The evolution of microRNAs in plants

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Abstract

MicroRNAs (miRNAs) are a central player in post-transcriptional regulation of gene expression and are involved in numerous biological processes in eukaryotes. Knowledge of the origins and divergence of miRNAs paves the way for a better understanding of the complexity of the regulatory networks that they participate in. The biogenesis, degradation, and regulatory activities of miRNAs are relatively better understood, but the evolutionary history of miRNAs still needs more exploration. Inverted duplication of target genes, random hairpin sequences and small transposable elements constitute three main models that explain the origination of miRNA genes (*MIR*). Both inter- and intra-species divergence of miRNAs exhibits functional adaptation and adaptation to changing environments in evolution. Here we summarize recent progress in studies on the evolution of *MIR* and related genes.

Short title: *MIR* gene evolution

Introduction

MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are central players in RNA silencing. SiRNAs are derived en mass from long double-stranded RNAs, and silence transgenes, viruses, and transposable elements [1]. MiRNAs are processed from short hairpin precursors encoded by *MIR* genes and regulate endogenous genes to impact development and stress responses [2,3]. RNA silencing as a mechanism to silence foreign genetic material (such as viruses) is conserved in all domains of life in eukaryotes, thus it is possible that it existed in the common eukaryotic ancestor. MiRNAs are found in all plant and animal lineages [4,5], and share similar biogenesis, function and turnover pathways in diverse lineages [2,6,7]. In this review, we summarize findings from studies on the origin and evolution of *MIR* genes and genes related to miRNA biogenesis and function, with a focus on plants.

The evolution of genes related to small RNA biogenesis and function

In miRNA biogenesis, Drosha generates pre-miRNAs from pri-miRNAs and Dicer then processes pre-miRNAs into mature miRNAs [7]. In plants, DICER-LIKE proteins (DCLs) perform the functions of both Drosha and

Dicer [7,8]. A comprehensive phylogenetic study on DCLs implicated the same origin but independent evolution of DCLs in plants and animals, and suggested that antiviral immunity served as a driving force in the evolution of plant DCLs [6]. In the green alga *Chlamydomonas reinhardtii*, which separated early from higher plants, miRNA biogenesis is likely conducted by *Cr*DCL3 [9]. Intriguingly, *Cr*DCL3 shows some Drosha-like features, possessing a proline-rich domain and lacking the PAZ domain. This is in contrast to the absence of Drosha-like proteins in higher plants; these differences in algal and higher plant DCLs indicate parallel evolution of the miRNA machinery in different plant lineages. On the other hand, the presence of a Drosha-like protein in *Chlamydomonas* suggests a single origin of DCL genes in animals and plants [9]. Echoing the similarity in miRNA biogenesis between *Chlamydomonas* and animals, a recent study found that *Chlamydomonas* miRNAs have similar features to animal miRNAs in terms of target recognition [10]. In animals, a miRNA recognizes its target transcripts via complementarity between the target and nucleotides 2 to 8 of the miRNA, namely the seed region. The seed region of a *Chlamydomonas* miRNA is sufficient to render miRNA-based repression of a target transcript [10]. This is different from the requirement for near-perfect base pairing for target recognition by higher plant miRNAs [11].

ARGONAUTE (AGO) family proteins associate with small RNAs and

mediate their activities. The plant AGO family can be divided into 3 major higher plant lineages, which are AGO1/5/10, AGO2/3/7, and AGO4/6/8/9, plus an algal lineage [12]. There exists a grass-specific AGO subfamily, designated AGO18, which is close to the AGO1/5/10 lineage, but its origination is unclear [13]. AGO18 in rice sequesters miR168 and acts as the decoy of AGO1, which is the target of miR168 [14]. In maize, however, *Zm*AGO18b binds 24-nt phased secondary siRNAs in male reproductive development [15]. In *Arabidopsis*, AGO10 serves as the decoy of AGO1 in binding miR165/166 [16,17]. Although AGO18 is grass-specific, its miRNA partner (i.e. miR168) and the miRNA target gene (i.e. *AGO1*) are conserved in land plants [4,18], implying a later origin of AGO18. Considering their similar functions in blocking AGO1-miRNA interactions but their different miRNA partners as well as different biological functions, AGO18 in cereals and AGO10 in *Arabidopsis* may have resulted from parallel evolution. The earlier emergence of miRNAs that are currently specifically bound by AGO proteins that emerged later in evolution, such as miR165/166, miR168 and miR390 [4,13], implies that the miRNAs initially perform some vital regulatory functions common in all plant lineages and the expansion in the AGOs adds additional layers to the regulatory networks or endow new biological functions to the miRNAs in specific lineages. These findings also suggest that the enormous variety of small RNAs contributes to the expansion and functional specialization of

AGO proteins.

In summary, the studies on the evolution of DCLs and AGOs suggest a single and ancient origin of the small RNA machinery in plants as well as in eukaryotes. The ancient DCLs and AGOs could be working with siRNAs in immunity, and those cooperating with miRNAs might have evolved later from them. It is unclear whether the miRNA machinery evolved prior to or after the divergence of plants and animals. The lack of conservation of miRNAs between plants and animals or between algae and higher plants indicates multiple independent origins of *MIR* genes themselves.

Identification of miRNAs in plants and insights into miRNA evolution

During the past decade, next-generation sequencing boosted the yield of genome and transcriptome sequences from a tremendous number of plants. Efforts to identify miRNAs from the ever-increasing databases of plant genomes and transcriptomes shed light on the overall features of *MIR* gene evolution. The numbers of miRNAs identified by these efforts are not related to the phylogeny but rather to the tissue type and developmental stages of the samples [19-21]. For example, 428 miRNAs were found in the liverwort *Pellia endiviifolia* [19] but only 129 were found in another liverwort *Marchantia polymorpha* [20]. The numbers of *MIR*

genes in species that underwent extra whole genome duplications are much higher than those in related species without such duplications. For example, currently, there are 2787 annotated *MIR* genes in *Glycine max* [22] vs. 216 in *Phaseolus vulgaris* [23] (two species in the Fabaceae family), and 680 in *Brassica rapa* [24,25] vs. 80 in *Capsella rubella* [21] (two species in the Brassicaceae family). The identification of miRNAs from various plants also provided an opportunity to understand the conservation of miRNAs over both large and small evolutionary distances. By comparing miRNA families identified in select angiosperms, it is evident that only a few miRNA families are conserved in land plants or even angiosperms (Figure 1A). Even in one family, such as Poaceae and Brassicaceae (Figure 1B,C), many more miRNAs are species-specific rather than conserved, suggesting rapid origination and divergence of *MIR* genes.

Origin of miRNAs

Three possible *de novo* origins of *MIR* genes have been uncovered by previous studies (reviewed by [8], Figure 2).

Gene inverted duplication

Studies on some young *MIR* genes suggest their evolution from inverted duplication of target genes, as sequences flanking the mature miRNAs in

MIR genes show similarity to those flanking the miRNA-binding sites in target genes [26]. Such inverted repeats could generate hairpin structures that initially produce many small RNAs (i.e. siRNAs). During evolution, only a key portion of the hairpin is retained to result in an *MIR* gene that encodes only one predominant small RNA species - the miRNA (Figure 2A). There are many examples showing sequence homology between *MIR* genes and target genes, thus supporting this model [26-29]. The original target inverted duplication hypothesis calls for neutral evolution of the sequences other than those of the mature miRNAs and miRNA stars in *MIR* genes [26]. However, this is not the case for some young miRNAs. For example, for *At*miR824, selection also affected the fold-back structure of the pre-miRNA rather than only the miR824/miR824* sequences in different *Arabidopsis thaliana* ecotypes [30].

Spontaneous evolution

Only half of *Arabidopsis lyrata MIR* gene families that can align to protein-coding genes yield miRNAs targeting the homologous genes [27]. This cannot be explained by the target inverted duplication hypothesis and implies that there must be other origins for *MIR* genes in plants. A source of miRNAs may be hairpin regions scattered in the genome, which may occasionally give rise to precursors of miRNAs upon acquiring promoters enabling transcription [31] (Figure 2B). For example, mpss05, a candidate

pre-miRNA foldback found in *Arabidopsis thaliana*, can align to two regions that may have originated by a duplication of a chromosomal fragment [31].

Miniature Inverted-repeat Transposable Elements (MITE)

Another potential source of miRNAs is MITEs, as MITE RNAs may be able to fold into stem loops that resemble miRNA precursors. 10 miRNAs in *Arabidopsis* and 38 in rice were reported to be derived from MITEs [32,33] (Figure 2C). However, MITEs tend to generate many small RNAs, which maybe better classified as siRNAs [32]. Thus, the MITE-origin hypothesis is still controversial. Recently, *Ta*miR1123 that functions in vernalization in wheat was found to be produced from a MITE locus and is an example that supports this model [34].

Functional divergence of miRNAs

Besides the *de novo* origination of *MIR* genes, duplications of existing *MIR* genes produce paralogous genes, enlarging the *MIR* gene family and potentially expanding the functions of the family. One clear case of functional divergence of paralogous miRNAs is *ArabidopsisMIR159/319*, which originated from the same ancestor but separated in the common ancestors of land plants and evolved to target two distinct gene families [4,35]. Despite few examples in the divergence of miRNA target specificity

as in the case of miR159/319, plenty of evidence supports tissue- and/or taxonomy-specific divergence in miRNA evolution. Two related genes, *MIR156* and *MIR529*, encode highly similar miRNAs but exhibit differences in their distribution among plant species, tissue expression patterns and evolutionary rates [36]. Intriguingly, even though there still exists some potentially functional miR529-responsive elements in the *Arabidopsis thaliana* genome, miR529 was lost in *Arabidopsis*. Members of the *MIR156* family may compensate for the regulatory functions of miR529 in *Arabidopsis* [36]. The divergent expression patterns of paraloguos miRNAs may have also contributed to plant adaptation to extreme environments, as suggested by a recent study on the small RNA transcriptome of mangroves, which grow under high salinity, poor oxygen and low nutrient conditions [37]. A study in *Brassica rapa* found that *MIR* genes are over retained compared with protein-coding genes after a species-specific whole genome triplication (WGT) event [24]. The retained multiple-copy *MIR* genes are under increased purifying selection as compared to protein-coding genes, suggesting functional importance of these *MIR* genes. A recent study on 22-nt miRNAs raised a new hypothesis that some miRNA (super) families are derived from existing miRNA families in a target-driven manner. Rapidly evolving target genes drive the evolution of miRNAs to maintain the miRNA-target relationship. For example, the *MIR7122* family targeting *TAS-LIKE* (*TASL*) genes

probably evolved from the conserved *MIR390* family targeting *TAS3* [38]. Divergence in miRNAs or targets may have played a role in crop domestication. Studies comparing cultivated rice with wild rice (*Oryza rufipogon*) found positively selected miRNAs and target genes in cultivated rice, suggesting that miRNAs were involved in or even drove rice domestication [39,40]. Another study suggested that a loss-of-function mutation in *MIR172p* improved fruit size during apple domestication [41].

Co-evolution of miRNAs and miRNA target genes

A mutual selection between miRNAs and their target genes probably occurs in evolution. A recent report shows that during the domestication of soybean, many factors influenced the evolution of *MIR* genes and miRNA targets, including the duplication status, expression level, and miRNA-target interactions [22]. A similar case is a study on *Populus trichocarpa* that experienced a recent whole genome duplication (WGD) event compared to other related species. The authors found the *de novo* emergence of new *Populus*-specific miRNAs after WGD as regulators of the newly formed duplicated genes in salicoids [42].

Some miRNA-target interactions result in small RNA amplification. Certain miRNA targets, such as *PPR*, *NBS-LRR*, *MYB*, and noncoding *TAS* loci, generate secondary, phased siRNAs (phasiRNAs) upon miRNA-guided RNA cleavage [43]. Such small RNA amplification is probably an

economic way to suppress a large number of similar genes with only one initial miRNA. This is a broadly adopted regulatory strategy during plant evolution [4,38,44]. The miRNA-target pairs exhibit patterns of co-evolution. In soybean, the miRNA targeting sites in *NBS-LRR* genes exhibit evolutionary patterns different from those of flanking sequences, suggesting that miRNAs influence the evolution of the targets [45]. On the other hand, a newly published report shows that *NBS-LRR* genes keep giving birth to new miRNAs targeting themselves in a convergent manner in diverse lineages. The evolution of these miRNAs appears to be driven by the amino acid diversity of their target sites, as diversity in miRNAs lies mainly in nucleotides corresponding to the third codon positions of the target site sequences [46].

Occasionally, conserved miRNA-target pairs could be lost in specific plant groups [4], and in some cases, re-construction of the regulation of the target genes is vital for their survival. For instance, *DCL1* is targeted by miR162 to form a feedback loop to achieve homeostasis of miRNA biogenesis. However, in some plants including *Physcomitrella patens*, *Selaginella moellendorffii*, and *Salvia miltiorrhiza*, either the binding site for miR162 is highly diverged or miR162 is poorly expressed. In *Salvia miltiorrhiza*, miR397 replaces miR162 to maintain the regulation [47]. Similarly, miR398 that targets *CSD2* is missing in *Physcomitrella patens*, but miR1073 targets *CSD2* in this species and this miRNA shares a

similar expression pattern with Arabidopsis miR398 [48].

Perspectives

MiRNAs and their target genes constitute a complex and dynamic gene regulatory network. From an evolutionary perspective, this network underwent parallel evolution in animals and plants, and in eudicots and monocots after the divergence of these lineages, leading to similar logistics but different flavors of the network in different lineages. When a new small RNA is produced, it may be retained and rewired into an existing regulatory network, making up a new miRNA and a new component of the network. The retained miRNA could also shape the target sequences, introducing even more divergence between lineages. With advanced sequencing technology, more and more lineage-specific miRNAs will be discovered from different plants and tissues, thus revealing more patterns of evolution, either supporting or opposing the current hypotheses.

Figure legends

Figure 1. miRNA families identified in selected plant species. (A) miRNA family distribution in selected plant species.Data are from miRBase v21 and [19-24,42,49]. The selected species are basal land plants *Physcomitrella patens*, *Marchantia polymorpha* and *Pellia*

endiviifolia, the basal vascular plant *Selaginella moellendorffii*, the basal angiosperm *Amborella trichopoda*, monocots *Brachypodium distachyum*, *Oryza sativa* and *Zea mays*, and eudicots *Glycine max*, *Phaseolus vulgaris*, *Populus trichocarpa*, *Moringa oleifera*, *Brassica rapa*, *Capsella rubella*, *Arabidopsis lyrata*, and *Arabidopsis thaliana*. Only 78 miRNA families are found in more than 4 species, most miRNA families are present in a smaller number of species. **(B, C)** Venn diagrams for shared miRNA families in closely related species in Brassicaceae (B) and Poaceae (C). The data sources are the same as those in (A). The number of shared miRNA families in closely related species is smaller than that of species-specific ones, indicating rapid and independent origination of miRNA families.

Figure 2. Origins of plant *MIR* genes. Three models that explain the *de novo* origination of miRNAs. **(A)** *MIR* genes stem from inverted duplication of target genes. The transcription of inverted duplicated founder genes leads to the emergence of proto *MIR*s. Proto *MIR*s often exhibit a long stem-loop structure fitting for the processing by DCL3 and DCL4 to generate siRNAs to suppress target gene expression. Shortening and mutations occur to the inverted repeats during evolution, resulting in the formation of pre-miRNA-like hairpins, which are compatible with processing by DCL1 to generate mature miRNAs. The transcript of the founder gene serves as the target of the mature miRNA. Red spots on the

transcript indicate mutations that also accumulate when the founder gene evolves. Occasionally, the target of the miRNA is not the founder gene, which implies divergence in the course of evolution. **(B)** Small random inverted sequences scattered in the genome provide a source of *MIR*genes. First, the random inverted repeats obtain a promoter element to enable transcription; then mutations and selections occur during evolution to form the precursor of a miRNA. **(C)** MITEs contribute to the origination of miRNAs. MITEs, a type of non-autonomous transposons, are composed of terminal inverted repeats (TIRs) and a short open reading frame (ORF), and are roughly 100~500bp in size. MITEs may serve as templates to produce miRNA precursors.

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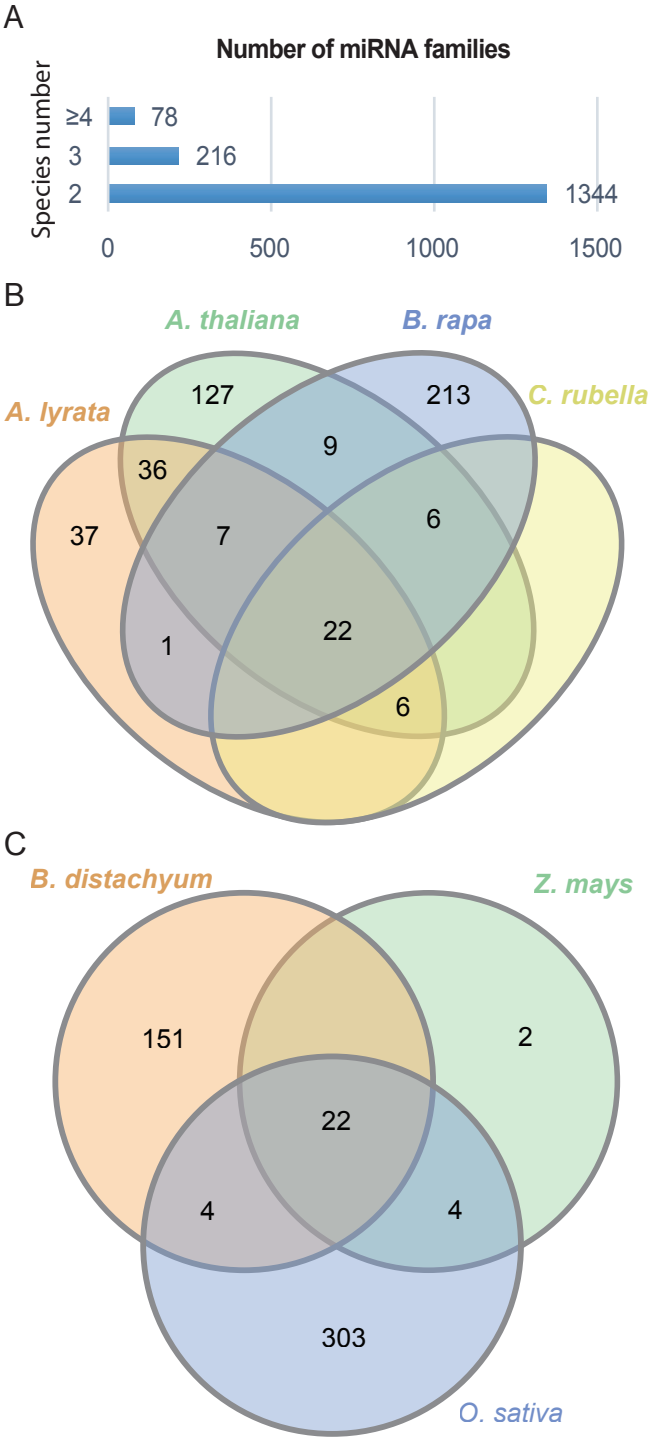
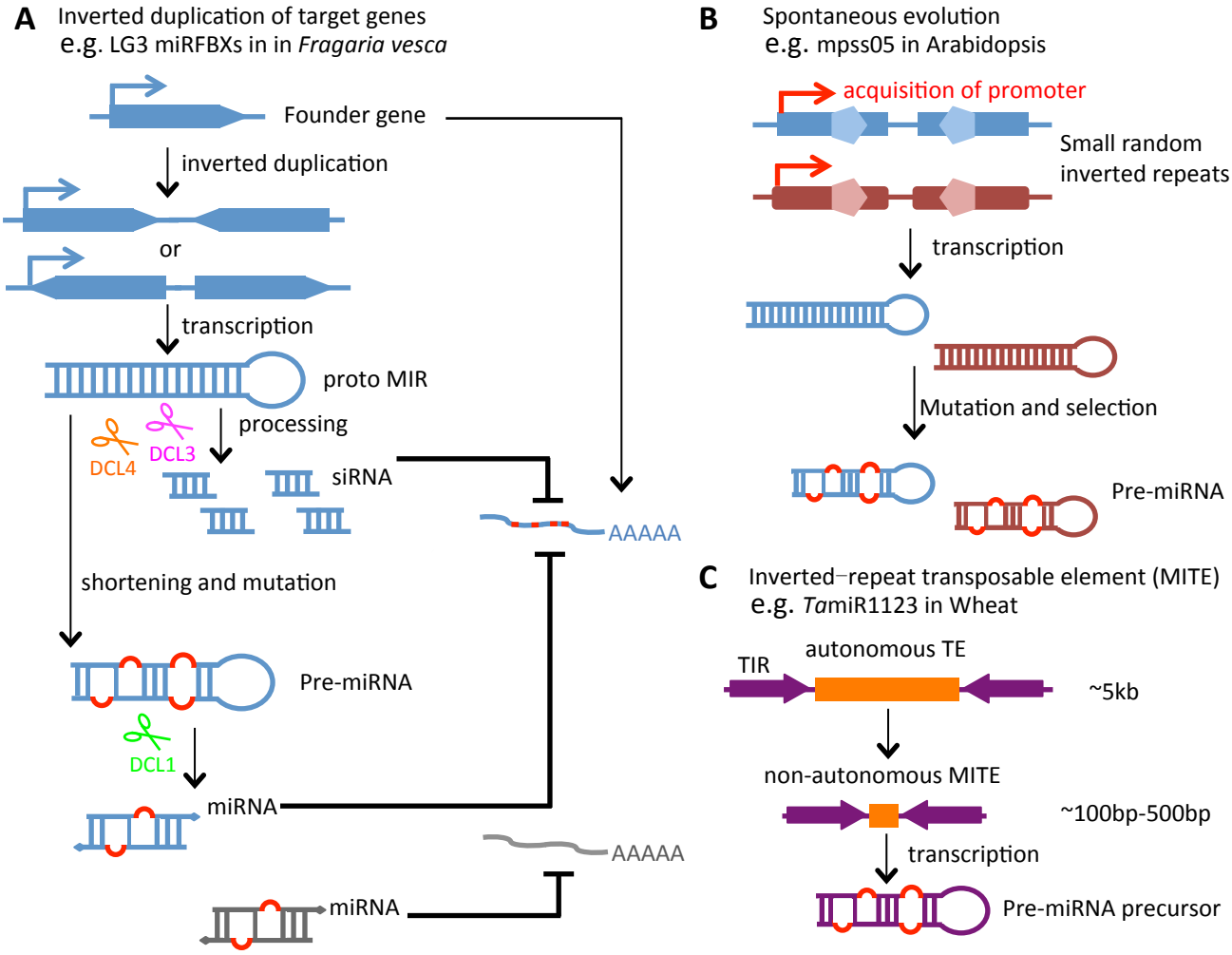

A
Number of miRNA families
Species number
≥4
3
2
78
216
1344
0
500
1000
1500
B
A. thaliana
B. rapa
A. lyrata
C. rubella
127
213
9
36
37
7
6
22
1
6
C
B. distachyum
Z. mays
151
2
22
4
4
303
O. sativa


A
Inverted duplication of target genes
e.g. LG3 miRFBXs in in *Fragaria vesca*
Founder gene
inverted duplication
or
transcription
proto MIR
processing
DCL4
DCL3
siRNA
AAAAA
shortening and mutation
Pre-miRNA
DCL1
miRNA
AAAAA
miRNA
B
Spontaneous evolution
e.g. mpss05 in Arabidopsis
acquisition of promoter
Small random
inverted repeats
transcription
Mutation and selection
Pre-miRNA
C
Inverted−repeat transposable element (MITE)
e.g. *Ta*miR1123 in Wheat
TIR
autonomous TE
~5kb
non-autonomous MITE
~100bp-500bp
transcription
Pre-miRNA precursor