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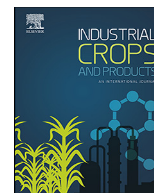

MicroRNAs and their targets by high-throughput sequencing and differentially expressed analysis in five *Miscanthus* species

Jiajing Sheng[a,1], Xingfei Zheng[a,1], Jia Wang[a], Shuguang Liu[a], Ming Pang[b], Fasong Zhou[a], Zhongli Hu[a], Ying Diao[b,*], Surong Jin[c,*]

[a] State Key Laboratory of Hybrid Rice, Lotus Engineering Research Center of Hubei Province, College of Life Sciences, Wuhan University, Wuhan, 430072, PR China
[b] College of Forestry and Life Sciences, Chongqing University of Arts and Sciences, Chongqing, PR China
[c] School of Chemistry, Chemical Engineering and Life Sciences, Wuhan University of Technology, Wuhan, 430070, PR China

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ABSTRACT

MicroRNAs (miRNAs) are noncoding small endogenous RNAs with approximate 22 nucleotides and have been identified as the key regulators in the translational level regulation of gene expression by either directly binding or cleaving target mRNAs. In plant, miRNAs have been shown to control numerous genes involved in a wide variety of metabolic and biological processes. Increasing extensive studies have been performed on model species and important agricultural crops including *Arabidopsis thaliana* L., *Oryza sativa* L. and *Zea mays* L., but miRNAs have not yet been complete identified in *Miscanthus*, a promising potential bioenergy candidate. In this study, miRNAs in *Miscanthus lutarioriparius* L., *Miscanthus sacchariflorus N.* , *Miscanthus floridulus W.*, *Miscanthus sinensis A.*, and *Miscanthus x giganteous* were constructed by Solexa high throughput sequencing and subsequently performed the bioinformatics analysis. Totally, 93 conserved miRNA families as well as 485 novel miRNA families were discovered in the five *Miscanthus* species libraries. Gene ontology (GO) analysis revealed these novel miRNAs' targets involved in 94 biological processes in *M. lutarioriparius*, which 25 of these processes participated in the metabolism of carbon, glucose, starch, fatty acid, and lignin formation. In the pathway enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG), 10 metabolism networks were found, including starch and sucrose metabolism, fatty acid metabolism, phenylpropanoid biosynthesis, oxidative phosphorylation, and the processing of other metabolites. This study provides a large scale identification of miRNAs and their potential targets in *Miscanthus* species. These miRNAs add the growing database of new miRNAs and lay the foundation for further understanding of miRNA evolution, diversification and regulation in *Miscanthus*.

1. Introduction

MicroRNAs are a class of non-coding endogenous small RNAs, consisting of 20–24 nucleotides in plants. It regulated gene expression at the post-transcriptional level, mainly through mRNA targets cleavage and translation inhibition or acted at transcriptional levels by inducing changes in DNA methylation of target loci (Bartel, 2004; Khraiwesh et al., 2010; Reinhart et al., 2002). Recently, numerous miRNAs along with their functions were identified and elucidated using bioinformatics tools and experimental methods including direct cloning, deep sequencing, and other approaches. According to the publicly available database miRBase Version 21.0 (ftp://mirbase.org/pub/mirbase), 24521 mature miRNA sequences from 206 species processed to produce 30 424 mature miRNA products have been identified, including the plant species of fully sequenced genomes *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor* and *Medicago truncatula* (Kozomara and Griffiths-Jones, 2014). In these researches, miRNAs have been shown to play pivotal roles in a wide variety of metabolic and biological processes, including plant growth, development, signal transduction, protein degradation and response to biotic and abiotic stress. In plants, miRNA genes are transcribed by a RNA polymerase II into a long primary miRNA (pri-miRNA) with a cap and a

Abbreviations: MiRNA, microRNA; EST, expressed sequence tag; NCBI, national center for biotechnology information; *M. lutarioriparius*, *Miscanthus lutarioriparius*; *M. sacchariflorus*, *Miscanthus sacchariflorus*; *M. floridulus*, *Miscanthus floridulus*; *M. sinensis*, *Miscanthus sinensis*; *M. giganteous*, *Miscanthus giganteous*; GO, gene ontology; Rfam, RNA family database; BLAST, basic local alignment tool; TPM, transcripts per million; eEF-1α, Eukaryotic elongation factor 1α; EIF-4α, eukaryotic initiation factor 4α; UBC, ubiquitin-conjugating enzyme; *UBQ*, ubiquitin; TUBB, β-tubulin; CYP, cyclophilin

* Corresponding authors.
E-mail addresses: ydiao@whu.edu.cn (Y. Diao), jinsr@163.com (S. Jin).

[1] These authors contributed equally to this work and should be considered co-first authors.

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poly (A) tail containing adopting hairpin-like regions. Such hairpin-like structures are processed into a precursor miRNA (pre-miRNA). These two steps are processed by the RNAse II enzyme Dicer-like1 (DCL1) and its double-strand RNA binding (DRB) partner HYL1 to release a double-stranded miRNA intermediate (duplex miR:miR*) (Barkan and Small, 2014; Bartel, 2004; Vazquez et al., 2008). MicroRNAs make functions in the cytoplasm by incorporating into an RNA-induced silencing complex (RISC), which will guide the targets cleavage and/or inhibit their translation by sequence-specific recognition of protein-coding mRNAs (Voinnet, 2009).

Miscanthus is a tall, rhizomatous, and perennial grass genus, which is native primarily to a wide range of climates from eastern Asia south to the Pacific islands (Hodkinson et al., 2002). It is the most valuable taxa of fiber biomass plant and used as a considerable candidate for promising alternative bioenergy crop. In recent years, scientists pay many interests in exploring the new plant resources for biomass crop breeding and genetic improvement. For example, the natural triploid species *Miscanthus* × *giganteus (Mxg)* has been evaluated for more than two decades as a biomass crop in both Europe and the United States (Hastings et al., 2009). Recent functional studies of miRNAs in *Arabidopsis* and *maize* have demonstrated that manipulation of specific miRNAs can improve biomass accumulation in transgenic plants, which could be the focus of several researches in *Miscanthus* (Schwab et al., 2005a; Wu and Poethig, 2006). To date, miRNAs in many crop species such as *Panicum virgatum* and *Oryza sativa*, have been uncovered to regulatory networks controlling crop yield and forage quality (Fu et al., 2012; Matts et al., 2010). The study on *Miscanthus* miRNAs was reported just in *Miscanthus x giganteus* (GEO ID: GSE28755, total reads: 12,400,937) (Kankshita et al., 2010). However, the miRNAs in other *Miscanthus* species have not been complete identified, especially in *Miscanthus lutarioriparius* (formerly called Triarrhena lutarioriparia), which is a native plant in China and consistently much taller than other *Miscanthus* taxa (300–700 cm height) and may indicate more biomass accumulation. *M. lutarioriparius* is also a traditional and excellent fiber crop for papermaking in China.

In the present study, the deep sequencing technique was employed to identify miRNAs of five *Miscanthus* species and also combined the computational analysis to predict and annotate miRNAs and their targets. The miRNA expression profiles of *Miscanthus* vary with different species were explored. More the reports of small RNA data have been done, higher would be the ability to identify conserved miRNAs and predicted the novel miRNA. Further experimental studies with the different *Miscanthus* species would be helpful for understanding the biology of *Miscanthus* and its regulatory systems. The results of this study would enrich the database of novel miRNAs, promote the researches in the genetic improvement of biofuel plants and provide new tools for plant breeders.

2. Materials and methods

2.1. Plant material

Plant materials of *M. lutarioriparius, M. sacchariflorus, M. floridulus* and *M. sinensis* were collected in the field (Yueyang, Hunan Province, China) and authenticated by Prof. Fasong Zhou. *M.* × *giganteus* were kindly provided by Professor Wu Ju-ying (Beijing Research Development Center for Grass and Environment). Rhizome of each individual was separate planted in green house at 30 °C with a 12 h high/12 h dark photoperiod for one month. The leaves, which were collected from each representative clones were cleaned in sterilized water and frozen in liquid nitrogen and store at −80 °C before use.

2.2. Small RNA library preparation and sequencing

Five libraries for *M. lutarioriparius* , *M. sacchariflorus* , *M. floridulus*, *M. sinensis*, and *M. giganteous* were constructed in this study. Total RNAs in each species were extracted combined samples with at least three clones germinated according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The small RNA fragments of 18–30 nt were isolated using 15% denaturing polyacrylamide gel electrophoresis. Then, these fragments were ligated to a 5′ and 3′ adaptor sequentially and reverse transcribed to DNA by RT-PCR. The reversed products were sequenced using Illumina HiSeq 2000, which was performed at Beijing Genomics Institute (BGI), Shenzhen, China. The sequenced short reads data are deposited in NCBI (accession numbers: SRA057895; SRP094670).

2.3. Small RNA analysis and conserved miRNA identification

The raw sequence data were first screened to eliminate the low-quality tags without 3′adapter, without the insert tag and those with poly-A tails. The remaining reads that sequences longer than 16nt were used to the further computational analysis. First, the sequences matching non-coding RNAs such as rRNA, tRNA, snRNA, snoRNA that available in Rfam (http://www.sanger.ac.uk/software/Rfam) and the GenBank noncoding RNA database (http://www.ncbi.nlm.nih.gov/) were removed. Then length distribution of clean reads was summarized and remaining reads were used to the further processed. The remaining sequences were aligned to published datasets (miRBase 21.0) ((http://www.mirbase.org/) and the conserved miRNAs in the five *Miscanthus* were identified. Only small RNAs those mature and precursor sequences with no more than two mismatch to known plant miRNAs and not located in the seed region were considered an evolutionary conservation. All small RNA fragments and the identified orthologs of conserved miRNAs from miRBase were screened using the program SOAP 2.0.

2.4. Statistical analysis for differential expression of miRNAs

In order to identify the differentially expressed miRNAs in the five libraries, miRNA expression levels were analyzed using following statistical methods. First, the expression level of miRNAs in each library was normalized to get the value of RPM (matching reads per 1 million total reads). Then, the differences of expression abundance between the five libraries (Fold-change) for each miRNA were calculated using DEGseq software (http://telethon.bio.unipd.it/bioinfo/IDEG6/). Finally, the Log2 (Fold-change) and P-value were calculated every two libraries. The criteria to identify differentially expressed miRNAs between every two libraries as follows: (1) *P*-value between the two libraries was less than 0.05 and (2) log2 ratio was greater than 1or less than −1.

2.5. Prediction of novel miRNAs

The prediction of potentially novel miRNAs was conducted using Mireap program (Beijing Genome Institute, http://sourceforge.net/projects/mireap/) according to their characteristic hairpin structure, the Dicer cleavage site, the matches between miRNA and opposite miRNA* and the minimum free energy. Identified mature miRNAs sequences were used to do Blast searches against sorghum genome from NCBI (Paterson et al., 2009) and only reads above five could be used to predict novel miRNAs. Briefly, the following strategies were used: (1) candidate miRNA sites were screened out from breakpoints defined by mapping small RNAs, (2) a minimally stringent criterion described by Stocks et al. (2012) was used to select miRNA candidate and a maximum of three mismatches between the miRNA and miRNA* was allowed, and (3) the RNA secondary structure was checked using Mfold at *p*-value less than 0.1 and mature miRNAs residing in one arm of the stem region with a free-folding energy lower than or equal to −20 kcal mol-1. (Zuker, 2003).

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