Review TRENDS in Biotechnology Vol.22 No.9 September 2004

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Using RNAi to improve plant nutritional value: from mechanism to application

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RNA interference (RNAi) is an ancient mechanism of gene suppression, whose machinery and biological functions are only partially understood. Intensive studies have focused on developing RNAi technologies for treating human diseases and for improving plant traits. Yet application of RNAi to improving the nutritional value of plants for human and animal nutrition, and development of the related RNAi technologies are still in their infancy. Here we discuss current knowledge of plant RNAi function, as well as concepts and strategies for the improvement of plant nutritional value through the development of plant RNAi technologies.

Although total yield is still the first priority for both traditional plant breeding and contemporary plant genetic engineering in developing countries, the goal of improving the nutritional value of plants is receiving increasing attention [1,2]. Some major diseases, such as heart disease and cancer, can be prevented by dietary supplements of specific nutrients [3]. In particular, essential amino acids, minerals, fatty acids and vitamins are key factors for robust human health and growth [2], and a diet of plant foods rich in essential nutrients can significantly improve human health and life expectancy [4].

Traditional breeding has been tremendously successful in improving the nutritional value of food and feed [5]; however, this process is time-consuming and the limited genetic resources of most crops have left little room for continued improvement by these means. Over the past few decades, the possibilities for improvement have been broadened by extensive gene mapping and identification, whole-genome sequencing of model plants and crops, and the development of gene transfer technologies. Directed efforts are now underway to use genetic engineering of metabolic pathways to alter plant nutrients [2]. These efforts depend on a detailed understanding of plant metabolic pathways and their constituent enzymes.

Currently, the principal strategy for transgenic enhancement of plant nutrients involves increasing the expression of anabolic biosynthetic genes. Unfortunately, the efficacy of this approach for crop engineering has been restricted by two main obstructions. First, the introduction of extra gene copies can have the non-intuitive effect of decreasing expression from both the introduced and homologous endogenous loci – a phenomenon of gene silencing known as CO-SUPPRESSION (See Glossary) [6]. Second, feedback metabolic loops tend to maintain homeostatic levels of nutrients. For example, plants might respond to an increase in nutrient production induced by overexpression of biosynthetic genes by activating a degradation pathway that either negates nutrient accumulation or converts nutrients into undesirable metabolites [7]. It is therefore necessary to consider schemes for metabolic engineering that decrease the levels of catabolic enzymes; effective and expedient methods to achieve this, however, have been wanting in the past.

Ironically, the very phenomenon of co-suppression that plagues some overexpression efforts might be useful for realizing such a reduction in catabolic enzymes. Co-suppression has been recently recognized as a manifestation of RNA INTERFERENCE (RNAi) – an endogenous pathway of negative posttranscriptional regulation. RNAi has revolutionized the possibilities for creating customized 'knock-down' of gene activity. RNAi operates in both plants and animals and uses double-stranded RNA (dsRNA) as a trigger that targets homologous mRNAs for degradation. Methods that introduce dsRNA into plant and animal cells have been enormously successful in decreasing cognate gene expression *in vivo* [8–11].

In this review, we first examine current understanding of the endogenous RNAi pathway in plants. We then discuss strategies and applications of RNAi for improving plant nutritional value via the coordinated overexpression and suppression of genes in plants. Finally, we discuss the development of plant RNAi technologies.

The RNAi pathway in plants

The phenomenon of plant co-suppression was accidentally discovered during attempts to alter the pigmentation of commercial petunia flowers [6]. It was supposed that deeper flower colors might result from the overexpression of a chalcone synthase gene driven by the constitutive 35S promoter. Instead, both endogenous and transgenic chalcone synthase genes were silenced in these plants,

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Available online 29 July 2004

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Glossary

Antisense technology: In mammalian cells, an antisense molecule can be RNA, DNA or their modified forms. In plants, antisense molecules refer to RNAs that are produced by overexpressing the antisense strands of specific genes. These antisense RNAs were proposed to anneal with specific mRNAs or to recruit RNA-dependent RNA polymerase (RdRP) enzyme to produce dsRNAs. Antisense technology was originally used in mammalian cells and thought to target the cellular mRNA molecules by repressing mRNA translation. The mechanism of antisense-RNA-mediated gene silencing in plants is related to RNAi by the feature that dsRNAs are produced as triggers for RNA degradation. The difference between antisense- and dsRNA-triggered RNAi technology is that the latter efficiently produces dsRNA for degradation of target mRNA and that formation of dsRNAs in the former might be a slow process.

Co-suppression: The silencing of an endogenous plant gene triggered by extra copies of a transgene with the same sequence. Co-suppression can reflect either transcriptional repression (transcriptional gene silencing) or posttranscriptional gene silencing. The latter process is the plant version of RNAi.

Dicer: The term Dicer was initially coined by Greg Hannon to describe a *Drosophila* multidomain enzyme of the RNase III family. *Drosophila* Dicer cuts long dsRNA into small dsRNAs of 21–23 nucleotides with a 3′ overhang, known as siRNAs. In plants, at least two types of detectable Dicer activity are responsible for the production of siRNAs with distinct sizes of 21 and 24 nucleotides. These plant enzymes are known as 'Dicer-like enzymes'. Because plants have several Dicer homologs, they are sometimes referred to individually as Dicer-like I, Dicer-like II, and so on.

MicroRNA (miRNA) pathway: miRNAs are an abundant class of non-coding small RNAs (of 21–24 nucleotides) that are present in diverse eukaryotes and formed by Dicer or Dicer-like enzymes. In animals most miRNAs function by repressing the translation of specific target mRNAs, but most plant miRNAs function like natural siRNAs to target specific mRNAs for cleavage. The only difference between plant and animal miRNAs is the extent of their complementarity to target mRNAs. Whereas plant miRNAs have extensive complementarity to their target mRNAs, animal miRNAs are much less homologous and, when paired to their targets, form bulges that are proposed to block the translation of the target mRNA.

miRNA precursor: An imperfect stem–loop RNA structure generated from a long transcript, known as primary miRNA, that is encoded by a non-protein-coding region in the genome. Dicer or Dicer-like enzyme cleaves a miRNA precursor and produces a mature miRNA.

RNA-dependent RNA polymerase (RdRP): An RNA polymerase that is involved in RNA silencing in plants, worm, *Neurospora* and *Dictyostelium*, but not in *Drosophila* or human cells. RdRPs have been proposed to use cellular aberrant RNAs as templates and to copy them into cRNAs to form dsRNA. This newly synthesized dsRNA is thought to act as a substrate for Dicer-like enzymes.

RNA-induced silencing complex (RISC): An siRNA– or miRNA–protein complex that acts as an endonuclease and cleaves the complementary target mRNA, or as a repressor and blocks the target mRNA translation.

RNA interference (RNAi): A specific form of RNA silencing that reflects posttranscriptional RNA degradation induced by exogenous dsRNA. The term RNAi is now used widely to describe RNA silencing in both plants and animals.

RNA silencing: A group of related phenomena in diverse eukaryotes in which aberrant or dsRNA triggers a marked reduction in either transcription of the corresponding gene or direct degradation of the corresponding mRNA.

Small interfering RNA (siRNA): Small RNAs (21–25 nucleotides) that is produced from long dsRNA by Dicer or Dicer-like enzymes or chemically synthesized, and can be recruited by multiple cellular proteins to form an RNA-induced silencing complex (RISC) that interferes with mRNA stability or mRNA translation.

Virus-induced gene silencing (VIGS): A technology that exploits an RNA-mediated antiviral defense mechanism. It describes a phenomenon in which both a viral gene and its host homologous gene are silenced upon the viral infection of its host. A recombinant virus engineered with a piece of its host gene can efficiently silence this host gene by viral infection.

resulting in mosaic flowers with unpigmented white sectors [6].

Eight years later, Craig Mello and Andrew Fire found that traces of dsRNA in *Caenorhabditis elegans* triggered a marked silencing of genes containing sequences identical to the dsRNA [12,13]. They called this unconventional gene silencing 'RNA interference'. Re-examination of plant co-suppression, as well as plant gene silencing mediated by ANTISENSE TECHNOLOGY, showed that both processes led to the cellular production of dsRNAs. These RNA species activated the RNAi pathway, resulting in the observed silencing of both homologous endogenous and introduced loci [14–17].

The process of RNAi has been dissected biochemically in both plants and animals and found to be conserved among diverse eukaryotes. Zamore *et al.* [18] empirically showed that small RNAs of 21–23 nucleotides, named SMALL INTERFERING RNAs (siRNAs), are the key factors in mediating specific RNA degradation in an *in vitro Drosophila* system. siRNAs were predicted and confirmed to be the direct products of dsRNA cleavage by the multidomain RNase III enzyme DICER [19,20]. siRNAs are extremely similar in length to those discovered previously in VIRUS-INDUCED GENE SILENCING (VIGS) in plants [15], and indeed DICER ACTIVITY is readily detected in wheat germ and cauliflower extracts [14]. siRNAs are subsequently assembled first into a multiprotein complex called an siRNP, and then into an active RNA-INDUCED SILENCING COMPLEX (RISC); these complexes seek out and cleave target mRNAs that are complementary to the siRNA [20–22] (Figure 1).

An endogenous negative gene regulatory pathway, known as the MICRORNA (MIRNA) PATHWAY, also uses small RNAs of about 22 nucleotides [14,23–28]. miRNAs are universally derived from longer precursor transcripts that adopt a stem–loop structure with significant, but imperfect, double-stranded character. Dicer is responsible for cleaving the MIRNA PRECURSOR to produce the mature miRNA [23,29–31]. Both siRNAs and miRNAs are thought to be assembled into similar RISC structures that regulate complementary RNA (cRNA) targets by targeting them either for cleavage or for translation repression [14,32,33] (Figure 1). In plants, most identified miRNAs show extensive or complete complementarity to their presumed target mRNAs, and many of these mRNAs have been shown to be subject to miRNA-mediated cleavage [14,33,34].

The endogenous plant RNA SILENCING machinery also involves an RNA-DEPENDENT RNA POLYMERASE (RdRP). This polymerase uses RNA templates to synthesize cRNAs in either a primed or non-primed manner [14]; the cRNAs anneal to form dsRNAs, which are then processed by Dicer to generate siRNAs (Figure 1). Transgenic plants designed to overexpress exogenous or extra copies of endogenous genes often produce aberrant mRNAs with incomplete coding regions. These aberrant RNA species have been proposed to function as templates for RdRP, which might mediate their clearance via activation of the RNAi pathway [35]. The requirement for RdRP in plant RNAi can be bypassed by the expression of dsRNA in the form of RNA containing long inverted repeats [36], and RNAi in some animal species, such as *Drosophila* and human, does not seem to involve RdRP at all, because these species lack the corresponding genes [37]. Notably, RNA silencing can spread over the plants from one region to another, and RdRP has been proposed to have a role in this 'spread of silencing' (Figure 1) [38,39].

Approaches to reduce the expression of undesirable genes

Two general approaches are commonly used to reduce the levels of undesirable gene products: recessive gene

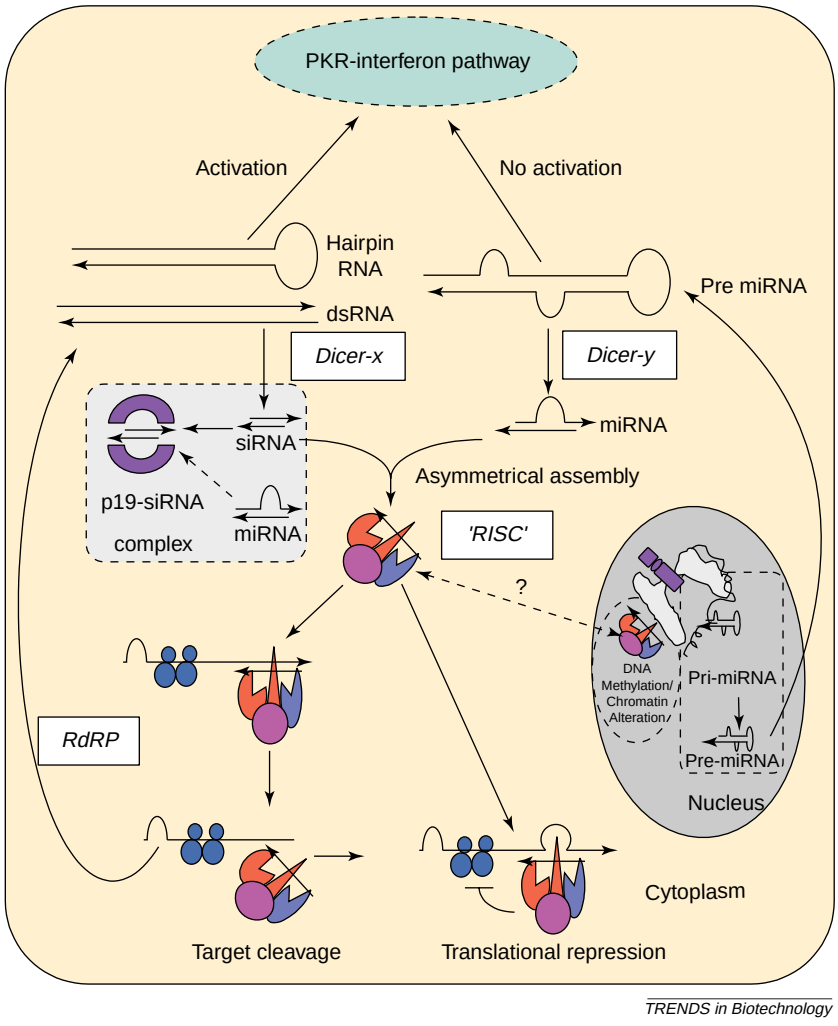

Figure 1. RNA interference (RNAi) and microRNA (miRNA) pathways and their interaction with the PKR–interferon pathway and the viral silencing suppressor p19. Double-stranded RNA (dsRNA) or hairpin RNA is processed by a multidomain RNase-III-like enzyme, Dicer-x, into siRNAs of 21–24 nucleotides; similarly, an miRNA precursor (Pre-miRNA), derived from a primary miRNA (Pri-miRNA) transcript, is converted perhaps by a different type of RNase-III-like enzyme, Dicer-y, into a highly asymmetrical miRNA duplex. Both small interfering RNA (siRNA) and miRNA duplexes might be assembled into 'RNA-induced silencing complex (RISC)-like complexes according to asymmetrical rules, with one strand favorably in the RISC and the other strand possibly degraded. The assembled RISCs find their specific target mRNAs for either cleavage or translational repression on the basis of complementarity between the siRNAs or miRNAs and their targets. The cleaved RNA products, like the hypothetical 'aberrant RNAs', might act as templates for RNA-dependent RNA polymerase (RdRP) to synthesize new populations of dsRNAs in an unprimed or primed manner, which would result in silencing spreading from the 3′ to 5′ end of a gene transcript. RISCs might also be able to traverse the nucleus and trigger transcriptional silencing of specific genes via chromatin alteration or DNA methylation. In addition, the viral silencing suppressor p19 binds siRNA duplex with extremely high affinity, and thereby blocks siRNA-programmed RISC assembly, resulting in the silencing of silencing (counter-silencing). Similarly, p19 might also bind the hypothetical miRNA duplex and block miRNA function, causing marked changes in development. Finally, long dsRNA and hairpin RNA, and perhaps siRNA duplexes, could activate the host PKR–interferon pathway, leading to nonspecific cell death. By contrast, miRNA precursor or miRNA duplexes with unpaired bulges and/or mismatches might be fairly benign in cells and inert in activating PKR–interferon pathways, which is the base for designing second-generation silencing vectors or miRNA vectors.

disruption and dominant gene silencing. In gene disruption approaches the target sequence is mutated to eliminate either expression or function, whereas in dominant gene silencing either destruction of the gene transcript or inhibition of transcription is induced. The advantages of the dominant gene silencing methodologies over the gene disruption approach are twofold.

First, dominant gene silencing is easier to bring about genetically and screening of the resultant transgenic plants is also more straightforward. Second, in contrast to the gene disruption approach, dominant gene silencing can be done in a spatial and temporal manner by using specific promoters.

Of the dominant gene silencing approaches, dsRNA-triggered RNAi is apparently the most powerful [40]: it is the most efficient in terms of the extent of gene silencing, and the resulting silencing is almost as complete as that achieved in a gene knockout approach. It seems that dsRNA-triggered RNAi directly bypasses the requirement for dsRNA synthesis via RdRP, which is probably the rate-limiting step in the plant RNAi pathway.

Development of plant RNAi technologies

In the first case study of gene silencing via dsRNA-triggered RNAi technology in plants, inverted repeats were used to overexpress dsRNAs that triggered highly efficient silencing of flower identity genes [41]. This vector-based RNAi technology was further improved by Peter Waterhouse and colleagues [40] by introducing an intron as the linker. These RNAi vectors are specifically designed

to generate long dsRNA species that have the same sequence as the target genes.

Similarly, vectors designed to express hairpin RNAs have also been successfully applied to silence the corresponding target genes [42]. Constitutive expression of dsRNA or hairpin RNA often leads to unexpected adverse effects on plant growth and development. Consequently, chemically inducible RNAi silencing vectors have been developed to enable temporal and spatial control of gene silencing [43,44]. The application of such chemically inducible silencing systems to the study of plant functional genomics is significant, but large-scale use of chemicals for plant improvement is impractical and harmful to the environment. Tissue- or organ-specific control of gene silencing might be a better choice for the development of plant RNAi technologies.

Another approach to silence genes in plants is VIGS [45,46]. In this approach, target genes can be transiently inactivated by infecting the plants with a recombinant virus that expresses fragments of the endogenous plant gene transcripts. In essence, VIGS achieves RNAi via viral induction without introducing any genetic change in plants. VIGS is very useful for gene functional studies [45], but it can do nothing to change genetic information for plant improvement.

Strategies for improving plant nutritional value: advantages of RNAi

Because plants represent the principal source of human foods and livestock feeds, many efforts to improve the nutritional content of plants have focused on plant breeding. This can be done either by classical breeding based on selection of the natural or induced genetic variations, or by genetic engineering of transgenic plants. Genetic engineering technologies have advantages over classical breeding not only because they increase the scope of genes and the types of mutation that can be manipulated, but also because they have the ability to control the spatial and temporal expression patterns of the genes of interest.

Why is the control of the spatial and temporal expression so important for crop improvement? In many crops, the tissue that is consumed as food (mainly seeds) is distinct from the tissues that control plant growth and productivity (mainly roots and shoots). In many cases, however, genes controlling specific traits do not operate in a tissue-specific manner, but function in all or most plant organs. Therefore, a mutation of a given gene that is beneficial for improving seed quality is often deleterious for the growth of other plant organs.

This problem is well illustrated by efforts to increase the level of lysine in plants. Lysine synthesis is strongly regulated by lysine-mediated feedback inhibition of the activity of dihydrodipicolinate synthase (DHPS), the first enzyme specifically committed to lysine biosynthesis. Genetic mutations in plant DHPS genes to render them insensitive to lysine cause the overproduction of lysine in all plant organs [47,48]. But although an increase in lysine in seeds is beneficial, an increase in vegetative tissues is undesirable, because high concentrations of lysine cause abnormal vegetative growth and flower development, which in turn reduce seed yield [47,48]. Targeted expression of transgenic DHPS in seeds of several crop plants by using seed-specific promoters eliminates its undesirable effects in vegetative tissues, resulting in plants with good growth characteristics that accumulate high concentrations of lysine in their seeds [49,50].

Tissue-specific manipulation holds importance not only for gene overexpression, but also for gene suppression approaches. The accumulation of desirable metabolites in plant seeds might also be negatively regulated by enzymes that either degrade or convert them into undesirable metabolites. Such enzymes could be suppressed, but their constitutive suppression might also have adverse effects on plant growth and reproduction. Again, the essential amino acid lysine provides a good example to illustrate this problem, because the accumulation of lysine in plants is negatively affected by its catabolism (degradation) into glutamate and acetyl coenzyme A (CoA) [7,51]. When combined with the seed-specific expression of a feedback-insensitive DHPS mutant, constitutive knockout of lysine catabolism by a gene insertion knockout approach accelerates lysine accumulation in seeds [52]; however, the seeds of plants that accumulate increased amounts of lysine germinate poorly, apparently because the excess lysine produced in the seeds is not degraded efficiently during seed germination [52]. Reduction of lysine catabolism specifically during seed development by an RNAi approach indeed improves seed germination [53].

RNAi technology has also been used in several other plants to improve their nutritional quality. For example, caffeine content in coffee plants has been markedly reduced by RNAi-mediated suppression of the caffeine synthase gene [54]. In another study, RNAi has been successfully used to generate a dominant high-lysine maize variant by knocking out the expression of the 22-kD maize zein storage protein, a protein that is poor in lysine content [55]. Traditional breeding has been successful only for the screening of a recessive lysine-rich mutant called *opaque 2* (*O2*). The *O2* gene encodes a maize 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 2* mutant is not very useful in agriculture because of its adverse effects on seed quality and yield. By contrast, downregulation 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.

RNAi technology has also been successful in genetic modification of the fatty acid composition of oil. RNAi mediated by a hairpin RNA has been used in cotton to downregulate two key fatty acid desaturase genes encoding stearoyl-acyl-carrier protein Δ9-desaturase and oleoyl-phosphatidylcholine ω6-desaturase [56]. Knockdown of these two genes in cotton leads to an increase in nutritionally improved high-oleic and high-stearic cottonseed oils, which are essential fatty acids for health of the human heart. Almost certainly, more examples of crops improved by dsRNA-triggered RNAi technology will be reported in the coming years.

Counter-silencing to overexpress valuable genes in plants

It is generally thought that RNAi originally evolved as a defense mechanism against invasive nucleic acids, including those of viruses and transposons. For example, during viral propagation most plant RNA viruses form a dsRNA intermediate, which can be cleaved by Dicer to generate siRNAs that will target the viral RNA genome for degradation. A logical counter-defense by many viruses has been to evolve RNAi inhibitors, which allow them to infect plants productively even in the presence of an active RNAi pathway [57–59]. This event seems to have happened often during evolution, and some 20 different RNA silencing suppressors have been identified from different plant viruses in recent years.

These suppressors of RNA silencing might prove to be useful tools not only for dissecting the biochemical steps of RNAi [60,61], but also for promoting the expression of plant transgenes. For example, the p19 protein encoded by tombusviruses was recently found to inhibit RNAi by binding siRNAs with high affinity [61–65] (Figure 1). When the host silencing response was suppressed by p19 in tobacco plants, the expression of various transgenes in a transient expression assay was enhanced by more than 50-fold [66]. Transgenic plants expressing p19 or other RNA silencing suppressors might be therefore able to overexpress desired genes that up until now have been found to be mysteriously suppressed.

Ironically, then, it could be that both RNAi and inhibitors of RNAi, which are themselves the product of host–virus coevolution, could be ultimately co-opted by people to engineer crops for human benefit. Because viral silencing suppressors might also affect the biogenesis of endogenous miRNAs [67], however, this co-option will also depend on fine-tuning the expression of the silencing suppressors to a proper developmental stage to avoid any possible interference with normal development pathways programmed by endogenous miRNAs.

Next-generation RNAi vectors: what can we learn from miRNAs?

The current RNAi vectors were designed to produce either short siRNAs, such as those produced by animal RNAi vectors, or long dsRNAs, such as produced by plant RNAi vectors. Animal RNAi vectors generally use U6 or H1 RNA polymerase III promoters to express RNAs with small stem–loop or hairpin structures to silence endogenous genes [68–70]; however, siRNAs and long dsRNAs produced by these RNAi vectors tend to activate RNA-dependent protein kinase (PKR) pathways and cause nonspecific cell death in mammalian cells [71–73]. Plants also express PKR genes and might have a similar pathway that could function as part of the plant stress response [74]. Avoiding activation of the PKR pathway in cells remains a major challenge to the development of RNAi technologies.

By contrast, the endogenous expression of extensive miRNAs in plants and animals does not show adverse effects other than the programmed roles of these miRNAs in gene regulation for proper development [24,75,76]. It seems that the structure of miRNAs has been selectively evolved to avoid triggering the PKR pathway. Developing next-generation RNAi vectors with characteristics of miRNA structures might provide a safer alternative and be more advantageous to controlling gene expression for several reasons.

First, miRNAs do not trigger the PKR pathway. Second, miRNAs accurately target gene transcripts for destruction or translational repression with high efficiency [14,33,75]. A recent study has shown that siRNA delivered on the backbone of human miR30 was 80% more effective in reducing the target gene products than was siRNA expressed by conventional short hairpin RNA [77]. Third, miRNA expression is subject to temporal and spatial regulation [23,78–80]. Dissecting miRNA gene structures will provide more choice for the development of tissue-specific RNAi vectors. Fourth, a single miRNA can target several different genes or members of a gene family, such as the miR165/miR166-directed cleavage of *PHV* and *PBH* [14,34]. Last, single-stranded miRNAs are initially generated as siRNA-like duplexes, whose structures are highly asymmetrical in energy at the duplex ends [22,81]. These asymmetrical duplex structures predestine one strand to enter the RISC to its maximum, while the other strand is probably destroyed [22]. As a result, miRNA-like small RNAs produced by miRNA-based siRNA vectors should show higher preference for RISC assembly and should direct efficient cleavage of their target mRNAs.

Although the miRNA-based vector has not been developed, it shows great potential for use in both plants and animals. More studies are needed for the successful development of miRNA vectors: for example, the miRNA duplex structure should be dissected to derive rules for designing miRNA-like siRNAs; and miRNA gene elements should be tested for the temporal and spatial expression of siRNAs.

Conclusions and perspective

The nutritional value of human vegetable foods has an increasing role in the prevention of various human diseases associated with malnutrition. Tremendous efforts have been invested in improving the nutritional value of human plant foods and livestock feeds. RNAi triggered by dsRNA has great advantages over antisense and co-suppression approaches, owing to its higher gene silencing efficiency and the shorter time needed to screen the targeted plants.

Tissue- or organ-specific RNAi vectors are needed to achieve targeted gene silencing in particular plant tissues and organs with minimal interference to the normal plant lifecycle. Future dissection of miRNA gene structures will greatly facilitate the development of RNAi vectors with high silencing efficiency and fewer side-effects in plants. Genetic engineering of highly nutritional food crops requires both gene silencing and counter-silencing technologies. Developing vectors that can suppress the RNAi pathway, while overexpressing transgenes will revolutionize this field. Such vectors might be based on viral suppressors of RNA silencing. Future research will focus on the development and fine-tuning of RNAi-based gene silencing vectors that can operate in a temporally and spatially controlled manner (Figure 2).

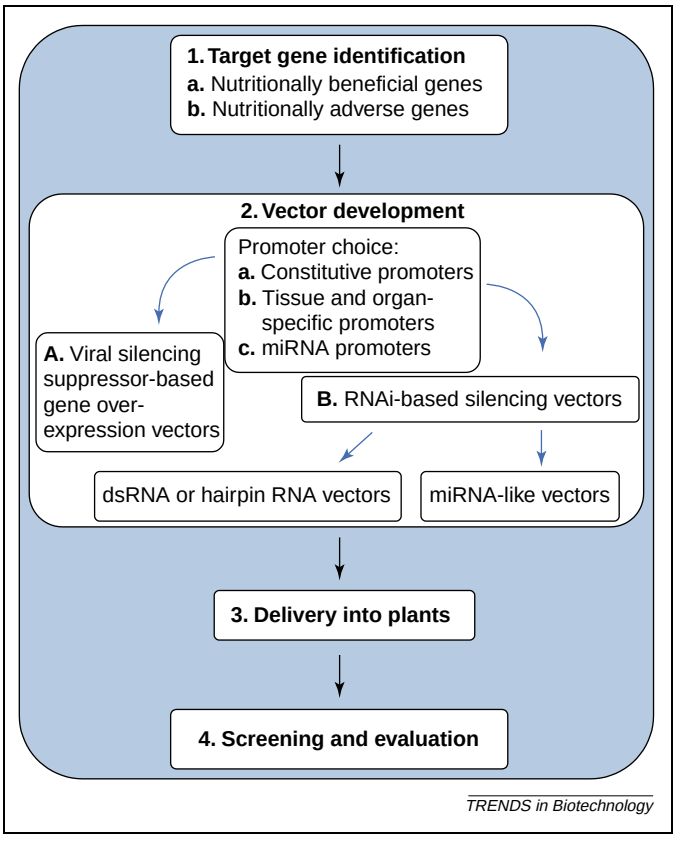

Figure 2. RNA interference (RNAi)-based technologies for improving the nutritional qualities of plants. (1) The first step is to identify and to clone the target genes, including nutritionally beneficial genes and adverse genes. (2) The second step is to coordinatively overexpress nutritionally beneficial genes and to silence antinutritional and adverse genes in a spatially and temporally controlled manner by designing various RNAi suppressor or enhancer vectors. (3) The third step is to produce transgenic plants by delivering into the plants the designed RNAi vectors specific for the designated target genes. (iv) Last, the desired transgenic plants are screened for improved nutritional value and for safety.

Acknowledgements

We especially thank Phillip D. Zamore for vital support and discussions during our studies on plant RNAi. We also thank members the Zamore laboratory for encouragement; Eric Lai, Qinghua Liu and Yu Xiang for critical reading and comments on this review; and Shelley Schwarzbaum for editing of this review. G.T. is a Charles A. King Trust Research Fellow of the Medical Foundation with funding from the Charles A. King Trust (Fleet National Bank, Co-trustee) and the June Rockwell Levy Foundation (Boston, MA). G.T. is supported by funding from the National Institutes of Health (GM62862–01 to P.D.Z.). G.G. is an incumbent of the Bronfman Chair of Plant Sciences with funding from BARD (Binational Israel/USA Science Foundation), the Israel Academy of Sciences and Humanities, National Council for Research and Development, DIP (German/Israeli Foundation) and the Framework Program of the Commission of the European Communities.

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