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Recent studies on non-coding small RNAs in plants

Shen Enhui, Liu Yang, Ye Chuyu, Fan Longjiang* (*Zhejiang Key Laboratory of Crop Germplasm/Department of Agronomy, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, China*)

Summary There are many non-coding small RNAs in plants and animals, which regulate gene expression via direct cleavage of target mRNAs or via inhibition of translation at the post-transcriptional level. In this paper, recent studies on microRNA (miRNA)-mediated phased siRNAs (phasiRNA) and endogenous target mimics (eTMs) were reviewed: 1) phasiRNAs can be generated both in coding and non-coding loci, and some require miRNA-mediated cleavage for their biogenesis. Pattern and evolutionary mechanisms of miRNA-mediated phasiRNAs were discussed. 2) Genome-wide identification and application of eTMs as miRNA decoy targets were recommended, including the use of artificial target mimics to validate the functions of miRNAs and employing bioinformatics methods to identify eTMs in the whole-genome level.

Key words microRNA; small interfering RNA; phased small interfering RNA; endogenous target mimics; bioinformatics
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植物非编码小 RNA 研究进展(英文).浙江大学学报(农业与生命科学版),2014,40(4):370－378

沈恩惠, 刘扬, 叶楚玉, 樊龙江*(浙江大学农业与生物技术学院农学系/浙江省作物种质资源重点实验室,杭州 310058)

摘要 在植物和动物中存在着大量非编码小 RNA,它们通过对靶标 mRNA 直接切割或在转录后抑制其翻译对基因表达起调控作用.本文主要综述了该领域在以下 2 方面的研究进展.1)微 RNA(miRNA)介导的具有相位排列的小干扰 RNA(phasiRNA):由 miRNA 介导的 phasiRNA 可由编码和非编码位点产生;介绍了 phasiRNA 产生的模式特征,以及 phasiRNA 和 miRNA 的进化机制.2)介绍了最近出现的内源 miRNA 诱捕靶标(endogenous target mimics,eTMs)研究进展,包括利用人工诱捕靶标检验 miRNA 的功能,通过生物信息学方法在全基因组水平计算识别 eTMs 等.

关键词 微 RNA; 小干扰 RNA; 相位排列的小干扰 RNA; 内源诱捕靶标; 生物信息学

There are many small non-coding RNAs in plants, which regulate gene expression through direct cleavage of the target mRNAs or through suppression of translation at the post-transcriptional level. Small

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* **Corresponding author:** Fan Longjiang, Tel: +86－571－88982730; E-mail: fanlj@zju.edu.cn

Biography: Shen Enhui, E-mail: ttxsenhui@gmail.com

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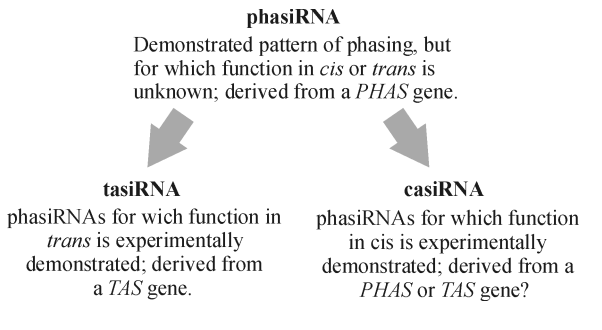
RNAs in plants are divided into two main categories: miRNA (microRNA) and siRNA (small interfering RNA). In this review, we present recent progress on the miRNA-mediated phased siRNAs (phasiRNA), which can be generated by *cis* and *trans*-regulation through miRNA. phasiRNA originates from *trans*-regulation, which targets more genes, and thus has received much attention recently. phasiRNA can be generated in both coding and non-coding loci with a specific pattern feature that makes it possible to develop bioinformatics methods for large-scale identification. Some studies focused on the evolutionary mechanism between the miRNA and phasiRNA, which was helpful in studying the functions of phasiRNA. In addition, this review introduces the research on the recently identified endogenous target mimics (eTMs). It is known that miRNA can target genes and play an inhibitory effect on specific mRNA, however, during the plants' development, a class of long non-coding RNAs named eTMs bind to miRNA and block its functions by segregating miRNA. Currently, we are able to identify the eTMs at the whole-genome level.

1　Researches about miRNA-mediated phasiRNA

1.1　phasiRNA

In plants, some target sites or genes of miRNAs (such as miR173, miR390, miR393) can trigger the production of phased secondary siRNAs from either non-coding (such as *TAS* and miR390) or protein-coding genes (such as *AFB* and miR393). With some obvious structural features of phasiRNAs and known reference genomes, we can use bioinformatics methods to detect such the types of siRNAs with the combination of large-scale small RNA data. Howell *et al.* [1] identified the *TAS* candidate gene loci based on a remarkable feature of small RNAs that consolidates and starts the coordination of a given 21-nucleotide phase within an eight-cycle window that generates phasiRNA loci. *P* value was used as a parameter to describe the phased score, and it was influenced by both small RNA abundance and the number of positions occupied. *P* values were applied in a scrolling window format with single nucleotide advances, and scores were assigned to the fourth cycle position in phasing plots. Chen *et al.* [2] also developed a method to detect phasiRNA, and similar to Howell *et al.* [1], they took into main consideration the distribution of phasiRNAs and constructed the *P* value to search the candidate loci with the use of a hypergeometric distribution[3]. A larger *P* value score indicated a more significant phased structure. In addition, Rajagopalan *et al.* [4] have the same core principle but a looser standard that detects the phased loci with the permission of a two base difference. Based on the above methods, Liu *et al.* [5] identified hundreds of phasiRNA loci in wild rice, and further research found that these sites could be important under the selection pressure during rice domestication. Many phasiRNA sites have been identified in other species[1-2,5-6], which indicates that it may be a conservative pathway to regulate the growth of plants.

miRNA-mediated siRNAs have two regulatory pathways: one is *cis*-regulatory and the other is *trans*-regulatory (Fig. 1). We pay more attention to the *trans*-regulatory siRNAs, which can target one or more downstream genes, especially the phasiRNA loci originated from protein-coding genes.



phasiRNA-generating loci are called *PHAS* genes, as tasiRNA-generating loci are *TAS* genes[6].

Fig. 1　Definition of phased small interfering RNA classes in plants

1.2　phasiRNA can be generated from both non-coding and coding regions

1.2.1　miRNA-mediated phasiRNA from non-coding loci　There are many types of siRNAs, some of

which are specific in plants with a phased array structure. Phased arrays are a phenomenon where the primary transcript produces a continuous specific length of small RNA products with the participation of some transcription factors. One typical type was tasiRNA, which is a type of siRNAs found in some research to take part in the changing development stage of leaves in the model plant *Arabidopsis*[7-9] (Table 1). The synthesis of tasiRNA starts from a single-stranded RNA that is then cleaved by a specific miRNA. The cleavage products are formed into a double-stranded RNA by RDR6 (RNA-dependent RNA polymerase 6), and then the DCL (Dicer-like) enzymes act on complexes to catalyze the formation of small RNAs. Although the role of the binding sites with miRNA is still not clear[10], different tasiRNAs have different miRNA binding sites, such as miR173 (*TAS1* and *TAS2*), miR390 (*TAS3*), and miR828 (*TAS4*)[4,7-8,10-12]. Among them *TAS3* is the most conservative and has been found in many terrestrial plants (such as rice)[10,13-14]. Furthermore, the expression of different phased siRNAs changed significantly, and parts of them can be formed into complexes by the bind of the ARGONAUTE protein to cleave the target mRNAs[13]. Zhao *et al.*[15] found some phased arrays of siRNAs in *Chlamydomonas reinhardtii* but did not find RDR homologous genes in its genome; therefore, the mechanism that produced tasiRNAs was unclear. Heisel *et al.*[16] and Zhu *et al.*[17] have found a specific type of siRNAs, which is called long miRNA-like hairpin and is related to the development of rice seeds in the 6th and the 12th chromosomes. The single-stranded RNA can be formed into a long miRNA-like precursor structure, but when the loop is quite large (>1 kb), the long stem can form into 21-nucleotide phased arrays. This formation is similar to tasiRNA duplexes, and also supports the mentioned regulation of the DCL*4* in rice, which can influence the formation of siRNAs. These long miRNA-like sites were also found in other plants[4,18], and they were considered to be derived from the duplication of progenitor genes[16].

Table 1 phasiRNA identified in non-coding loci in plants

miRNA	Length of miRNA/nt	phasiRNA loci	Species	References
miR173	22	TAS1a-c, TAS2	*Arabidopsis thaliana*	Vazquez *et al.*[7]; Peragine *et al.*[8]; Axell *et al.*[10]
miR390	21	TAS3	*A. thaliana*	Fahlgren *et al.*[11]; Willams *et al.*[12]
miR828	22	TAS4	*A. thaliana*	Rajagopalan *et al.*[4]
miR529, miR156	21,20	TAS6	*Physcomitrella patens*	Arif *et al.*[19]

1.2.2 miRNA-mediated phasiRNA from protein-coding loci Some miRNA-mediated phasiRNAs are from protein-coding genes. Howell *et al.*[1] and Chen *et al.*[2] first reported this phenomenon (Table 2) in several resistance genes (such as NBS-LRR genes). Due to the distribution of small RNAs in the genome as hotspots or clusters, Johnson *et al.*[20] generated a large data set of small RNAs from different tissues and developmental stages in rice and identified a large number of phased 21-mer clusters, which were flanked by a degenerate 22-nt motif that was offset by 12-nt from the main phase of the cluster and were preferentially expressed in inflorescences with a specific pattern. The miR2118 was one member of the 22-nt motif pattern, and the 24-mer clusters also had a specific expression pattern. In dicot, miR2118 was also discovered and was shown to target NBS-LRR disease resistance genes, and it made this gene locus generate phasiRNA, which can act as both *cis* and *trans* regulators[6,21-23]. Xia *et al.*[23] found that miR828 targeted *MYB* genes in apples, while some of the miR828-targeted *MYBs* can generate over 100 sequence-distinct siRNAs that potentially target over 70 diverse genes. Källman *et al.*[24] found that many sRNA (small RNA) sequences in spruce can be assigned to a 21-nt siRNA sequence, which originated from the degradation of NBS-LRR genes. Further results showed that this phased degradation of resistance-related genes that contained either a TIR or NBS domain was initiated from an abundant 22-nt miRNA-guided cleavage.

Intriguingly, the known miRNA-mediated phasiRNA loci, which were generated from protein-coding genes, were associated more or less with NBS-LRR resistance genes (Table 2). Ii is suggested that the regulation mechanism of miRNA-mediated phasiRNA may play an important role in the regulation of plant resistance.

Table 2　phasiRNA identified in protein-coding loci in plants

miRNA	Length of miRNA/nt	phasiRNA loci	Species	References
miR828, miR159, miR858	22,21,22	MYB	*Malus domestica*	Xia *et al*.[23]
miR161/miR400	21	PPP clade	*A. thaliana*	Howell *et al*.[1]; Chen *et al*.[2]
miR393	22	siRAAR	*A. thaliana*	Howell *et al*.[1]; Windels *et al*.[25]
miR780/miR856	21	ATCHX18	*A. thaliana*	Howell *et al*.[1]
miR472	22	NBS-LRR	*A. thaliana*	Howell *et al*.[1]
miR2109	22	NBS-LRR	*Medicago truncatula*	Zhai *et al*.[6]
miR1507	22	NBS-LRR; DCL2	*M. truncatula*	Zhai *et al*.[6]
miR1509	22	Predicted transcription factor	*M. truncatula*	Zhai *et al*.[6]
miR5754	22	Predicted protein kinase	*M. truncatula*	Zhai *et al*.[6]
miR156/miR172	21	AP2-like	*M. truncatula*	Zhai *et al*.[6]
miR4376	22	ACA10	*Solanum lycopersium*	Wang *et al*.[26]
miR482	22	NBS-LRR	*S. lycopersium*	Shivaprasad *et al*.[21]
miR2118	22	NBS-LRR; SGS3	Plant	Shivaprasad *et al*.[21]; Zhai *et al*.[6]; Johnson *et al*.[20]
miR6019	22	NBS-LRR	*Nicotiana tabacum*	Li *et al*.[27]
miR6020	21	NBS-LRR	*N. tabacum*	Li *et al*.[27]
miR7122	22	PPR	Plant	Xia *et al*.[28]

1.3　Pattern of miRNA-mediated phasiRNA

miRNA complementary sites exist upstream and downstream of the region that generates small RNAs, which can be divided into two types: One is a single-hit model (*i.e.*, *TAS1/2/4*)[4,7-8,10] and the other is two-hit model (*i. e.*, *TAS3/6*)[11-12,19]. The length of miRNA in the single-hit mode was 22-nt, and 21-nt in the two-hit model; the formation direction of the single-hit mode phasiRNA started at the 5′ cleavage site, and at the 3′ cleavage site in the two-hit model. There were also some variants in the single-hit mode, such as generating 24-nt phasiRNA (miRNA2275)[29]; similarly, some variants had miRNA complementary sites both upstream and downstream, but the 3′ site was not cleavable in the two-hit model (miR1509, miR7122)[6]. Another existing variant (*TAS6*) was that both 5′ and 3′ ends were cleavable in order to generate phasiRNA in the two-hit model. In addition, the cleavage sites of miRNA-mediated phasiRNA tended to occur in the 10th - 11th bases of the 5′ end of miRNA, which made it possible to identify phasiRNA sites on a large scale.

1.4　Evolution mechanism of miRNA and phasiRNA

Learning the evolution mechanism of phasiRNA and its mediated miRNA would contribute to the identification and analysis of phasiRNA. According to the sequence similarity of the binding sits between miR161/miR163 genes and their target genes in *Arabidopsis*, Allen *et al*.[18] proposed that genes encoding miRNAs in plants originated in the inverted duplication of target gene sequences. Due to progenitor sequences of miRNA, the inverted duplication events occur and result in head-to-head or tail-to-tail orientations of complete or partial gene sequences. With the constraints of the foldback

transcript and DCL enzymes, the miRNA genes were formed to regulate the expression of their founder genes or other members. Rajagopalan *et al.*[4] also found the similar sequence of six miRNA genes in *Arabidopsis*, excluding miR161 and miR163, between stem structure and the corresponding region of the target genes. Heisel *et al.*[16] showed that miRNA-like hairpin genes also share a similar evolutionary mechanism and regarded the *POT* gene as the progenitor of *Os06g21900* gene. However, the sites of tasiRNA did not have the same foldback transcript as miRNA did, and the formation of duplexes was depended on RDR rather than the DCL, indicating that the origin of tasiRNA could be different[18]. In fact, when they first discovered tasiRNA in *Arabidopsis*, Vazquez *et al.*[7] pointed out that the lack of similar sequences in the tasiRNA site and its target gene would make the tasiRNA originate from another mechanism. Axtell *et al.*[10] proposed a "two-hit trigger" mechanism in the study of the universally conserved *TAS3* of tasiRNA genes in higher plants in which both sites were cleaved to generate two transcripts containing miR390 complementary sites, which resulted in the formation of phased siRNA. Howell *et al.*[1] thought that *TAS* genes may have originated from the regulatory gene of miRNA. PPR-P genes, as a rapidly expansive gene family, were similar to the known *TAS* loci. They generated many 21-nt phased small RNAs, which were responsible for the regulation of PPR-P at the post-transcriptional level. The genetic drifts or recombination events resulting in the loss of coding potential and the reservation of the small RNA binding sites and phasiRNA producing regions may have arisen as an evolutionary mechanism for the new *TAS* sites[1]. Shen *et al.*[30] made a phyletic evolution study on *TAS3* sites, and they found that the duplication events of genes or chromosomes led to the expansion of *TAS3* members, though some members were lost in some species. Johnson *et al.*[20] found some differences between phased small RNA and tasiRNA associated with inflorescence stages in rice. The number of tasiRNA members was very few but they were expressed in nearly every tissue, while there were hundreds of such small RNA loci distributed in several small RNA clusters that were only expressed at the developmental stage of inflorescence. Moreover, such types of small RNA only existed in a 22-nt phased array with the 5′ cleavage site either with the length of 21-nt or 24-nt, which was similar to the specific *TAS1/TAS2* genes in *Arabidopsis*[13] and different from the single-hit model of *TAS3*. The latest findings showed that these miRNA-mediated phasiRNAs may have originated from a common progenitor sequence and would be conserved during the evolution process[28].

2 Research about eTMs of miRNA

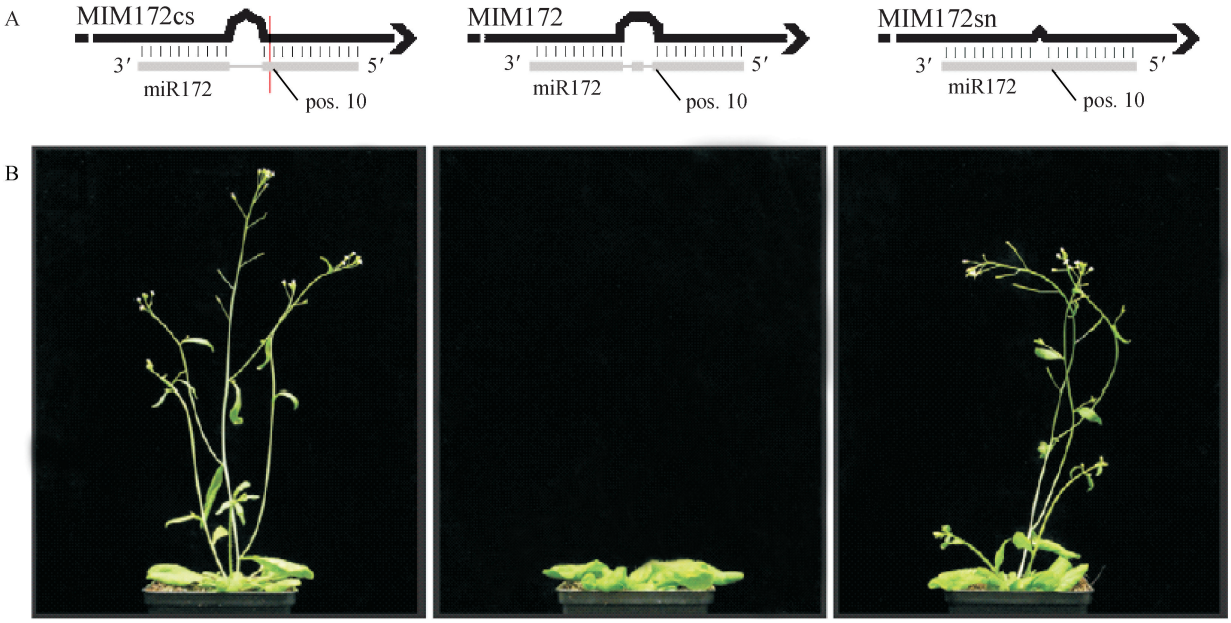
2.1 Definition of eTMs

miRNAs play an important role in the growth and development of animals and plants. In order to realize their regulation functions, miRNAs first bind to the AGO protein to form a complex and then target the specific mRNA through complementary base pairing in order to inhibit the expression of the target mRNA[31-32]. Franco-Zorrilla *et al.*[33] discovered the non-protein coding gene *IPS1* (induced by phosphate starvation1) from *A. thaliana*. The *IPS1* gene contained a motif with a sequence complementary to miR399 due to the mismatched loop at the expected miRNA cleavage site, which led to the sequestration of miRNA instead of cleavage. The authentic gene of miR399 is *PHO2*, which encodes the ubiquitin-conjugating enzyme to maintain homeostasis and to perform normal physiological function in the cells[34]. The presence of *IPS1* blocks miR399 from targeting *PHO2*; this type of long non-coding RNA was defined as eTMs.

2.2 Making use of artificial target mimics to analyze the function of miRNA

In plants, many miRNAs have been identified but the majority of their functions were unknown. Discovering the functions of miRNA is increasingly important. In previous research, eTMs showed a negative regulation correlation with its specific miRNA and sequestered the mRNA instead of cleaving it.

According to this mechanism, Todesco *et al.*[35] developed some artificial sequences to target mimics in *Arabidopsis* and to forecast the miRNA function. The results showed that 14 functions of all 71 miRNA families were inhibited, resulting in abnormal morphology in *A. thaliana*. Additionally, they found that the highly conserved miRNA tended to have a great impact on the growth of plants, and the impact of novel miRNAs on plant growth was not obvious. Furthermore, the 3-nt bulge arose between the 10th and 11th bases in the miRNA 5′ cleavage site, otherwise the function of target mimics would lose (Fig.2).



A: Three artificial target mimics. A target mimic with an unmodified central sequence (MIM172cs), which retained complementary to the central portion of miR172 across the cleavage site (red line) opposite position 10 to 11 of the miRNA, did not change flowering time. Modification of the central sequence (TCTA to GAGT; MIM172) restored a three nucleotide bulge found in *IPS1* and generated a functional target mimic, which caused a delay in flowering. However, a single nucleotide mismatch introduced into the center of an authentic miR172 target site (MIM172sn), but without a bulge, was not sufficient to reduce miR172 activity.

B: Four-week old plants grown at 23 ℃ in long days[35].

Fig.2　Required bulge at the cleavage site for target mimicry

2.3 Identifying eTMs on a large-scale with bioinformatics methods

The use of artificial target mimics can identify the functions of specific miRNA; however, the identification of eTMs in plants is still at the early stages. The research on the binding rules between miRNA and eTMs has laid the foundation for the identification of eTMs on a large scale. Meng *et al.*[36] first identified eTMs in the transcriptomes of *Arabidopsis* TAIR 10 (The *Arabidopsis* Information Resource, release 10) and rice TIGR 6.1 (The Institute for Genome Research, release 6.1). The results found 300 and 260 potential eTMs in *Arabidopsis* and rice, respectively. The following methods were used: First, Ssearch from the FASTA3 program package [downloaded from the FTP site of EBI (European Bioinformatics Institute), ftp://ftp.ebi.ac.uk/pub/software/unix/fasta/][37-38] was used to find the sites in the cDNA sequences that were reverse complementary to the miRNAs. Second, Perl scripts were written to screen the results from Ssearch, obeying the following rules: 1) The 3- to 5-nt bulges must exist within the complementary sites of the cDNAs, and the bulges should be located in the middle of the corresponding miRNAs (definition of the middle positions: 9th to 11th-nt of the 19-nt-long miRNAs, 10th to 11th-nt of the 20-nt ones,

10th to 12th-nt of the 21-nt ones, 11th to 12th-nt of the 22-nt ones, 11th to 13th-nt of the 23-nt ones, and 12th to 13th-nt of the 24-nt ones); 2) for the number of mismatches within the non-middle region of each miRNA, no more than four were allowed, and the consecutive mismatches could not exceed 2 nt; and 3) no bulge was permitted within the non-middle regions of the miRNAs. In addition, they also studied the distribution of the eTMs sites in *Arabidopsis* and rice and found that most of these sites were in the UTR region while only a small portion was located in the CDS region. GO was applied to do the enrichment analysis for eTMs, and degradome was sequenced to recognize the authentic targets of miRNA, thus the regulatory networks including eTMs, miRNAs, and target genes were successfully constructed.

Banks *et al*.[39] applied computational analysis to identify a numerous endogenous sequences with potential miRNA decoy activity for conserved miRNAs in several plant species. Their data suggested that eTMs can be widespread and may be a component of the global gene expression regulatory network in plants.

Several previous studies identified eTMs through the annotated genome sequences, and Wu *et al*.[40] developed a new computational method to predict the distribution of eTMs in the intergenic regions and non-coding genes. According to the RNA-Seq data and RT-PCR experiments, they found that more than 40% of the detected predicted eTMs were expressed. They also proved that the eTMs of several miRNA could effectively inhibit the functions of their corresponding miRNAs. Non-coding RNAs, short ORF (open reading frame)-encoding genes (with coding potential of less than 100 amino acids), and intergenic sequences in the *Arabidopsis* Information Resource (TAIR) 10 for *Arabidopsis* and the Michigan State University Rice Genome Annotation Project (release 7) annotations were collected and used as the eTMs prediction libraries. eTMs for the 20 selected miRNAs were predicted using local scripts with the following rules: 1) Bulges were only permitted at the 59 end from the 9th to 12th positions of the miRNA sequence; 2) the bulge in eTMs should be composed of only three nucleotides; 3) a perfect nucleotide pairing was required at the 59 end from the 2nd to 8th positions of the miRNA sequence; and 4) except for the central bulge, the total mismatches and G/U pairs within the eTMs and miRNA pairing regions should be no more than three. The distance between qualified intergenic eTMs and their upstream/downstream genes should be longer than 200 nucleotides. As a result, 25 of 36 detected eTMs and 94 of 186 detected eTMs were indeed transcribed in *Arabidopsis* and rice, respectively. The eTMs for an miRNA can be more than one, with the sequences conservation existing in the target sites.

3 Conclusions

This review describes the recent progress in phasiRNA and eTMs, which are closely related to miRNA. The existence of phasiRNA and eTMs is important in maintaining the homeostasis of plant cells; currently, only a small portion of these was proved to be functional, and more resources should be used to clarify the many mysteries of phasiRNA and eTMs. Fortunately, the bioinformatics was developed quickly and was crucial in the identification of phasiRNA and eTMs. The development of this sequencing technique and its continuous production of high-throughput data have laid a good foundation for future analysis and research. In addition, the research about small RNA in plants is developing rapidly and occupies a very important area in the plant kingdom. However, the mechanisms of its formation and evolution still remain to be solved.

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