis* is not a host of PVX.

Plant RNAi transformation vector

Various types of gene silencing constructs have been reported from different laboratories all over the world but gene construct encoding intron-spliced RNA with a hairpin structure can induce PTGS with almost 100% efficiency (Smith et al. 2000; Sinha et al. 2006). Wesley et al. (2001) made a generic vector pHANNIBAL that allows a simple, single PCR product from a gene of interest to be easily converted into a highly effective ihp RNA (intron-spliced hairpin RNA) silencing construct. They have also created a high throughput vector, pHELLSGATE; that facilitate the cloning of gene libraries of large numbers of defined genes, such as those in EST collections, using an in vitro recombinase system. Apart from these two, many such as pSTARLING, pSTARGATE, pWATERGATE, pOpOff hairpin RNAi vectors have been developed at CSIRO Plant Industry (<http://www.pi.csiro.au/RNAi/vectors.htm>). Miki and Shimamoto (2004) developed a Gateway vector, pANDA, for RNA interference of rice genes. In the construct, hairpin RNA derived from a given gene is transcribed from a strong maize ubiquitin promoter, and an

intron is placed 5' upstream of inverted repeats to enhance RNA expression. A similar vector, pANDA-mini, was also developed for direct transfer into leaf cells or protoplast by this group. Wang et al. (2004) also reported a novel PCR based RNAi vector, pTCK303 with a maize ubiquitin promoter, two specific multienzyme sites and a rice intron for monocot gene silencing. This vector requires less time for its creation as it requires only one PCR product amplified by a single pair of primers and two ligation reactions.

Hirai et al. (2007) constructed an RNAi vector and studied the efficacy of construct using either the standard Cauliflower mosaic virus (CaMV) 35S promoter or an expression enhancing promoter, the E12Ω sequence. This experiment showed an increase in gene silencing with the use of the E12Ω sequence containing vector as opposed to the standard CaMV 35S transcription driven vector. The E12Ω sequence has two tandemly repeated enhancer sequence of CaMV 35S promoter and hence confers enhanced level of expression as compared to CaMV 35S promoter.

Higuchi and coworkers addressed a current challenge in RNAi vector technology that can ensure the proper inverted repeat which can successfully make dsRNA and cause a silencing effect once inside the plant. They made this construct using two partially complementary long oligonucleotides. Multiple cloning steps are unnecessary beyond this point, which is an advantage when developing time sensitive commercial products not only because multiple cloning steps are eliminated but also because the inverted repeats are constructed before cloning into the vector. This method proves that it is possible to transform plants with inverted repeats that can produce stable dsRNAs for RNAi plants (Higuchi et al. 2009).

Another type of RNAi vector useful for the functional gene analysis has been reported by Tzfira and coworkers, is pSAT-RNAi vector. They demonstrated use of such vectors for the simultaneous silencing of multiple transgenes in transgenic plants (Dafny-Yelin et al. 2007). These pSAT-RNAi plasmids feature a *ChsA* intron sequence which is flanked by two unique multiple cloning sites (MCSs) suitable for insertion of target gene sequences in reverse orientations. Expression of hpRNA in different pSAT-RNAi plasmids is controlled by various promoter and terminator sequences. Several such hpRNA expression cassettes can be assembled into a single *Agrobacterium* binary vector. Luo et al. (2008) developed a novel T-vector, pGFPm-T for convenient cloning of the RT-PCR products to generate bidirectional restriction fragments as intermediates for ihpRNAi vector.

Waterhouse group reported that it is necessary to include a spacer region between the arms of hpRNA constructs for stability of the inverted repeat DNA in *E. coli* (Wesley et al.

2001). They made a construct encoding a single self-complementary hairpin RNA (hpRNA) of the Niacin protease (*Pro*) gene sequence of potato virus Y (PVY). The construct contained sense and antisense *Pro* sequence flanking an 800-nucleotide spacer fragment derived from the *uidA* (GUS) gene. The spacer fragment contributed only to the stability of the perfect inverted-repeats and was not required for specificity of the PTGS. However, replacing the spacer (loop) region of hpRNA constructs with a functional intron sequence increased the proportion of independent silenced lines recovered from approximately 50 to about 100% (Smith et al. 2000). The constructs were designed such that their pre-mRNA should splice to form hpRNAs with small loops. To test the effect of removing the loop region of hpRNA, they replaced the spacer with intron sequence. As a control, they made a sister construct in which the intron sequence was inserted in the reverse, non-splicing, orientation. When transformed into tobacco, 22 of 34 (65%) reverse-intron plants developed immunity to PVY, a frequency similar to plants transformed with the GUS spacer construct. But with the construct containing the functional intron they found that 22 of 23 plants were immune to the virus. In another report, intronic sequence of *Gm fad 2-1* (*Glycine max* L. *fad2-1*) was engineered in spliceable orientation between the 3' untranslated region of *Gm fad 2-1* as the arms of hpRNA instead of spacer, along with 6 nucleotide exonic sequence at 5' splice site and 9 nucleotide exonic sequence at 3' splice site. ihp construct was thus designed to produce a 212 nt stem and a 15 nt loop after splicing of ihp RNA (Sinha 2006; Sinha et al. 2006).

Several intron-less hpRNA, antisense and co-suppression constructs have been used in many of the gene/host combinations (Sinha et al. 2008; Gupta et al. 2009). The ihpRNA constructs were effective, with arm length ranging from 98 to 853 nt, giving 66–100% (average 30%) independent silenced transformants (Wesley et al. 2001). Intron-free hpRNA constructs gave 48–69% (average 58%) silenced transformants, and conventional co-suppression or antisense constructs gave 0–30% (average 13 and 12% respectively) silenced transformants (Wesley et al. 2001).

Apart from different RNAi vectors reported from several laboratories, RNAi vectors for *Arabidopsis* and maize are freely available through the Arabidopsis Biological Resource Center (ABRC, <http://www.arabidopsis.org>) which are actually donated by the Functional Genomics of Plant Chromatin consortium (<http://www.chromdb.org>). Details and complete sequences for these ChromDB RNAi vectors for instance pFGC5941 and pMCG16 are available at the ChromDB website (<http://www.chromdb.org>). The ChromDB vectors are based on pCambia plasmids developed by the Center for Application of Molecular Biology to International Agriculture (Cambia), which

contain two origins of replication, one for replication in *Agrobacterium tumefaciens* and another for replication in *Escherichia coli* (<http://www.cambia.org/>).

Despite of high specificity and efficiency of siRNA based RNAi vectors, now new generation micro RNA based RNAi vectors with high silencing accuracy and fewer side effects in plants are preferred as it does not trigger PKR pathways. PKR pathway causes, in mammalian cells, a non specific cell death by long dsRNA produced by siRNA induced pathway (Marques and Williams 2005). Plants also express PKR genes in response to different stresses and probably involve the similar mammalian cell type of PKR pathway. Furthermore, siRNAs can also affect RNAs that are not perfectly complementary, generally considered off targets (Jackson et al. 2003). In addition long double stranded precursors generate a multitude of siRNA with varying 5' and 3' ends, which make the prediction of off targets particularly difficult (Schwab et al. 2006). It has now been well understood that alteration of several nucleotides within miRNA sequence does not affects its biogenesis unless and until the initial base pairing in stem loop structure of the precursor is changed (Hervé et al. 2004). This has made possible to use modified version of natural miRNA sequences i.e. artificial miRNA (amiRNA), to target any gene of interest. The amiRNA technology was first used for gene knockdown in human cell lines, and it was successfully employed to down regulate gene expression without affecting the expression of the other unrelated genes in transgenic plants (see review Ossowski et al. 2008). These plants amiRNAs are expressed from vectors derived from precursors of ath-miR159a, ath-miR164d, ath-miR174a, ath-miR319a and Osa-miR528 (Liu et al. 2010). Animal miRNAs typically cause translational arrest of target mRNA that only partially complementary to the miRNA. Complementarity to the seed region (position 2 to 8) of the miRNA is often sufficient for effective regulation, allowing an animal miRNA to control large number of targets (Schwab et al. 2006; Lewis et al. 2005). Whereas, plant miRNAs have few (zero to five) mismatches to their targets and trigger local transcript cleavage and subsequent degradation (Llave et al. 2002). According to correct findings, a specific miRNA can only control up to 10 targets in plants (Schwab et al. 2005). To find a gene-specific gene silencing tool, a series of amiRNAs were designed to compare their effects with those of natural miRNAs. The results show that amiRNAs have only limited autonomous effects and are, therefore, quite suitable for gene-specific gene silencing in plants. Here very recently developed amiRNA based gene silencing vectors are discussed. Wang et al. (2010) reported generation of multipurpose 'Highly Efficient gene Silencing Compatible vector (HESC vector) for rice amiRNA from the precursor of Osa-amiR 528 by eliminating the central

miRNA-containing region. The method not only reduces the time for vector construction, but also saves the cost of reagent which makes it suitable for large-scale gene knockout experiments. Liu and coworkers (2010) developed simple amiRNA vector called pAmiR169d, based on the ath-miR169d precursor having two restriction sites in stem region of precursor pre-miRNA which allows to clone synthetic oligonucleotides or amplicons. They have assessed the efficacy of vector by transient assays of targeting GUS-GFP sequence in *N. benthamiana*. Warthman et al. (2008) exploited the amiRNA technology in both japonica and indica rice varieties to target silencing of three genes phytoene desaturase (*pds*), spotted leaf11 (*spl11*) and elongated uppermost internode (*Eui/CYP714D1*) using osa-miR528 precursor. This was the first report of amiRNA based gene silencing in any monocot plants to down regulate the expression of agronomically important traits. Now tasiRNA based gene silencing have also been reported as another effective approach to create dominant knockouts (Felippes and Weigel 2009).

Application of RNAi in crop improvement

Scientists all over the world working with RNAi have played a very important role in demonstrating its diverse applications as gene silencing tool in developing improved crop varieties in terms of insects and pest resistance, enhancement of their nutritional quality etc. Some of those applications are briefly discussed here.

Gene silencing: modifying metabolic pathways

In recent years, genes have been cloned for all the major enzymes that control fatty acid biosynthesis in oilseeds, including Δ -9 and Δ -12 desaturases that determine the relative proportions of C-18 saturated, monounsaturated and polyunsaturated fatty acids. Post-transcriptional gene silencing (PTGS) has been proved as a potent method to precisely down-regulate the expression of these genes during oil synthesis in the developing seed, without affecting their expression in other parts of the plant. The first successful alteration of fatty acid composition in storage oil was the enhancement of stearic acid content in rapeseed. A seed-specific antisense gene construct of *B. rapa* Δ -9 desaturase was used to reduce the enzyme activity in developing rapeseed embryos. The resulting transgenic plants showed dramatic increase in stearate levels upto 40% in seeds (Knutzon et al. 1992). High oleate transgenics were developed from the soybean transformed with *fad2-1* gene (encoding Δ -12 desaturase) transcript construct comprising of the coding region of the target gene in the sense orientation under control of a strong seed-

specific promoter (Kinney 1994). Similar results (79% increase in C18:1 fatty acid as compared with 22% in controls) were obtained with antisense expression of a Δ -12 desaturase in soybean (Kinney 1996). Also in soybean, linoleic acid content was increased by 10% by antisense expression of linoleate desaturase (Δ -15 desaturase). These findings indicate that it might be possible to increase oleic acid content upto 90% by seed-specific antisense inhibition of the respective desaturase gene. Both underexpression (by antisense or co-suppression) and overexpression strategies have been used to activate or inhibit the expression of the microsomal Δ -12 and Δ -15 desaturase genes, specifically in *Arabidopsis* seeds, in order to reduce or increase the levels of oleic acid, linoleic acid and α -linolenic acid respectively, (Cartea et al. 1998). Silencing of the endogenous Δ -12 desaturase gene by the sense suppression has resulted in substantial increase of oleic acid levels upto 89% in *B. napus* and 73% in *B. juncea* (Stoutjesdijk et al. 1999). In cotton, the siRNA approach directed against the seed-specifically expressed *fad 2-1* gene has resulted in varying increases of Δ -9:18:1 upto 77% from a starting proportion of 15% (Liu et al. 1999). Similarly, inverted repeat constructs encoding hpRNA (hairpin RNA) targeted against *sad-1* (encoding Δ -9 desaturase) driven by the seed specific soybean lectin promoter were transformed into Coker 315 cotton. Increase in the level of stearic acid was observed in over half of 26 individual transgenic lines, the highest level being 40% was approximately 20 fold greater than the 2% present in untransformed control plants (Liu et al. 2000).

Chintapakorn and Hamill (2003) used antisense approach to down regulate the activity of the important enzyme putrescine N-methyl transferase (PMT) in roots of *N. tabacum* to determine effects upon alkaloid metabolism. Transformed root lines produced markedly reduced PMT activity with a concomitant reduction in nicotine content as compared to control. Chen et al. (2004) reported transgenic down-regulation of the caffeic acid O-methyltransferase (COMT), a key lignin biosynthesis enzyme, led to an improved digestibility in tall fescue (*Festuca arundinacea*), using sense and antisense approach. Consistent and closely related molecular and biochemical data demonstrated that two co-suppressed transgenic lines were down regulated in their lignin biosynthesis. The rice mutant line LGC-1 (Low Glutelin Content-1) was the first commercially useful cultivar produced by hpRNA mediated silencing. This low protein rice is useful for patients with kidney disease whose protein intake is restricted. This dominant mutant produced hpRNA from an inverted repeat for glutelin, the gene for the major storage protein glutelin that leads to lower glutelin content in the rice through RNAi. Interestingly, this mutant was isolated in the 1970s and the mutant trait was found to stable for over 20 generations (Kusaba et al. 2003).

One of the most interesting applications of the transgene mediated RNAi for the genetic improvement of crop plants was the production of decaffeinated coffee plants. Ogita et al. (2003) described the construction of transgenic coffee plants in which expression of the gene encoding theobromine synthase (CaMXMT1) is repressed by RNA interference (RNAi) using 3' UTR (untranslated region) of CaMXMT1. Young leaves of transgenic lines expressing RNAi showed a 30–80% reduction in the theobromine content and a 50–70% reduction in the caffeine content as compare to control. Abott et al. (2002) reported a novel method of achieving a coordinated suppression using single chimeric constructs by incorporating partial sense sequence for multiple genes to target suppression of two or three lignin biosynthesis enzymes. Single chimeric transgenes was produced by fusing partial sense sequences for all possible combinations of two, or of all three, of lignin genes caffeate/5-hydroxyferulate O-methyltransferase (COMT), cinnamoyl-coenzyme A reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD). Line C0C6 (CAD-COMT-CCR) stood out as having substantial reductions in all three enzymes, with CAD reduced to 4%, COMT to 24% and CCR to 18% of wild type levels. Allen et al. (2004) reported RNAi-mediated replacement of morphine with the non narcotic alkaloid reticuline in opium poppy. They showed silencing of codeinone reductase (COR) in the opium poppy, *Papaver somniferum* using a chimeric hairpin RNA construct designed to silence all members of the multigene COR family through RNA interference. After gene silencing the precursor alkaloid (S)-reticuline, seven enzymatic steps upstream of codeinone, accumulated in transgenic plants at the expense of morphine, codeine, oripavine and thebaine.

RNAi has also been successfully used to generate a dominant high-lysine maize variant by knocking out the expression of the 22-kD maize zein storage protein that is poor in lysine content (Segal et al. 2003). Traditional breeding has been successful only for the screening of a recessive lysine-rich mutant called opaque-2 (O2). The O2 gene encodes maize with the basic leucine zipper transcriptional factor that controls the expression of a subset of storage proteins, including the 22-kDa zein storage protein. Although it is rich in lysine, the opaque mutant is not very useful in agriculture because of its adverse effect on seed quality and yield. In contrast, down regulation of the maize lysine-poor 22-kDa zein gene via RNAi does not alter the general functions of O2, but generates quality and normal maize seeds with high levels of lysine rich proteins.

Kalamaki et al. (2003) reported simultaneous transgenic suppression of LePG (*Lycopersicon esculentum*; polygalacturonase) and LeExp1 (*Lycopersicon esculentum*; expansin) which influenced rheological properties of juice and concentrates from a processing tomato variety. Their aim

of work was to examine the effects on the paste physical characteristics of suppressing LePG and LeExp1 together in a tomato variety optimized for processing, to combine improved serum properties with improved particle properties. Two types of constructs containing inverted repeats were employed to bring about gene silencing to examine their relative effectiveness. Crossing the silenced *LePG* and *LeExp1* transgenic inbred lines together resulted in the hybrid which depending upon the segregation of the two transgenes, is of four genotypes. The rheological properties of concentrates prepared from lines suppressed in both LePG and LeExp1 gene expression were compared with those of concentrates produced from hybrid fruits suppressed in only one of these genes and with azygous controls containing neither transgene.

Regina et al. (2006) reported the use of hpRNA constructs to silence an isoform of a starch-branching enzyme for the production of high amylose transgenic wheat (*Triticum aestivum*) line thereby altering its amylose-amylopectin ratio. Such lines can have wide implications in reducing the incidence of cardiovascular disease and colon cancers through its resistant starch content.

In the developing countries, it is a general practice that cotton seeds remain after fiber extraction are not utilized properly for their protein and calories content because they contain a toxic gossypol terpenoid. Transgenic cotton plants expressing RNAi construct of the δ -cadinene synthase gene of gossypol synthesis under the control of a seed-specific promoter caused seed-specific reduction of this metabolite, making cotton seeds with high nutritional value (Sunilkumar et al. 2006). Carotenoids and chlorophyll are synthesized by a common pathway hence genes controlling the light-mediated regulation of the photosynthetic machinery can alter the levels of carotenoids and flavonoids. Due to mutation in one of the regulatory gene *DE-ETIOLATED* (*DET1*), the high pigment (*hp-2*) phenotype of tomato, accumulates elevated levels of carotenoids and flavonoids. Such mutants possess abnormal growth and various vegetative phenotypes because it represses several light-dependent signaling pathways (Levin et al. 2003; Mustilli et al. 1999). RNAi-mediated suppression of *DET1* expression under fruit-specific promoter has recently shown to improve carotenoid and flavonoid levels in tomato fruits with minimal effects on plant growth and other fruit quality parameters (Davuluri et al. 2005).

Eaddy et al. (2008) produced tearless onion by silencing the lachrymatory factor synthase gene using RNA interference, so that when the onion is wounded it produced significantly reduced levels of tear-inducing lachrymatory factor. In onion (*Allium cepa*), propanthial S-oxide (lachrymatory factor [LF]), 1-propenyl methane thiosulfinate, and di-propyl disulfide are dominant sulfoxides (Block et al. 1992a, b) and chemicals responsible for inducing tear in

human, an undesirable irritant. Imai et al. (2002) discovered that the conversion of 1-propenyl sulfenic acid to LF is mediated by an enzyme called lachrymatory factor synthase (LFS). By reducing LFS and stopping the conversion of 1-propenyl sulfenic acid to the undesirable LF, onion thus generated, produced significantly reduced level of tear-inducing lachrymatory factor.

Gene silencing: protecting plants against attacks

Conferring insect-pest resistance to crop plants is another area where RNAi technology has been widely used. In this direction, Tenllado et al. (2004) showed a unique observation at least in plants that even a spray containing crude preparations of dsRNA can straightway cause gene silencing of plant viruses. Killing insects is desired to effectively decrease crop damage. Therefore, essential genes involved in metabolism are key RNAi targets because without these, insects cannot survive. A successful study showed that transgenic maize producing dsRNAs targeting a essential gene encoding a subunit of the midgut enzyme vacuolar ATPase A of western corn rootworm were effective at stunting growth and killing of this insect pest and hence gave protection against WCR infestation (Baum et al. 2007). On the other hand insects confer resistance to natural toxins, *Bt* resistance for instance. Gossypol is a compound produced by cotton plants that is toxic to insects. It has been identified that cytochrome P450 monooxygenase gene, *CYP6AE14*, which is highly expressed in the insect midgut and whose expression is correlated with larval growth when gossypol is added to artificial diet. RNAi mediated transgenic cotton producing dsRNAs targeting the *CYP6AE14* gene have been successful at partial protection against *Helicoverpa armigera* (Mao et al. 2007). Kumar et al. (2009) demonstrated potential use of acetylcholinesterase gene (*AChE*) as a target for producing RNAi mediated insect resistant plants. They have used conserved region of two homologues of AChE i.e. *AChE1* and *AChE2*, of *Helicoverpa armigera* for making siRNA to silence specifically the *AChE* gene. They observed mortality, growth inhibition of larvae, reduction in the pupal weight, malformation and fecundity when chemically synthesized siRNA molecules were directly fed to *H. armigera* larvae along with the artificial diet. Similar results have been demonstrated for sap-sucking insects wherein as much as five different putative genes (*Gene1* to *Gene5*) have been targeted for RNAi mediated silencing (Borgio 2010). Among those five genes, *Gene2* and *Gene3* showed 88.46 and 88.55% mortality after 7 days of incubation respectively when siRNA of respective gene were directly fed to adult insects along with artificial diets. However, the similar results are yet to be translated for producing insect resistance in transgenic plants using these genes.

Nematodes, such as root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes are other types of plant parasites which cause substantial loss to important crops like cereals, pulses, and vegetables in most part of the world. RNAi showed its potential to overcome these losses by silencing either plant genes which are involved in infection process or the essential genes within the nematode. Fairbairn and colleagues (2007) reported the silencing of putative transcription factor, *MjTis11* (*Meloidogyne javanica*, Tis11 Zinc finger CX₈CX₅CX₃H type putative transcription factor) of a root knot nematode, *Meloidogyne javanica*. They have produced transgenic tobacco expressing dsRNA targeting *MjTis11*. However, in this case, downregulation of *MjTis11* transcript levels in *M. javanica* did not results in a significant decrease in fecundity or egg hatching rate in any of the lines analyzed. In another report splicing factor and integrase gene of *M. incognita* were targeted to bring about host derived dsRNA mediated plant protection from this root-knot nematode infection in tobacco (Yadav et al. 2006). Huang et al. (2006) targeted *16D10* parasitism gene which is conserved across root knot nematode (RKN) spp. (*M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*) to achieve a broad spectrum resistance against these four major RKN species through host derived RNAi mediated gene silencing in *Arabidopsis*. In case of *Heterodera glycines*, a soybean cyst nematode (SCN), a major sperm protein (MSP) gene was used as target gene for producing host-derived dsRNA mediated gene silencing in transgenic soybean plants resistant against SCN (Steeves et al. 2006). Preliminary bioassay results of T₀ transgenic soybean lines indicated that the transgenic soybean were capable of suppressing the reproduction capabilities of *H. glycines* as MSP is essential for sperm motility and reproduction in this nematode.

In an attempt to generate RNAi mediated bacterial resistant plants, Escobar et al. (2001) demonstrated such silencing by targeting *iaaM* and *ipt* oncogenes of *Agrobacterium tumefaciens* which causes crown gall disease in many perennial fruit, nut and ornamental crops worldwide, by producing dsRNA mediated silencing in *A. thaliana* and *L. esculentum*. In an initial primary screening of transgenic, they observed complete abolition of tumorigenesis in 60% (3 out of 5) and 50% (3 out of 6) of tested tomato and *Arabidopsis* transformants respectively.

To confer protection against viral diseases to various crop plants, pathogen derived resistance has been linked to genetic manipulation of plants involving use of virus-derived genes or genome fragments with the purpose to interfere with a specific step during virus multiplication in the plants (Sanford and Johnston 1985). Under this approach plants transformed with constructs that produce self complementary RNAs capable of duplex formation can produce virus immunity with almost 100% efficiency when

targeted against viruses. There have been many such examples of viral resistant transgenic crops using this approach and some of them are discussed here. Waterhouse group first time exploited the deliberate use of RNA silencing to generate transgenic barley (*Hordeum vulgare*) against *Barley yellow dwarf virus* (BYDV). They have utilized hpRNA-encoding construct using maize ubiquitin promoter and targeting 5' end of the virus to transform barley. Out of 25 independent transformed barley lines with the BYDV hpRNA construct, nine lines showed extreme resistance to the virus and the majority of these contained a single transgene (Wang et al. 2000). Bonfim and colleagues (2007) reported RNAi approach to silence *ACI* gene in common bean (*Phaseolus vulgaris*) to produce transgenic lines with strong resistance to *Bean golden mosaic virus*. They used an intron spliced hpRNA vector (pBGMVRNAiAHAS) into which an *ACI* gene fragment was directionally cloned to generate sense and antisense arm. In a new approach 150 bp of N gene of four major tomato-infecting tospovirus (*Tomato spotted wilt virus*, TSWV; *Groundnut ring spot virus*, GRSV; *Tomato chlorotic spot virus*, TCSV and *Watermelon silver mottle virus*, WSMV) was taken in a single small chimeric hairpin RNA construct using intronic sequence of *A. thaliana* actin-2 gene to achieve multiple virus resistance (Bucher et al. 2006). Artificial miRNA based vectors have now attracted scientists to impart gene silencing. Qu et al. (2007) demonstrated the application of artificial miRNA using *A. thaliana* pre-miRNA 171a background to silence suppressor 2b sequence of *Cucumber mosaic virus* (CMV). They have observed effective resistance to CMV infection in transgenic tobacco lines in transient assay. In another report amiR-P69¹⁵⁹ was designed to target *Turnip yellow mosaic virus* (TYMV) suppressor, amiR-HC-Pro¹⁵⁹ using 273 bp *A. thaliana* pre-miR159a precursor. Transgenic lines carrying both ami-RNA showed specific resistance to TYMV and TuMV depending on the expression of the cognate amiRNA (Niu et al. 2006).

Future prospects

Apart from its utility in developing crop varieties resistant to stresses caused by virus, bacteria, fungi, insects, or natural disasters it has tremendous potential to be used in development of nutritionally rich, and anti-nutritional constituents (ANCs) free varieties of less utilized crops which otherwise grow well in marginal and less suited land and thrives well in conditions like flood, drought, when no other food crops survive well. Since this technology promises to impart organ/tissue specific silencing, this could be utilized in silencing or down regulation of the expression of ANCs producing gene in the plant parts of human interest of such neglected crops. However, sequences used as dsRNA trigger should be carefully selected as

long hairpin based siRNA are more likely to generate a diverse set of optimally effective siRNA. They have an increased potential to produce siRNA with off target effects. Therefore, minimal sequence determinants are highly imperative for the effectiveness of siRNA. Moreover, deployment of synthetic genes in the development of nutritionally improved and stress resistant crops should be done with great care and human exposure to such modified products should be done only after adequate testing accompanied with full disclosure to the end users.

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References

- Abott JC, Barakate A, Pincon G, Legrand M, Lapierr C, Mila I, Schuch W, Halpin C (2002) Simultaneous suppression of multiple genes by single transgenes. Down-regulation of three correlated lignin biosynthetic genes in tobacco. *Plant Physiol* 128:844–853
- Allen RS, Milligate AG, Chitty JA, Thisleton J, Miller JAC, Fist AJ, Gerlach WL, Larkin P (2004) RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. *Nat Biotechnol* 22:1559–1566
- Angell SM, Baulcombe DC (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO J* 16:3675–3684
- Baulcombe DC (1999) Fast forward genetics based on virus induced gene silencing. *Curr Opin Plant Biol* 2:109–113
- Baum JA, Bogaert T, Clinton W, Heck GR, Feld-mann P, Illagan O, Johnson S, Plaetinck G, Mu-nyikwa T, Pleau M, Vaughan TY, Robert J (2007) Control of coleopteran insect pests through RNA interference. *Nat Biotechnol* 25:1322–1326
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363–366
- Block E, Naganathan S, Putman D, Zhao SH (1992a) Allium chemistry: HPLC analysis of thiosulfates from onion, garlic, wild garlic (ramosoms), leek, scallion, shallot, elephant (great-headed) garlic, chive, and Chinese chive. Uniquely high allyl to methyl ratios in some garlic samples. *J Agric Food Chem* 40:2418–2430
- Block E, Putman D, Zhao SH (1992b) Allium chemistry: GC-MS analysis of thiosulfates and related compounds from onion, leek, scallion, shallot, chive, and Chinese chive. *J Agric Food Chem* 40:2431–2438
- Bonfim K, Faria JC, Nogueira Elsa OPL, Mendes ÉA, Aragão FJL (2007) RNAi-mediated resistance to *Bean golden mosaic virus* in genetically engineered common bean (*Phaseolus vulgaris*). *Mol Plant Microb Interact* 20:717–726
- Borgio JF (2010) RNAi mediated gene knockdown in sucking and chewing insect pests. *J Biopesticides* 3:386–393
- Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell* 123:1279–1291
- Bouche N, Lauressergues D, Gascioli V, Vaucheret H (2006) An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J* 25:3347–3356

- Bucher E, Lohuis D, van Poppel PM, Geerts-Dimitriadou C, Goldbach R, Prins M (2006) Multiple virus resistance at a high frequency using a single transgene construct. *J Gen Virol* 87:3697–3701
- Cao X, Aufsatz W, Zilberman D, Mette MF, Huang MS, Matzke M, Jacobsen SE (2003) Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. *Curr Biol* 13:2212–2217
- Cartea ME, Migdal M, Galle AM, Pelletier G, Guerche P (1998) Comparison of sense and antisense methodologies for modifying the fatty acid composition of *Arabidopsis thaliana* oilseed. *Plant Sci* 136:181–194
- Chan SW, Henderson IR, Jacobsen SE (2005) Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat Rev Genet* 6:351–360
- Chen L, Auh CK, Dowling P, Bell J, Lehmann D, Wang ZY (2004) Transgenic down-regulation of caffeic acid O-methyltransferase (COMT) led to improved digestibility in tall fescue (*Festuca arundinacea*). *Funct Plant Biol* 31:235–245
- Chintapakorn Y, Hamill JD (2003) Antisense-mediated down-regulation of putrescine N-methyltransferase activity in transgenic *Nicotiana tabacum* L. can lead to elevated levels of anatabine at the expense of nicotine. *Plant Mol Biol* 53:87–105
- Dafny-Yelin M, Chung SM, Frankman EL, Tzfira T (2007) pSAT RNA interference vectors: a modular series for multiple gene down-regulation in plants. *Plant Physiol* 145:1272–1281
- Dalmay T, Hamilton A, Mueller E, Baulcombe DC (2000a) Potato virus X amplicons in *Arabidopsis* mediate genetic and epigenetic gene silencing. *Plant Cell* 12:369–379
- Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (2000b) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101:543–553
- Davuluri GR, van Tuinen A, Fraser PD, Manfredonia A, Newman R, Burgess D, Brummell DA, King SR, Palys J, Uhlig J (2005) Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat Biotechnol* 23:890–895
- Diaz-Pendon JA, Li F, Li W-X, Ding S-W (2006) Suppression of antiviral silencing by cucumber mosaic virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. *Plant Cell* 19:2053–2063
- Donaire L, Barajas D, Martinez-Garcia B, Martinez-Priego L, Pagan I, Llave C (2008) Structural and genetic requirements for the biogenesis of tobacco rattle virus-derived small interfering RNAs. *J Virol* 82:5167–5177
- Eaddy CC, Kamoi T, Kato M, Porter NG, Davis S, Shaw M, Kamoi A, Imai S (2008) Silencing onion lachrymatory factor synthase causes a significant change in the sulfure secondary metabolite profile. *Plant Physiol* 147:2096–2106
- Eamens A, Wang M-B, Smith NA, Waterhouse PM (2008) RNA silencing in plants: yesterday, today, and tomorrow. *Plant Physiol* 147:456–468
- Escobar MA, Civerolo EL, Summerfelt KR, Dandekar AM (2001) RNAi mediated oncogene silencing confers resistance to crown gall tumorigenesis. *Proc Natl Acad Sci USA* 98:13437–13442
- Fairbairn DJ, Cavalloro AS, Bernard M, Mahalinga-Iyer J, Graham MW, Botella JR (2007) Host-delivered RNAi: an effective strategy to silence genes in plant parasite nematodes. *Planta* 226:1525–1533
- Felippes FF, Weigel D (2009) Triggering the formation of tasiRNAs in *Arabidopsis thaliana*: the role of microRNA miR173. *EMBO Rep* 10:264–270
- Gascioli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr Biol* 15:1494–1500
- Goselle V, Fache I, Meulewaeter F, Cornelissen M, Metzlaiff M (2002) SVISS- novel transient gene silencing system for gene function discovery and validation in tobacco plants. *Plant J* 32:859–866
- Gupta NC, Sinha SK, Jolly M, Dubey N, Sachdev A (2009) Antisense RNA-mediated inhibition of *GmFAD2-1* encoding omega-6-desaturase. *Indian J Plant Physiol* 14:336–343
- Hervé V, Vazquez F, Crété P, Bartel DP (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* 18:1187–1197
- Higuchi M, Yoshizumi T, Kuriyama T, Hara H, Akagi C, Shimada H, Matsui M (2009) Simple construction of plant RNAi vectors using long oligonucleotides. *J Plant Res* 122:477–482
- Hirai S, Oka S, Adachi E, Kodama H (2007) The effects of spacer sequences on silencing efficiency of plant RNAi vectors. *Plant Cell Rep* 26:651–659
- <http://www.pi.csiro.au/RNAi/vectors.htm/hairpin> RNAi Vectors for Plants. Accessed 20 Jan 2010
- Huang G, Allen R, Davis EL, Baum TJ, Hussey RS (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proc Natl Acad Sci USA* 103:14302–14306
- Imai S, Tsuge N, Tomotake M, Nagatome Y, Sawada H, Nagata T, Kumagai H (2002) Plant biochemistry: an onion enzyme that makes the eyes water. *Nature* 419:685
- Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 21:635–637
- Kalamaki MS, Harpster MH, Palys JM, Labavitch JM, Reid DS, Brummell DA (2003) Simultaneous transgenic suppression of LePG and LeExp1 influences rheological properties of juice and concentrates from a processing tomato variety. *J Agric Food Chem* 51:7456–7464
- Kanno T, Mette MF, Kreil DP, Aufsatz W, Matzke M, Matzke AJM (2004) Involvement of a putative SNF2 chromatin remodeling protein DRD1 in RNA-directed DNA methylation. *Curr Biol* 14:801–805
- Kanno T, Huettel B, Mette MF, Aufsatz W, Jaligot E, Daxinger L, Kriel DP, Matzke M, Matzke AJM (2005) Atypical RNA polymerase subunits required for RNA-directed DNA methylation. *Nat Genet* 37:761–765
- Katiyar-Agarwal S, Gao S, Vivian-Smith A, Jin H (2007) A novel class of bacteria-induced small RNAs in *Arabidopsis*. *Genes Dev* 21:3123–3134
- Kennerdell JR, Carthew RW (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* 95:1017–1026
- Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115:209–216
- Kinney AJ (1994) Genetic modification of the storage lipids of plants. *Curr Opin Biotechnol* 5:144–151
- Kinney AJ (1996) Development of genetically engineered soybean oils for food application. *J Food Lipids* 3:273–292
- Knutzon DS, Thompson GA, Radke SE, Johnson WB, Knauf VC, Krill JC (1992) Modification of *Brassica* seed oil by antisense expression of a stearyl-acyl carrier protein desaturase gene. *Proc Natl Acad Sci USA* 89:11184–11188
- Kumar M, Gupta GP, Rajam MV (2009) Silencing of acetylcholinesterase gene of *Helicoverpa armigera* by siRNA affects larval growth and its life cycle. *J Insect Physiol* 55:273–278
- Kusaba M, Miyahara K, Lida S, Fukuoka H, Takano T, Sassa H, Nishimura M, Nishio T (2003) Low glutelin content 1: a dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice. *Plant Cell* 15:1455–1467

- Levin I, Frankel P, Gilboa N, Tanny S, Lalazar A (2003) The tomato dark green mutation is a novel allele of the tomato homolog of the DEETIOLATED1 gene. *Theor Appl Genet* 106:454–460
- Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15–20
- Lipardi C, Wei Q, Paterson BM (2001) RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* 107:297–307
- Liu Q, Singh SP, Brubaker CI, Sharp PJ, Green AG, Marshall DR (1999) Molecular cloning and expression of a cDNA encoding microsomal ω -6 desaturase from cotton (*Gossypium hirsutum*). *Aust J Plant Physiol* 26:101–106
- Liu Q, Singh S, Green A (2000) Genetic modification of cotton seed oil using inverted-repeat gene-silencing techniques. *Biochem Soc Trans* 28:927–929
- Liu C, Zhang L, Sun J, Luo Y, Wang MB, Fan YL, Wang L (2010) A simple artificial microRNA vector based on athmiR169d precursor from *Arabidopsis*. *Mol Biol Rep* 37:903–909
- Llave C, Kasschau KD, Rector MA, Carrington JC (2002) Endogenous and silencing-associated small RNAs in plants. *Plant Cell* 14:1605–1619
- Luo K, Harding SA, Tsai CJ (2008) A modified T-vector for simplified assembly of hairpin RNAi constructs. *Biotechnol Lett* 30:1271–1274
- Mao YB, Cai W, Wang J, Hong G, Tao X, Wang L, Huang Y, Chen X (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat Biotechnol* 25:1307–1313
- Marques JT, Williams BR (2005) Activation of the mammalian immune system by siRNAs. *Nat Biotechnol* 23:1399–1405
- Miki D, Shimamoto K (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol* 45:490–495
- Montgomery MK, Fire A (1998) Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. *Trends Genet* 14:255–258
- Mustilli AC, Fenzi F, Ciliento R, Alfano F, Bowler C (1999) Phenotype of the tomato high pigment-2 mutant is caused by a mutation in the tomato homolog of DEETIOLATED1. *Plant Cell* 11:145–157
- Napoli C, Lemieux C, Jorgensen R (1990) Introduction of chimeric chalcone synthase gene into *Petunia* results in reversible cosuppression of homologous genes in trans. *Plant Cell* 2:279–289
- Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat Biotechnol* 24:1420–1428
- Ogita S, Usefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) Producing decaffeinated coffee plants. *Nature* 423:823
- Ossowski S, Schwab R, Weigel D (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J* 53:674–690
- Peele C, Jordan CV, Muangsang N, Turnage M, Egelcrout E, Eagle P, Hanley-Bowdoin L, Robertson D (2001) Silencing of a meristem gene using geminivirus-derived vectors. *Plant J* 27:357–366
- Qu J, Ye J, Fang RX (2007) Artificial microRNA-mediated virus resistance in plants. *J Virol* 81:6690–6699
- Qu F, Ye X, Morris TJ (2008) *Arabidopsis* DRB4, AGO1, and AGO7 participate in a DCL4-initiated antiviral RNA silencing pathway that is negatively regulated by DCL1. *Proc Natl Acad Sci USA* 105:14732–14737
- Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, Kosar-Hashemi B, Li Z, Rahman S, Morell M (2006) High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. *Proc Natl Acad Sci USA* 103:3546–3551
- Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance. *J Theor Biol* 113:395–405
- Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of microRNAs on the plant transcriptome. *Dev Cell* 8:517–527
- Schwab R, Ossowski S, Riester M, Warthman N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. *Plant Cell* 18:1121–1133
- Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115:199–208
- Segal G, Song R, Messing J (2003) A new opaque variant of maize by a single dominant RNA-interference inducing transgene. *Genetics* 165:387–397
- Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107:465–476
- Sinha SK (2006) Designing of intron-spliced hairpin construct for silencing of *fad 2-1* gene of *Glycine max* L. PhD Thesis, Division of Biochemistry, Indian Agricultural Research Institute, New Delhi, India
- Sinha SK, Dubey N, Sachdev A (2006) Intron-spliced gene silencing construct for alteration of seed fatty acid composition by RNAi induced transgene silencing. *New Bot* 33:181–191
- Sinha SK, Dubey N, Sachdev A (2008) Gene silencing constructs for alteration of seed fatty acid composition by RNAi induced transgene silencing. *New Bot* 35:23–32
- Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* 407:319–320
- Steeves RM, Todd TC, Essig JS, Trick HN (2006) Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. *Funct Plant Biol* 33:991–999
- Stoutjesdijk PA, Hurlstone C, Singh SP, Green AG (1999) Proceedings of the 10th International Rapeseed Congress, Canberra Australia
- Sunilkumar G, Campbell LM, Puckhaber L, Stipanovic RD, Rathore KS (2006) Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. *Proc Natl Acad Sci USA* 103:18054–18059
- Tabara H, Grishok A, Mello CC (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* 282:430–431
- Tenllado F, Llave C, Diaz-Ruiz JR (2004) RNA interference as a new biotechnological tool for the control of virus diseases in plants. *Virus Res* 102:85–96
- Vaistij FE, Jones L, Baulcombe DC (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* 14:857–867
- Vaucheret H (2005) RNA polymerase IV and transcriptional silencing. *Nat Genet* 37:659–660
- Voinnet O, Vain P, Angell S, Baulcombe DC (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95:177–187
- Wang MB, Abbott DC, Waterhouse PM (2000) A single copy of a virus derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. *Mol Plant Pathol* 1:347–356
- Wang Z, Chen C, Xu Y, Jiang R, Han Y, Xu Z, Chong K (2004) A practical vector for efficient knockdown of gene expression in Rice (*Oryza sativa* L.). *Plant Mol Biol Rep* 22:409–417
- Wang X, Yang Y, Yu C, Zhou J, Cheng Y, Yan C, Chen J (2010) A highly efficient method for construction of rice artificial Micro-RNA vectors. *Mol Biotechnol*. doi:10.1007/s12033-010-9291-4

- Warthmann N, Chen H, Ossowski S, Weigel D, Herve P (2008) Highly specific gene silencing by artificial miRNAs in rice. *PLoS ONE* 3:e1829
- Waterhouse PM, Helliwell CA (2003) Exploring plant genomes by RNA-induced gene silencing. *Nat Rev Genet* 4:29–38
- Wesley V, Helliwell C, Smith N, Wang MB, Rouse D, Liu Q, Gooding P, Singh S, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and highthrough put gene silencing in plants. *Plant J* 27:581–590
- Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol* 2:E104
- Xie Z, Allen E, Wilken A, Carrington JC (2005) DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 102:12984–12989
- Yadav BC, Veluthambi K, Subramaniam K (2006) Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. *Mol Biochem Parasitol* 148:219–222
- Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101:25–33
- Zilberman D, Cao X, Jacobsen SE (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* 299:716–719