REVIEW ARTICLE

MicroRNAs and target mimics for crop improvement

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MicroRNAs (miRNAs) negatively regulate the expression of specific target messenger RNAs (mRNAs) within the cell. Artificial miRNAs have also been developed and found effective in gene silencing. The activity of individual miRNAs in turn can be regulated by endogenous target mimics, which are also described as miRNA sponges or decoys. Artificial miRNA target mimics have also been shown to function as 'miRNA inhibitors', thus leading to increased expression of the miRNA targets (mRNAs). Since miRNA target genes in plants are known to control a variety of traits, including yield/biomass, resistance to biotic/abiotic stresses and a number of other agronomically important characteristics, manipulation of miRNA and target mimics has the potential to be used for crop improvement programmes.

Keywords: Crop improvement, gene silencing, noncoding RNA, miRNA, ceRNA.

miRNAs and target mimics

MICRORNAs (miRNAs) are a class of small noncoding RNA (sncRNA) molecules, which represent a class of riboregulators and play a major role in regulation of gene expression in all eukaryotes, including plants and animals[1–4] (for sncRNAs and riboregulator; see Box 1). The genes encoding miRNAs are called *MIR* genes, which utilize RNA polymerase II (Pol II) to produce primary transcripts that are processed in the living cell into mature miRNA molecules (in animals, rarely Pol III is also utilized). Diverse plant processes that are regulated by these miRNA molecules include fate specification, development and metabolism[5]. The activity of miRNAs in a living organism, in turn, is precisely regulated by sophisticated mechanisms, since it has been shown that mis-regulation of miRNAs can lead to developmental abnormalities, causing diseases in humans[6] and phenotypic abnormalities in plants[7,8]. Apparently, a major mechanism that controls miRNA activity (both in animal and plant systems) involves a recently discovered robust endogenous system, which includes another class of RNAs described as endogenous target mimics or eTMs[9]. An eTM is generally a long non-coding RNA (lncRNA), but can also be part of a protein-coding transcript[10–13] (for lncRNA, see Box 1). In the literature, eTMs are also often described as miRNA decoys in plants[11–14] and miRNA sponges or competing endogenous RNA (ceRNA) in animals[15–18], although these terminologies are now being used interchangeably in plant and animal systems. Thus eTMs add a new class to the expanding repertoire of riboregulators and represent an essential component of competing endogenous RNA (ceRNA) regulatory network (ceRNET)[19,20] (for ceRNA and ceRNET, see Box 1).

A set of artificial TMs (aTMs) for inactivating miRNAs of *Arabidopsis thaliana* and soybean (*Glycine max*) has also been recently developed and the effects of these aTMs expressed as transgenes were examined. These effects were shown to resemble those observed in miRNA-resistant versions of the target genes in several plant species[11–14]. This collection of aTMs proved to be a useful resource to further investigate and manipulate the function/activity of individual miRNA families. In this review, a brief account of the different aspects of miRNAs (the miRNAome) and TMs is presented with special emphasis on their possible future use for crop improvement programmes.

Discovery of miRNAs

The year 2013 marked the 20th anniversary of the discovery of miRNA, since it was a forward genetics approach in 1993 used in two separate studies that identified the first miRNA *lin-4* in the worm *Caenorhabditis elegans*[21,22]. A few years later, the same approach was used for the discovery of another miRNA *let-7*, also in *C. elegans*[23]. Later, during 2000–2003, miRNAs were also discovered in plants, not through a forward genetics approach, but through a search for these molecules among the clones of one or more libraries of small fragments (22–24 nt), together with genetic and bioinformatics analyses[24–26].

Methods to study functions of miRNAs

It is now well established that miRNAs control many fundamental biological processes in plants through gene silencing (Figure 1). However, it has not always been easy to work out their biological roles due to lack of

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Box 1. Glossary

Argonaute: a protein (with two conserved RNA binding domains) that takes part in the formation of RNA-induced silencing complex, which is involved in microRNA (miRNA)-induced silencing of messenger RNA (mRNA).

ceRNA network: a network of RNA transcripts (both protein-coding and non-coding), including ceRNA (target mimic), which competes for microRNA binding (using miRNA response elements) and co-regulates each other in a complex manner.

Competing endogenous RNA: a functional coding or non-coding RNA that sequesters miRNA and thus works as a target mimic and miRNA sponge/decoy.

Long non-coding RNA: a functional non-coding RNA that is relatively long in size (~200–1000 nucleotides long) and is involved in regulation of gene expression.

MicroRNA sponge/decoy: another term used for TM.

miRNAome: a collection of all miRNAs from all tissues, expressed at any time during the life of an organism.

miRBase: a database with details of miRNAs (along with other related data) discovered in any species.

miRNA response element (MRE): a small matching complementary nucleotide sequence that is found in target mRNA; a number of MREs for the same or different miRNA molecules may be found within the same mRNA molecule.

Next generation sequencing: sequencing of nucleic acid molecules using new, ultrafast and cost-effective methods that have recently been developed (including 454 pyrosequencing, Illumina, etc.).

Small non-coding RNA: a functional small RNA molecule (21–24 nucleotides long) that is not translated into a protein, and is involved in regulation of gene expression.

Riboregulator: an RNA molecule that regulates its own expression or the expression of another nucleic acid molecule in response to a signalling event.

RNA-induced silencing complex (RISC): a multi-protein complex with miRNA (or any other small interfering RNA), which is used as a template for recognizing complementary mRNA. On finding this mRNA, it activates argonaute (a protein within RISC) and cleaves mRNA.

Short-tandem target mimicry technology: a technology for target mimicry for silencing miRNA, where two short sequences mimic small RNA target sites, separated by a linker, and leading to degradation of targeted miRNA.

TALEN (transcription activator-like effector nucleases): a class of artificial restriction enzymes generated as fusion proteins, by fusing a TAL effector DNA binding domain to a DNA cleavage domain, e.g., Fok1.

Target mimicry: a phenomenon involving a molecule that mimics target mRNA molecule and sequesters miRNA, thus facilitating translation of mRNA, whose expression would have otherwise been repressed due to miRNA.

Target mimics: regulatory RNA molecules which exhibit target mimicry.

Target mRNA: the mRNA whose expression is suppressed by miRNA in a specific manner using MREs.

Transcription factor: a protein that binds to a DNA sequence and activates transcription.

Virus-based miRNA silencing: a virus-based system used to silence endogenous miRNAs and dissect their functions in different plant species.

availability of the loss-of-function (*lf*) mutants, which is attributed to the small size of miRNAs and their functional redundancy, so that a loss of function mutation in a single member of an miRNA gene family sometimes has no perceptible phenotypic effect[27]. Therefore, the functions of miRNAs were mainly studied through either the miRNA genes with reduced miRNA activity, or the target genes that produce mRNAs, which are resistant to their corresponding miRNAs. Another approach involves overexpression of cloned miRNA genes in transgenic plants (for different approaches of miRNA discovery, see ref. 28 and references therein and also Box 2). Some of the recently developed important approaches for the discovery of miRNA function include the following: miRNA target mimicry (TM)[9], short tandem target mimicry (STTM)[29], virus-based miRNA silencing (VbMS)[30] and TALEN-based knockout of miRNAs[31] (see Box 1 for TM, STTM, VbMS and TALEN). The use of endogenous or artificial TMs, which can create loss-of-function effects by directly targeting and inactivating/sequestering/

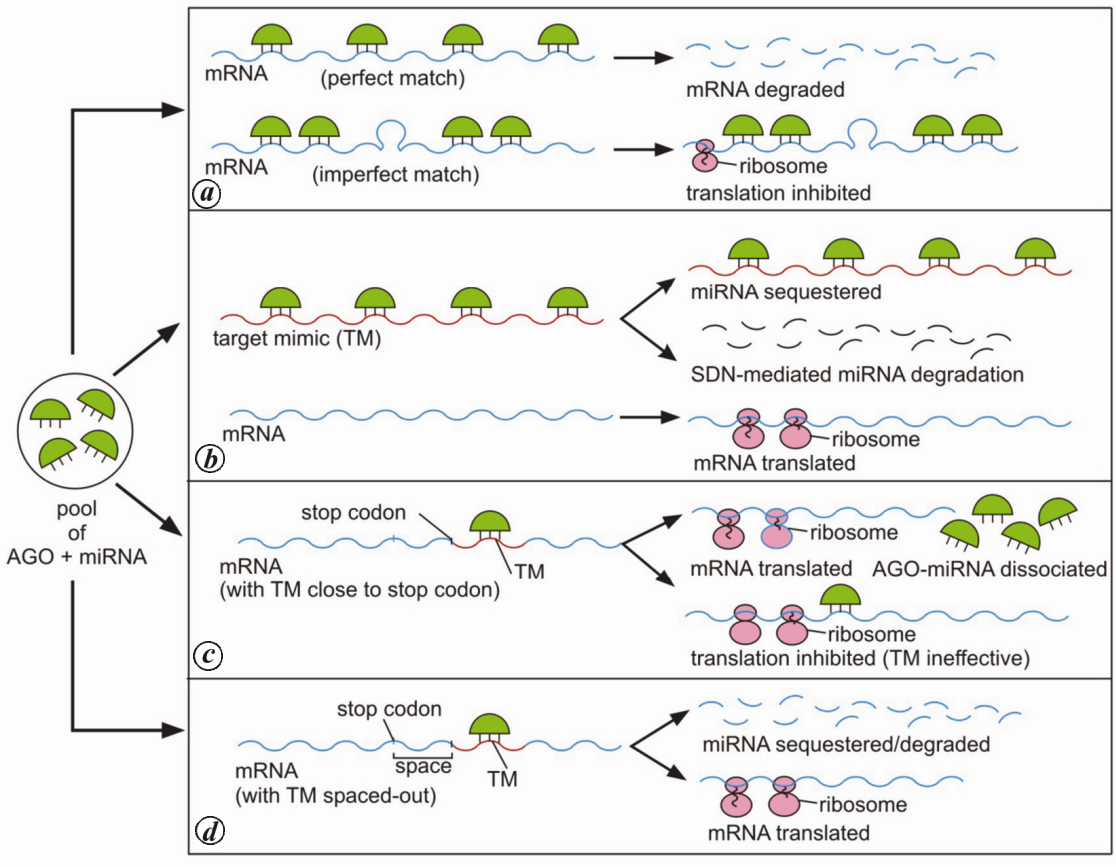

Figure 1. Interactions involving mRNA, microRNA (miRNA) and target mimic (TM). ***a***, Mature miRNAs loaded onto AGO (argonaute protein; see Box 1) bind to and inhibit target transcripts (mRNA) through either cleavage or via translational inhibition. ***b***, A TM and its corresponding mRNA are shown, where miRNA is sequestered by TM (miRNA decoy) or degraded due to SDN (small RNA degradation nuclease), permitting translation of mRNA. ***c***, An mRNA molecule with a TM in the 3′-UTR, very close to the stop codon in the open reading frame (ORF), so that either miRNA + AGO is dissociated, thus permitting translation of mRNA, or TM is rendered inefficient and miRNA remains effective, thus inhibiting translation. ***d***, An RNA molecule with a TM, where the TM is located in the 3′-UTR at an adequate distance from the stop codon (space between the stop codon and the TM), so that the TM is efficient in sequestering/degrading miRNA and translation of target mRNA will be affected.

degrading of mature miRNAs, is believed to be the most promising approach for the study of the function of miRNAs.

miRNAs and their target genes in plants

Genome-wide analysis of miRNAs has been conducted in a number of plant species, including rice, maize, barley and *Arabidopsis*[32–35]. Search for individual miRNAs has also been made in many plant species resulting in the discovery of hundreds of plant miRNAs, which are listed in miRNA databases, miRBase and PMRD[36,37]. The biogenesis and mechanism of action of these miRNAs has also been studied both in animal and plant systems (for reviews, see refs 38–41). It has been shown that miRNAs regulate gene expression post-transcriptionally through interactions with their target mRNAs, resulting either in transcript cleavage or translation inhibition[42]. These targets are often the genes encoding transcription factors (TFs; see Box 1) that are involved in regulating key developmental events.

MicroRNAs are evolutionarily ancient small RNAs, which are 19–24 nucleotides long and are generated by cleavage from larger, highly structured precursor molecules (pre-miRNA), both in plant and animal systems. Despite many similarities, plant miRNAs differ from animal miRNAs in several important features (for details, see refs 43 and 44). More recently, due to the availability of high-throughput next generation sequencing (NGS) technologies (see Box 1), and due to the realization that miRNAs perhaps influence many traits in one or the other plant species, a dramatic increase has been witnessed in the number of miRNA studies involving different traits in a variety of crops and tree species. The number of listed plant miRNAs in the database miRBase[36] increased from ~2000 in ~40 plant species in April 2010 (miRBase15) to ~6800 miRNAs in 62 plant species in June 2013 (miRBase v20). More recently, in June 2014, this frequency reached ~8500 miRNAs in 73 plant species

Box 2. Discovery, function, and use of microRNA (miRNA) and miRNA sponges

Methods for the discovery of miRNAs

Three different methods that have generally been used for miRNA discovery include the following[28]: (i) forward genetics approach, where a mutation visualized through phenotypic change leads to discovery of a miRNA; (ii) *de novo* discovery of miRNA using a reverse genetics approach, which involves cloning and sequencing of small cDNA molecules obtained from a size-fractionated cDNA library; and (iii) *in silico* discovery of miRNA using computational/bioinformatics tools, followed by experimental validation of candidate miRNAs.

Methods for the discovery of target mimics

Methods for discovery of target mimics (TM) is based on the fact that same sequence representing a specific miRNA response element (MRE; see Box 1) is common among TM and the corresponding mRNA targeted by a specific miRNA. Therefore, a TM can be predicted by examining the co-occurrence of miRNA response elements (MREs) in the mRNAs and TM (which would contain a bulge or a mismatch that is absent in mRNA) on a genome-wide basis. A software called TraceRNA has also been developed for exploration of TM. The following steps are involved: (i) select a gene of interest; (ii) select miRNAs that repress expression of the selected gene; (iii) select expression data for miRNA and (iv) generate regulatory network.

Principle involved in the function of miRNA and TM

The principle involved in the function of miRNA includes the following steps: (i) synthesis and processing of miRNA; (ii) loading of miRNA onto the protein argonaute (see Box 1), leading to the formation of RNA-induced silencing complex (RISC; see Box 1); (iii) association of RISC with the target mRNA (through pairing between miRNA and mRNA) and (iv) degradation on or inhibition of the expression of target mRNA. Similarly, TM pairs with miRNA with the help of MREs (see Box 1), without being degraded due to bulge/mismatch at positions 10 and 11, so that it functions as a miRNA sponge/decoy and sequesters miRNA (Figure B1).

Designing of target mimics (miRNA sponges/decoys) for crop improvement

A TM will generally have a sequence which is complementary to that of miRNA, so that the miRNA will pair with the corresponding TM, just as it pairs with mRNA, but without causing any cleavage. Two different types of TM that have been designed include: (i) bulge TM (which forms a bulge between nulceotides 10 and 11 while pairing with miRNA), and (ii) mismatch TM (with a mismatch at positions 10 and 11 between miRNA and the TM).

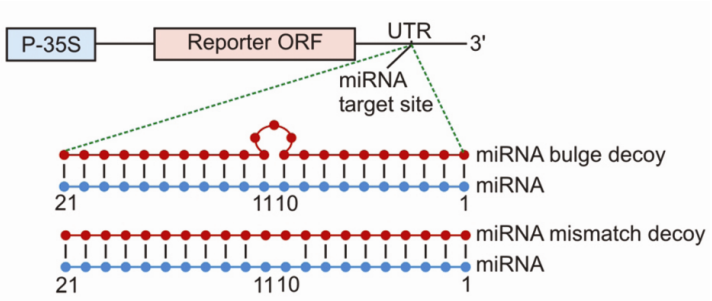

Figure B1. Two alternative designs of an artificial target mimic (aTM) inserted in 3′-UTR of a clone that carries a promoter sequence (P-35S) and a reporter ORF. The design of the aTM is shown at the top and pairing of miRNA with TM is shown below, where in the upper duplex pairing of miRNA is shown with a TM carrying a three-nucleotide bulge (3B), and in the lower duplex pairing of miRNA is shown with a TM carrying mismatch of two-nucleotides (2M).

(miRBase v21; http://mirbase.org/). A separate database for plant miRNAs (PMRD) was also developed[37], which had 8433 miRNAs from 121 plant species in 2010. PMRD had all the plant miRNAs included in miRBase at that time and a large number of additional miRNAs. Unfortunately this database has not been updated since 2010. However, one should be careful in using these databases, since some miRNAs in these databases may be spurious, as revealed in a recent study, when 1993 of a total of 6172 miRNAs in miRBase v20 were found to lack sufficient evidence to justify their annotation as genuine miRNAs. This was done on the basis of the criteria that are generally used for correctly identifying novel miRNAs[45]. In the latest version of miRBase (v.21) also,

Table 1. Examples of manipulation of expression of miRNA or its target

Plant species	Target gene	miRNA	Strategy used	Improved trait	Reference
Arabidopsis	*NFYA3*, *NFYA5*	miR169	Overexpression of target	Drought tolerance	92, 93
	CSD1, *CSD2*, *CCS*	miR398	Downregulation/overexpression of miRNA	Ferrooxidative stress/heat stress	94, 95
	ARF8	miR167	Aberrant ARF8	Tolerance parthenocarpy	96
Rice	*PCF5*, *PCF8*	miR319	RNAi	Cold tolerance	97
	SPL14, *SPL16*	miR156	Mutant target gene	Grain yield, grain size/shape/quality panicle branching	98–100
	LAC	miR397	Overexpression of miRNA	Grain yield, grain size/weight panicle length, more branches	50
Soybean	*NFYA3*	miR168	Overexpression of target gene	Drought tolerance	93
Tomato	*ARF8*	miR167	Aberrant ARF8	Parthenocarpy	96

more than 70 miRNAs were dropped due to wrong annotation and duplication.

Although the discovery of miRNAs has been relatively straightforward, the identification of the miRNA target genes has been challenging. Several algorithms and miRNA target prediction programmes with varied efficiencies have been developed, the latest[46,47] being the starBase v2.0. However, computational miRNA target prediction needs to be followed by validation of the predicted targets using wet-lab approaches. A plant mRNA target expression database (PMTED) has also been established to provide information on the known functional miRNA target genes[48].

Trait-specific miRNAs in plants

Trait-specific miRNAs have been identified and examined either as individual miRNAs or using a genome-wide approach. One or more of these individual plant miRNAs (or a group of miRNAs collectively) are involved in each individual process/trait such as organ morphogenesis, development, organ identity, responses to biotic/abiotic stresses, juvenile biomass and yield per se. Some of the important miRNAs, each with specific major activity and effect on individual traits, include the following: (i) miRNA319 is known to control general plant development[49]; (ii) miR156 and miRNA397 have recently been shown to affect the yield in rice[50] (Table 1); (iii) miR156 and miR172 are known to be involved in a change in the development phase and in the development of floral organs[51,52]; (iv) miR399, the most extensively studied miRNA, and a set of ~20 other miRNAs have been shown to be involved in phosphate homeostasis[9,53]; miR395 is involved in sulphur metabolism[54] and miR398 responds to Cu deficiency and oxidative stress[55]; (v) miR160 and miR164 are involved in auxin homeostasis and regulation of plant development (see ref. 7 and references therein); (vi) a number of miRNAs have also been shown to respond to low nitrate[56–58], biotic stress and abiotic stresses[59–64], including salinity[65] and drought[66–70]; (vii) an artificial miRNA was also shown to silence *OsMRP5* gene in transgenic rice plants, leading to a reduction of 35.8–71.9% in phytic acid, which negatively impacts human health and the environment[71] (for artificial miRNA, see next section); (viii) specific miRNAs affecting heterosis have also been reported in maize[72]. This information is now becoming increasingly useful for designing miRNA-based strategies for crop improvement (Table 1).

Artificial miRNAs (amiRNAs)

Artificial miRNAs (amiRNAs) have been developed and their utility in gene silencing was examined. An amiRNA functions in a way similar to endogenous miRNA, but can be designed to target virtually any gene in the plant genome. Software like Web-based MicroRNA Designer 3 (WMD3) and Designer Artificial miRNA Tool (DART) have also been developed to facilitate this research activity. Utilizing WMD, work on the development of amiRNAs was initially undertaken in *Arabidopsis*[73,74] and rice[75,76], but was later extended to >100 plant species[77,78]. A high-throughput method for the development of amiRNAs in *Arabidopsis* and other plant species has been reported

recently[79]. In *Arabidopsis thaliana*, a genome-wide library of amiRNAs for targeting 22,000 genes was also developed[80].

Discovery and development of target mimics

Endogenous and artificial target mimics

Two endogenous target mimics (eTMs) were discovered for the first time in 2007 in the model plant species *A. thaliana*[9] (for target mimics, see Box 1). It was shown that the transcripts derived from the genes *IPS1* (*INDUCED BY PHOSPHATE STARVATION 1*) and *At4* function as eTMs to control the inhibitory effect of miR399 on the expression of the gene *PHO2* (also known as *UBC24*, encoding ubiquitin E2 conjugation enzyme, UBC24). *At4* belongs to the TPS family of genes (induced due to P_i starvation; first described in tomato). The gene *PHO2* is known to be involved in P-homeostasis through its negative control on the expression of phosphate transporter Pht in the roots; miR399 is induced by PHR1 (a transcription factor) under conditions of low phosphate in the shoot. After its production, miR399 is transported to the roots, where it inhibits the expression of *PHO2*, thus neutralizing the negative effect of *PHO2* on Pht, leading to increase in the concentration of P_i in the shoot (sometimes reaching toxic levels). The concentration of miR399, in turn, is controlled by *IPS1* and *At4* transcripts, which function as eTMs, and provide a perfect binding site for miR399, thus facilitating sequestering of miR399. The *IPS1* and *At4* transcripts, on annealing with miR399, each forms a three-nucleotide bulge (3B) between nucleotides 9 and 12 (crucial for cleavage), thus avoiding cleavage of these transcripts by miR399. This phenomenon was described as target mimicry, since *IPS1* and *At4* transcripts mimic the *PHO2* transcript (the target) and sequester miR399.

Identification of eTMs in plants: Following the discovery of *IPS1* and *At4* transcripts as eTMs in *A. thaliana*, a computational search for eTMs for all miRNAs that were available in miRBase (see Box 1) was undertaken first in *Arabiodpsis*[11] and then in a number of other plant species[12–14,81] including the following: *A. thaliana*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Brachypodium distachyon*, *Glycine max*, *Vitis vinifera*, *Populus trichocarpa*, *Pinus taeda* and *Physcomitrella patens*. It was observed that while annealing with miRNA, many eTMs either formed a three-nucleotide bulge, or had a two-nucleotide mismatch (2M) at the middle of miRNA binding site (between nucleotides 9 and 12 at the 5′-end of miRNA) within the eTM sequence. This bulge or mismatch prevented miRNA-mediated cleavage of these eTMs. It was also noted that the eTMs could be derived either from coding or from non-coding genome sequences (although more frequently from the coding region, with higher frequency in the UTRs), and the sequence involved in the bulge/mismatch and the backbone of eTM was not always conserved even within a species. This variation may perhaps be involved in fine-tuning of the regulatory activity of eTMs. It was also observed that transgenic overexpression of an eTM leads to decreased abundance of the corresponding miRNA and increased expression of the miRNA target genes, resulting in altered phenotype of the plant. However, transgenic overexpression of heterologous eTMs did not always give the same results. Further, the expression of miRNA target was found to be dose-dependent, which varied in a temporal and spatial manner, suggesting a complex regulation of the expression of miRNA target genes by eTMs[11–13].

Development of artificial target mimics: Artificial target mimics (aTMs) were also developed using nucleotide sequences, which were complementary to corresponding miRNAs of interest, but carried bulges (3B) or mismatches (2M) to prevent miRNA-mediated cleavage (see Figure B1 in Box 2). The construct for an individual aTM and the corresponding transgenic plants has been described as MIM[9], each followed by the numeric identifier of the targeted miRNA family (e.g. MIM399 for miR399). The first two aTMs (MIM156 for miR156 and MIM319 for miR319) were developed using *IPS1* backbone (eTM backbone) from *A. thaliana*. Their ability to inhibit the activity of corresponding miRNAs was tested in transgenic *Arabidopsis* plants[9]. Later transgenic plants of *A. thaliana* expressing aTMs for 73 families of miRNAs were designed to test their activity against corresponding miRNAs[11]. A single aTM could be designed for each miRNA family, although for some miRNA families more than one aTMs, one each for a subfamily, were developed. A number of independent transgenics were obtained for each aTM designed. One-fifth of the above transgenic lines with aTMs had obvious morphological defects.

In another independent study, aTMs were also developed using scaffolds (backbone) from maize and soybean ncRNAs, and were tested using transgenics developed in *A. thaliana*, and also through a study of transient expression in *Nicotiana benthamiana* leaves achieved through *Agrobacterium*-mediated agroinfiltration[12]. These aTMs also had either the bulged (3B) or the mismatched (2M) structures to avoid their cleavage by the corresponding miRNAs (see Figure B1 in Box 2). Later, in an addendum to this earlier report, development, use and overexpression of aTMs with bulged (3B) and mismatched (2M) structures targeting several conserved miRNAs (e.g. miR160, miR171, miR172, miR319) were reported. In this subsequent study, aTMs were used for transforming soybean plants, and their effects on the activity of miRNAs were examined[13]. The level of target mRNAs and the phenotypes of the transgenic plants expressing these aTMs confirmed the functions of the corresponding

miRNAs[13]. The possibility of using both eTMs and aTMs for crop improvement was also discussed[12,13]. It is thus obvious that aTMs represent a flexible and robust tool, not only for studying miRNA function, but also for targeted engineering of gene expression in plants.

While developing an aTM, the transcript backbone and TM site were carefully chosen. In majority of studies, the transcript backbone of the construct belonged to the conserved miR399 MIM from *Arabidopsis IPS1* gene or its orthologue from maize (*Zma-miR399* MIM), although several other target genes and miRNA precursor molecules from soybean were also utilized[11,12]. It was also shown that an aTM sequence could be made a part of a protein coding transcript embedded in 3′-UTR sequence. The translation of the coding sequence itself would depend on the position of aTM site with respect to the ORF stop codon of the target gene, so that the aTM position needs to be carefully selected to avoid any undesirable effect on translation of coding gene[11]. It was found that an aTM site adjacent to the stop codon will interfere with translation, while an aTM site separated from the stop codon by at least ~75 bp spacer will not interfere with translation[12,13] (Figure 1 *c* and *d*).

Another class of aTMs, which was developed and used, included short tandem target mimics (STTMs), which were developed for the study of the function of miRNAs[29]. These STTMs were in general shown to be more potent than other types of aTMs in controlling the activity of miRNAs in *Arabidopsis*. The STTM for miRNA165 and miR166 (named STTM165/166-48) contained two copies of imperfect miR165/166 binding sites (24 nucleotides), one for miR165 and the other for miR166, with a 48-nucleotide RNA spacer between them, and also contained three additional nucleotides (CTA) to make a cleavage-preventive bulge during pairing of STTM with miRNAs. The transgenic plants, with constitutive expression of STTM, exhibited more dramatic alteration in phenotype of *Arabidopsis* than the corresponding transgenics carrying regular aTMs for miR165 and miR166, suggesting that the STTM approach may be more powerful than other aTM approaches for studying the functions of miRNAs.

miRNA and TMs for crop improvement

Many agronomically important traits in plants are the result of coordinated expression of multiple genes controlled by key regulators such as transcription factors and/or miRNAs. Several strategies have been developed to improve such traits through genetic engineering and molecular breeding to supplement conventional plant breeding. Although successful in general, this method is challenging as the engineering of complex traits such as yield may require tuning of expression of multiple genes during various phases of plant development. Precise manipulation of an endogenous RNA regulatory network affords a significant opportunity to achieve a desired outcome of enhanced plant development or response to environmental stresses for increased crop yield.

In theory, a number of major traits can be manipulated through one or more miRNAs or their TMs (Figure 2; Table 1). Several examples on the use of miRNAs will illustrate the potential of using this technology for crop improvement. First, an overexpression of OsmiR397 in rice has been shown to enlarge grain size and promote panicle length and branching by downregulating its target, *OsLAC* (encoding laccase-like protein), leading to 25% increase in grain yield[50]. Since miR397 is highly conserved across different species, it may prove useful for increasing grain yield not only in rice, but also in other cereal crops. Mutant version of rice miRNA target genes, *OsSPL14/16*, whose expression is controlled by miR156, has also been shown to give higher yield (Table 1). Second, overexpression of dominant mutant of *Corngrass 1* (*Cg1*) gene encoding miR156 promotes juvenile biomass (250% more starch) and inhibits flowering in bio energy crop switchgrass (*Panicum virgatum*), making it useful for improvement of biofuel crops[82]. Third, for developing tolerance against biotic stresses, including viral/bacterial/fungal diseases, the activity of one or more known miRNAs can be manipulated. These miRNAs also include the recently discovered miRNA cascade for plant defence, which involves miRNAs like miR482/2118; these miRNAs target genes encoding nucleotide-binding site-leucine-rich repeats (NBS-LRR), which are the major plant innate immune receptors[83–86]. In specific situations, one may also like to manipulate the defence system of the host to make it resistant to the onslaught of the pathogen, which sometimes uses its artillery in the form of miRNAs to silence the defence system of the host[87]. In such cases, the defence system of the host can be manipulated to become resistant to the miRNA of the pathogen. Fourth, for imparting tolerance against abiotic stresses to a crop, miRNAs that respond to drought, salinity, heat, oxidative stress, etc. can be manipulated. These miRNAs have been described in a number of studies and reviews (for a recent review, see ref. 60 and Table 1). For instance, miR399 and some other miRNAs are involved in responses to P-deprivation/starvation; miR826 and miR5090 are involved in N limitation; miR169, miR319 and miR398 are involved in drought (miR398 is also involved in heat/oxidative stress and copper deprivation). Use of artificial polycistronic miRNA for engineering resistance against streak mosaic virus in wheat has also been reported recently[88].

In majority of the examples listed above, transgenic approach has been used for overexpression or suppression of one or more specific miRNAs, depending upon whether the target gene(s) has a desirable or an undesirable effect (Figure 2). The overexpression of miRNAs or novel miRNAs can be achieved through amiRNAs

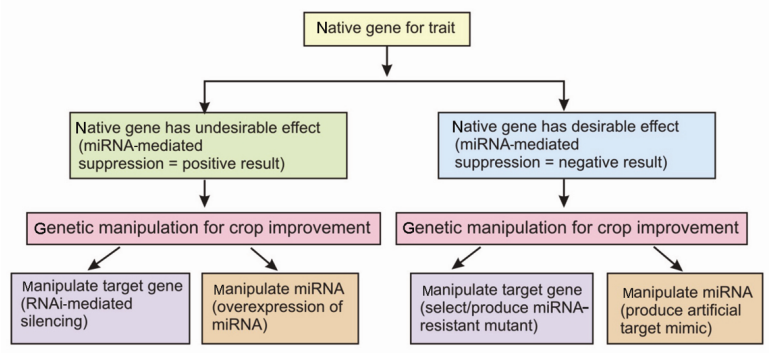

Figure 2. Two major strategies for developing miRNA/TM-based GM technology. (i) On the left, the native target gene has an undesirable effect, and the candidate miRNAs serve as positive regulators for target traits so that strategies, including RNAi-mediated silencing or knockout of miRNA target genes by RNAi/mutation, or overexpression of miRNA can be used for crop improvement. (ii) On the right, the target gene has a desirable effect on the trait of interest, so that the miRNAs potentially serve as negative regulators for target trait, leading to undesirable outcome; in this case strategies, including overexpression of target genes or selection of miRNA-resistant target genes or artificial target mimic (aTM, also called miRNA decoy) can be used for a desirable outcome. In some cases, in order to avoid side effects, utilization of stress-inducible or tissue-specific promoters to adjust expression of candidate miRNAs and/or their target genes in a controllable fashion could also be considered.

associated with high-expression promoters. Similarly, suppression of miRNA activity can be achieved using inhibitors in the form of aTMs. However, while using amiRNA-mediated or TM-mediated transgenic approach for crop improvement, one may like to keep the following in mind: (i) Many miRNAs in living cells have no visible effects due to inadequate concentration/expression, so that it may be necessary to know the actual concentration/expression of each of the native miRNAs in living cells, and the concentration/expression needed for visible effects, before designing a strategy to use either the miRNAs or the TMs for crop improvement. (ii) Multiple TMs may be used as cassettes for fine tuning of gene expression, which may involve orchestrated inactivation of multiple miRNAs rather than manipulating the activity of individual specific miRNAs, one at a time[89]. (iii) The sequences of TMs (including backbone and binding sites) may need to be carefully designed to allow a rheostat-like control of miRNA activity. For instance, sometimes a TM may need to be embedded in 3′-UTR of a protein coding transcript, by inserting a spacer between the stop codon and the 3B/2M to facilitate expression of the protein coding gene with simultaneous inactivation of another miRNA with negative role (Figure 1).

The potential of miRNA-mediated genetic engineering for crop improvement has been discussed in a recent review[90] and the potential of aTMs for crop improvement has been demonstrated in several recent studies[11–14]. Target mimics, each having sites for a number of miRNAs can also be used.

Conclusion and perspectives

This review presents an overview of the role of miRNA and TMs in regulation of the gene expression in plants, including those involved in controlling important agronomic traits. Strategies suggested in the recent past for their use in crop improvement have also been briefly reviewed. In many cases, it has been suggested that amiRNA and aTMs should be carefully designed, and specifically expressed to achieve a desirable effect. Possible use of this strategy for crop improvement has also been documented in several cases, although the development of improved cultivars using this approach leading to their commercialization has yet to be achieved. One may, however, speculate that allelic variation in any *MIR* gene for an individual miRNA and the corresponding TMs (including presence/absence and copy number variation) should also occur within the primary and secondary gene pools of individual crops. This aspect has not been examined so far, but should receive the attention of plant breeders in future, so that it will be possible to develop DNA-based molecular markers associated with desirable alleles of genes encoding individual miRNAs and TMs. These associated markers can then be used for marker-aided selection (MAS) for crop improvement in a time and cost-effective manner. MAS involving markers associated with unknown QTL/genes has already been used successfully in the past[91], but the use of markers associated with miRNAs/TMs will be a completely novel approach for crop improvement. This approach should be preferred over transgenic approaches, keeping in mind the limitations of regulation and opposition to transgenic approaches by activists. Therefore, we anticipate that in future conventional plant breeding will be aided through both genetic engineering and MAS, by making use of miRNAs and TMs for crop improvement. This will allow manipulation of the expression of endogenous genetic systems (including miRNAs and TMs) that control the target traits of economic importance in all major crops.

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