PmiREN2.0: from data annotation to functional exploration of plant microRNAs

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ABSTRACT

Nearly 200 plant genomes have been sequenced over the last two years, and new functions of plant microRNAs (miRNAs) have been revealed. Therefore, timely update of the plant miRNA databases by incorporating miRNAs from the newly sequenced species and functional information is required to provide useful resources for advancing plant miRNA research. Here we report the update of PmiREN2.0 (https://pmiren.com/) with an addition of 19 363 miRNA entries from 91 plants, doubling the amount of data in the original version. Meanwhile, abundant regulatory information centred on miRNAs was added, including predicted upstream transcription factors through binding motifs scanning and elaborate annotation of miRNA targets. As an example, a genome-wide regulatory network centred on miRNAs was constructed for Arabidopsis. Furthermore, phylogenetic trees of conserved miRNA families were built to expand the understanding of miRNA evolution across the plant lineages. These data are helpful to deduce the regulatory relationships concerning miRNA functions in diverse plants. Beside the new data, a suite of design tools was incorporated to facilitate experimental practice. Finally, a forum named 'PmiREN Community' was added for discussion and resource and new discovery sharing. With these upgrades, PmiREN2.0 should serve the community better and accelerate miRNA research in plants.

INTRODUCTION

MicroRNA (miRNA) is a class of endogenous non-coding regulatory RNA that mainly functions in post-transcriptional gene expression (1–3). Although miRNAs are widespread in almost all organisms, the current knowledge is that miRNA biogenesis mechanisms have independently evolved in animals and plants (4,5). Thus, miRNAs have evolved and perform important functions in a kingdom-specific manner (2–5). Studies performed in plants have shown that miRNAs perform diverse biological activities, including development, reproduction, and response to external stimuli (6–10). The past 20 years have recorded constant growth in miRNA research (11), manifesting the significance of miRNAs in organism function and behaviour. Consequently, the number of publications related to miRNAs has increased annually (Supplementary Figure S1).

The next-generation sequencing (NGS) technology has played a critical role in promoting miRNA research as its high throughput has prompted the discovery of thousands of miRNAs (12–16). The third-generation sequencing methods promise to further ease and enrich miRNA acquisition. In the last two years alone, nearly 200 plant genomes were sequenced, an equivalent of all sequenced plant genomes since the first genome of the model plant species *Arabidopsis thaliana* became available in 2000 (17). Thousands of new plant small RNA (sRNA) sequencing datasets were uploaded to NCBI within the same period, and numerous datasets generated by new techniques including parallel analysis of RNA-ends sequencing (PARE-Seq), chromatin immunoprecipitation sequencing (ChIP-Seq) and DNA affinity purification sequencing (DAP-Seq) were also made available. These new progresses underscore the need for a comprehensive miRNA knowledgebase

capable of timely organizing and processing the rapidly expanding miRNA-related large datasets.

In recent years, there have been new trends in the functional study of plant miRNAs (9,10,15,18). First, continuous studies of miRNAs have uncovered new functions through revealing more upstream and downstream regulatory relationships. For example, new findings on more target genes have broadly extended the functions of miR156 to fruit ripening (19), seed dormancy (20), anthocyanin biosynthesis (21), stress responses (22) and secondary metabolism (23), from the original role in juvenile to adult phase transition (24,25). Elucidation of the unique spatiotemporal expression pattern of individual members of the miR172 family in *Arabidopsis* revealed divergent functions of them in plant development (26,27). Second, the availability of a large amount of data from NGS techniques such as sRNA-Seq, PARE-Seq, ChIP-Seq and DAP-Seq, together with the correlation of genomic scale expression between miRNAs and targets, enabled the comprehensive study of the miRNA-based regulatory network in a species, which will unravel the whole perspective of miRNA functions and the potential new functions of individual miRNAs (15,28). Third, expansion of experimental studies of miRNAs from model plants to non-model plants has gained new insights into miRNA functions. For instance, miRNAs in the parasitic plant *Cuscuta pentagona* have been shown to act as trans-species regulators of host–gene expression and virulence factors during parasitism (29). However, inadequate and inaccurate annotations of the miRNA loci in non-model plants have restricted the research progress.

As a database specializing in comprehensive identification and uniform annotation of plant miRNAs, the first version of PmiREN (Plant miRNA ENcyclopedia, PmiREN1.0) was released two years ago, containing 20 388 miRNA loci from 88 species (14), and has received over 160 000 worldwide page views since its debut in June of 2019. In the updated PmiREN2.0, annotation of 19 363 additional miRNAs in another 91 plants with newly sequenced genomes was added. Importantly, PmiREN2.0 contains multiple types of regulatory information for the miRNA loci towards the goal of constructing a miRNA-based regulatory network for all plant species. The evolution module of PmiREN2.0 contains polymorphism data and phylogenetic trees of conserved miRNA families across multiple plant lineages. The upgraded features of PmiREN2.0 also include a suite of built-in tools for retrieving custom information and accurately targeting the intended miRNA loci in experimental design. These miRNA annotations, datasets, and features of PmiREN2.0 should make it instrumental for exploring miRNA function and evolution in diverse plants.

MATERIALS AND METHODS

Data sources

PmiREN2.0 was updated with miRNA entries from 91 species/subspecies, including 26 lycophytes and ferns, six monocotyledons and 59 eudicotyledons. Detailed information of whole-genome references and gene annotations were available in Supplementary Table S1. sRNA-Seq datasets were obtained from the NCBI GEO DataSets (30) (Supplementary Table S2).

Data analysis pipelines

Identification and annotation of miRNAs. A standardized workflow for miRNA identification based on miRDeep-P2 (31) and the newly updated criteria (12) was employed to annotate miRNAs such as defining miRNA conservation, as previously described in PmiREN1.0 (14).

Prediction of cis-acting regulatory elements. *Cis*-acting regulatory elements of miRNA loci were identified and extracted from potential promoters of miRNA precursors (3000 bp upstream sequences of miRNA precursors). Two standalone software, PlantCare (32), and PlantRegMap (33) was used to predict *cis*-acting regulatory elements, and PlantRegMap outputs were filtered at P value <0.001.

Annotation of target genes. The functions of miRNA target genes were predicted using InterProScan (34) based on sequence similarity. Each predicted target gene was assigned an HTML page with detailed information on the family, domain, Gene Ontology (GO) terms (35), etc. In addition, BlastKOALA (36) was employed to generate a Kyoto Encyclopedia of Genes and Genomes (KEGG) (37) orthology annotation of the miRNA targets, and a hyperlink of the relevant KEGG pathway was provided.

Polymorphisms on miRNA loci. Genome variants were downloaded from Phytozome v12 (38) with the variant call format. Variants (SNPs and small InDels) located on miRNA loci were extracted by VCFtools (0.1.15) (39). Using an in-house Perl script, the variants were annotated on five parts of the miRNA locus, including the mature region, star region, 5′ arm region (20 bp), 3′ arm region (20 bp), and internal loop region.

Phylogenetic trees of conserved miRNA families. Phylogenetic trees were constructed for 28 deeply conserved miRNA families based on respective precursor sequences. Sequence alignments were performed using the MAFFT (v7.310) program (40), and phylogenetic trees were built using the IQTREE (v1.6.12) (41) following the extended model selection and tree inference. Bootstrap values were estimated from 1000 ultrafast bootstrap replications.

Regulatory network centred on miRNAs. The miRNA-target interactions and transcription factor (TF)-miRNA interactions were obtained in the model plant *Arabidopsis*, following the protocol described by Gao (28). Further, the regulatory network was constructed using Cytoscape (v3.4.0) (42). The Edge-weighted Spring Embedded Layout method and HIGHCHARTS (https://www.highcharts.com/) were employed to display the regulatory network so that users can select a miRNA or TF hub to display the preferred regulatory module.

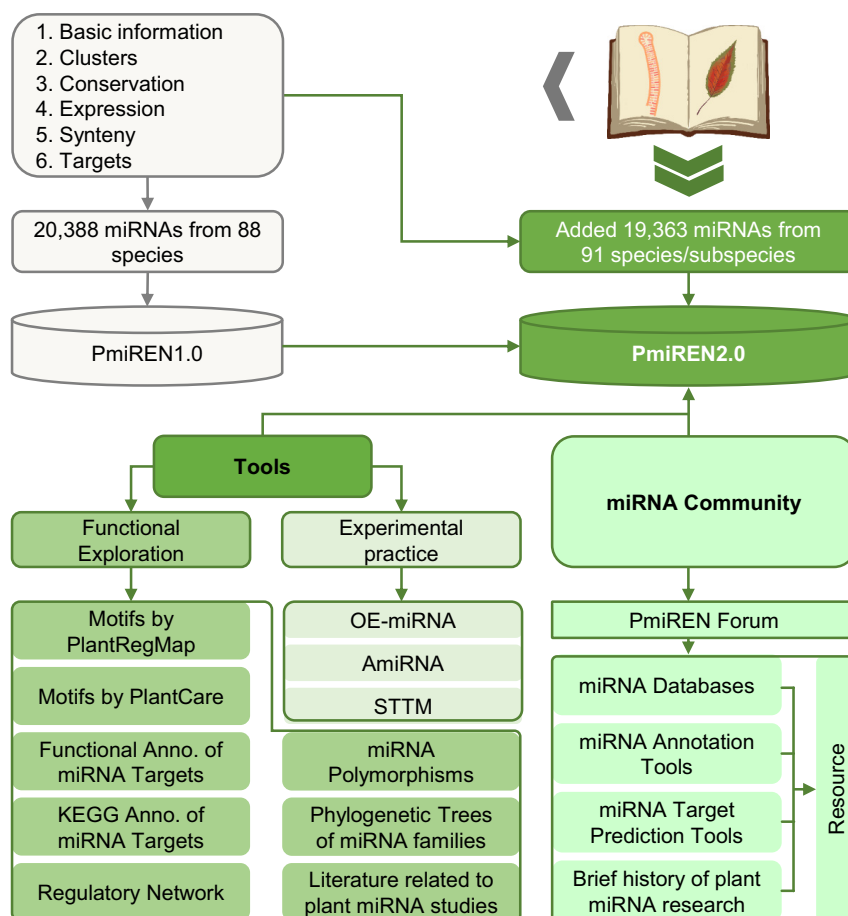*Development of three experimental design tools.* OE-miRNA was developed based on the open-source Primer3

Figure 1. Overview for the updated content in PmiREN2.0. Grey colour represents data in PmiREN1.0 while different green colours show updated data and features in PmiREN2.0. The book icon in the top right indicates the website logo of PmiREN.

software (43) using sequences of miRNA precursors flanking 500 bp as inputs. For AmiRNA and STTM, the procedures for constructing artificial miRNAs and mimic targets followed previous reports (44–48) and were implemented by PHP. The interfaces of these tools were created using HTML, CSS and JavaScript.

RESULTS

Updated data content of PmiREN2.0

A major source for new miRNA loci in PmiREN2.0 was 579 sRNA-Seq datasets from 66 plant species/subspecies (Supplementary Tables S1 and S2). Up to 17 798 high confidence miRNA loci (designated with three stars in PmiREN2.0) were identified by parsing these sRNA-Seq datasets using miRDeep-P2 (31) with the newly updated miRNA annotation criteria (12) as previously described (14). In addition, to increase representation of the miRNAs in the lycophyte and fern lineages, which would aid the understanding of evolutionary trajectories of miRNAs, 1565 unique miRNA loci retrieved from 25 lycophyte and fern species from previous work (18) were added to the PmiREN2.0 database after quality control. In total, 19 363 new miRNA entries from 91 species/subspecies were added to PmiREN2.0 (Figure 1).

Table 1. Summary of updated data content in PmiREN2.0

Data Items	PmiREN1.0	PmiREN2.0
Species/subspecies	88	179
miRNAs	20 388	39 751
miRNA families	5757	7838
sRNA-Seq libraries	1537	2116

Collectively, the updated PmiREN2.0 contains a total of 39 751 miRNA entries belonging to 7838 families from 179 species/subspecies phylogenetically ranging from chlorophytes to angiosperms (Table 1, Supplementary Figure S2). In comparison to version 1.0 of PmiREN, the amount of miRNA data in PmiREN2.0 almost doubled. Meanwhile, the same high and uniform annotation standards characterizing PmiREN1.0 were faithfully inherited. Detailed information on the identified and annotated miRNA loci, including the genomic location, sequences and secondary structures of miRNA precursors, mature and star miRNAs, clusters, etc., is provided for easy access and download. Relevant information on miRNA conservation in different species, synteny blocks, expression patterns across organ types and developmental stages, and target genes based on prediction and validation from PARE-Seq data is provided whenever possible.
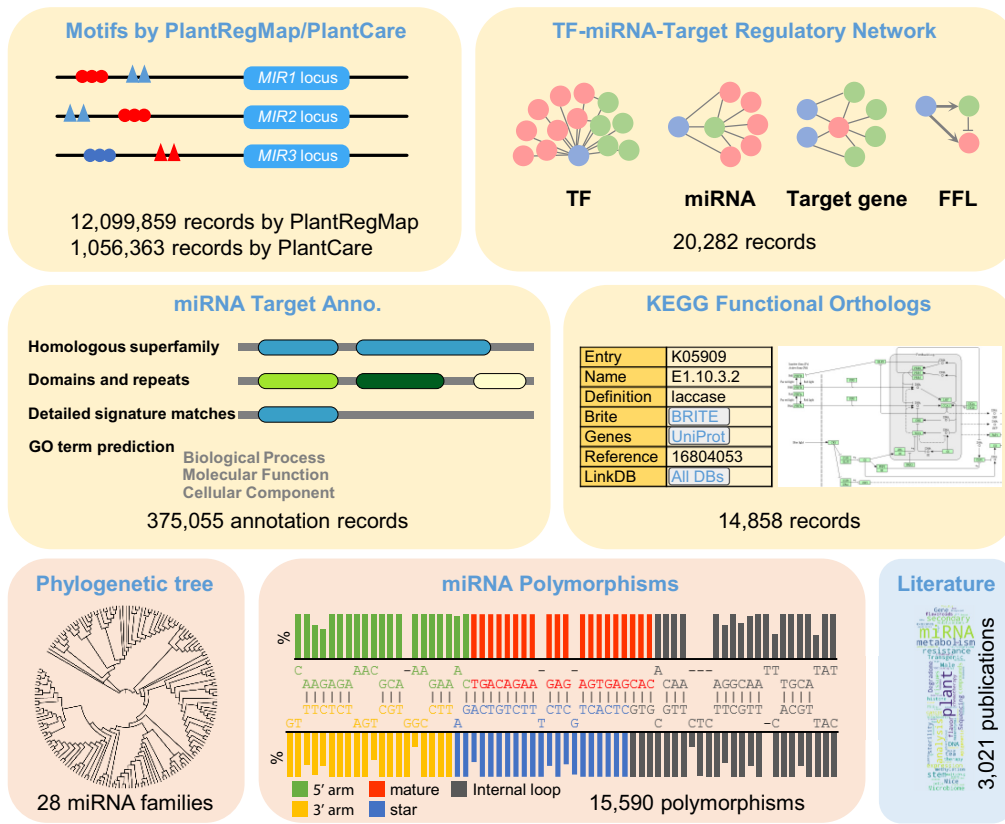
Figure 2. Schematic diagram for new features of PmiREN2.0 assisting miRNA functional exploration. TF represents transcription factor. FFL represents feed-forward loop.

Table 2. Summary of new features in PmiREN2.0

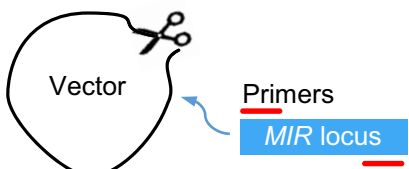| Types	Tools	Brief Introduction	Records
Functional exploration	Motifs by PlantRegMap	Prediction of *cis*-acting regulatory elements by PlantRegMap	12 099 859
	Motifs by PlantCare	Prediction of *cis*-acting regulatory elements by PlantCare	1 056 363
	Function of miRNA Targets	InterProScan annotation for individual miRNA target	375 055
	KEGG Annotation of miRNA Targets	KEGG annotation for individual miRNA target	14 858
	Regulation Network	Regulatory network of transcription factor-miRNA-targets	20 282
Evolutionary exploration	miRNA Polymorphisms	Variant browser for miRNAs	15 590 InDels from 4 species
	miRNA Phylogenetic Tree	Phylogenetic tree of specific miRNA family	28 families
Information collection	Literature	Comprehensive literature on plant miRNAs published in the last decade	3021

New features for miRNA functional exploration

To facilitate miRNA research especially for functional and evolutionary exploration, two sets of analytic tools or data collections were added in the PmiREN2.0 (Figures 1 and 2, Table 2). The first set of tools focus on exploring upstream transcriptional regulation and downstream target genes to facilitate building miRNA regulatory networks. The new functions of PmiREN2.0, 'Motif by PlantCare' and 'Motif by PlantRegMap', are two standalone tools for predicting *cis*-regulatory elements in promoters of miRNA loci (Figure 2, Table 2). For instance, previous studies in Arabidopsis

have discovered a number of TF-miRNA modules, including HY5-miR408 and SPL7-miR408 in regulating copper homeostasis (49,50) and SPLs-miR172 (25) in regulating flowering. By panoramically analysing DNA binding motifs in the miRNA promoters in 24 representative species, we found that the HY5-miR408 and SPL7-miR408 modules are likely conserved in land plants while the SPLs-miR172 module is conserved only in flowering plants, consistent with the biological functions of these modules (Supplementary Figure S3). 'Function of miRNA Targets' and 'KEGG' are two functions of PmiREN2.0 used to retrieve functional annotation of target genes generated by InterProScan
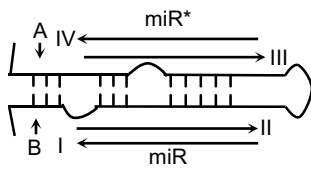
Figure 3. Schematic diagram for three tools assisting design of molecular and genetic experiments. (**A**) OE-miRNA for designing experiments to overexpress selected miRNAs. (**B**) AmiRNA for designing artificial miRNAs. (**C**) STTM for the design of short tandem target mimics.

(34) and BlastKOALA (36) (Figure 2, Table 2). These functions generate detailed information on each target gene, including gene structure, protein domains, annotation based on GO and KEGG ontology, and description of functions. 'Regulatory Network' is a function developed for constructing a miRNA-centred regulatory network including interactions between TFs-miRNAs and miRNAs-targets in the model plant *Arabidopsis* as previously described (28) (Figure 2, Table 2). An interactive map via JavaScript

and CSS was embedded to select and display the network modules.

The second sets of tools are for evolutionary exploration, consisted of the functions 'miRNA Polymorphisms' and 'miRNA Phylogenetic Tree' (Figures 1 and 2, Table 2). 'miRNA Polymorphisms' is used to scan variants in five parts of the miRNA loci, including the mature region, star region, 5' arm region (20 bp), 3' arm region (20 bp), and internal loop region, which potentially reflect the selection

pressure during evolution. Currently, this function includes over 15 000 records in four species, and a search engine was deployed. 'miRNA Phylogenetic Tree' is used to display the phylogenetic trees of 28 deeply conserved miRNA families generated by a uniform pipeline (details in Material and Methods). Additionally, over 3000 literature related to plant miRNAs in the last decade were collected to provide detailed references on miRNA function and evolution. 'Literature', a conditional search engine on title, abstract, keyword, journal, author and year, was developed and deployed in PmiREN2.0 (Figure 2, Table 2).

New features assisting genetic experimental design

Genetic manipulation of miRNAs by tuning their expression is a powerful means to explore miRNA functions. Successful experimental designs require accurate targeting of the correct miRNA loci. To meet this practical need and provide user-friendly output, PmiREN2.0 developed three tools, including 'OE-miRNA', 'AmiRNA', and 'STTM', to assist effective molecular and genetic experimental design (Figure 3A–C). 'OE-miRNA' is used to design primers for cloning the entire pre-miRNAs that is a prerequisite for successfully overexpressing the corresponding miRNAs. Likewise, 'AmiRNA' and 'STTM' assist in designing miRNA silencing experiments by the artificial miRNA and target mimic approaches.

PmiREN community and information sharing

The forum named 'PmiREN Community' was created to promote and facilitate data sharing and communications within the plant miRNA research community (Supplementary Figure S4). In this forum, new discoveries, discussions on technical issues, and all plant miRNA-related information are welcome for sharing. Additional tools and resources are integrated into the forum, such as other miRNA databases, tools for identification and annotation of plant miRNAs, miRNA target prediction methods, and miRNA research history in the last two decades. A brief introduction and hyperlinks for these resources are provided.

DISCUSSION AND PERSPECTIVES

Compared with the first version of PmiREN, PmiREN2.0 has added 91 species/subspecies and annotated 19,363 new miRNA entries from recent genomes and sRNA-Seq datasets, almost doubling the data size of the original version (Figure 1, Tables 1 and 2, Supplementary Figure S2). Even all the miRNA entries were carefully selected as previously described (14), it should be noted that they are still miRNA candidates since not fully experimentally validated. PmiREN2.0 also added relevant regulatory information centred on miRNAs, including TF binding motifs in the miRNA promoters, and detailed annotations of miRNA targets. In addition, tools facilitating experimental designs were developed (Figure 3A–C). These data and tools should be useful to deduce the gene regulatory relationships involving miRNAs and aid the exploration of miRNA functions across different plant lineages (Figure 1, Table 2).

Building a miRNA regulatory network for different plant species that contains credible information is highly desired for the understanding of miRNA function and evolution. Curation and integration of multi-omics datasets including CHIP-Seq, DAP-Seq and PARE-Seq in PmiREN2.0 represent a solid step towards this goal. As an example, the distribution of three types of well-studied DNA binding motifs in the promoters of *MIR408* (49,50) and *MIR172* (25) successfully identified in 24 representative species is shown in Supplementary Figure S3. Of course systematic sorting is required to increase the reliability of the newly identified regulatory relationships in PmiREN2.0. Users are encouraged to submit confirmed regulatory relationships in due time. After integrating all verified regulatory relationships, a search engine in PmiREN could be established for studying these regulatory relationships in other species and accelerating miRNA function exploration.

We expect PmiREN to become an all-in-one service platform for plant miRNA research. To do so, PmiREN will integrate different functions, including miRNA identification and annotation, miRNA target gene prediction, miRNA regulatory network construction, and more in future versions (15). A series of tools corresponding to these functions will be added. With the continuously increasing amount of data, PmiREN will also develop self-learning and self-improvement capabilities through artificial intelligence technology to be more accurate for miRNA identification and annotation. Similar ideas are extendable to target gene prediction, TF-miRNA interaction exploration, and miRNA regulatory network construction. Eventually, it is hoped that PmiREN will become a one-stop platform instrumental for plant miRNA research.

DATA AVAILABILITY

PmiREN2.0 is freely available at https://pmiren.com.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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