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Identification and Characterization of Drought Stress-Responsive Novel microRNAs in Dongxiang Wild Rice

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Abstract: MicroRNAs (miRNAs) are non-coding small RNAs, which play important regulatory roles in response to biotic and abiotic stresses. Dongxiang wild rice (*Oryza rufipogon*, DXWR) can survive in extreme drought environment, but its molecular mechanism of drought resistance is still largely unknown. To further explore miRNA regulatory mechanisms involved in drought resistance, we identified 138 novel miRNAs in DXWR using small RNA sequencing and bioinformatics approaches, and found that the expression levels of 67 novel miRNAs were significantly affected by drought stress. In total, 200 candidate target genes were predicted and annotated for the drought stress-responsive novel miRNAs. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways suggested that most of the target genes were related to metabolism. Stem-loop quantitative real-time PCR (qRT-PCR) results exhibited high concordance with sequencing data, which confirmed that miRNA expression patterns based on small RNA sequencing in the present study were reliable. Meanwhile, qRT-PCR validated the inverse expression patterns between several miRNAs and their target genes. These results will enhance our understanding of miRNA regulatory mechanisms in response to drought stress in DXWR, and can serve as an important reference for the protection and utilization of this valuable genetic resource.

Key words: Dongxiang wild rice; drought stress; genetic resource; novel microRNA; small RNA sequencing

Rice (*Oryza sativa*), a cereal crop, is the most widely consumed staple food for a large part of the world's human population (Fukao and Xiong, 2013). Drought, one of the most serious abiotic stresses, has severely impaired rice growth and productivity in recent years (Krannich et al, 2015; Li and Yang, 2017). The development of rice cultivars with increased drought resistance has been regarded as an efficient strategy to stabilize and improve production (Luo, 2010; Ding et al, 2016).

Dongxiang wild rice (*O. rufipogon*, DXWR) is a common wild rice that originates from Dongxiang County (28°14′ N), Jiangxi Province, China, which is the northernmost region where *O. rufipogon* is found in the world (Mao et al, 2015; Zhang et al, 2016). DXWR possesses high drought resistance and is a valuable genetic resource for the improvement of rice drought resistance (Zhang et al, 2006; Zhang et al, 2016). To date, however, little is known about the molecular mechanisms underlying the drought resistance of DXWR.

In plants, many studies have revealed that microRNAs (miRNAs) play important roles in response to biotic and abiotic stresses (Khraiwesh et al, 2012; Pei et al, 2013; Sailaja et al, 2014; Shriram et al, 2016). However, most of the current studies focus on model plant species and major crops (Tang and Chu, 2017). For example, Xia et al (2012) reported that over-expression of *OsmiR393* results in the reduction of resistance to drought and salt stress in rice. Yan et al (2016) showed

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that the reduction in the expression of *miR165/166* confers drought and cold resistance to *Arabidopsis*. Li et al (2016) reported that overexpression of soybean *miR172c* confers resistance to water deficit and salt stress in *Arabidopsis*. According to the latest release of miRBase (Release 21, http://www.mirbase.org), a total of 35 828 mature miRNAs have been identified in 223 species. However, it is estimated that the total number of miRNAs is approximately 1% of all the genes in each genome, implying that a myriad of miRNAs remain unidentified (Lim et al, 2003). To further understand the functions of plant miRNAs, more studies should be performed using various plant species or closely related species containing novel miRNAs specific to genetic and developmental features.

In the present study, using small RNA sequencing and bioinformatic approaches, we identified and characterized novel miRNAs in DXWR, analyzed their expression patterns under drought stress, predicted and annotated target genes for the drought stress-responsive novel miRNAs. In addition, classification and enrichment of the target genes were conducted using Gene Ontology (GO) analysis, and the functions of the target gene products and associated pathways were predicted using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. These results provide novel insights into the regulatory mechanisms underlying plant resistance to drought stress.

MATERIALS AND METHODS

Identification of novel miRNAs by bioinformatics

Two small RNA libraries were constructed from seedlings of DXWR with and without drought stress, and they were named DY-D and DY-N, respectively (Zhang et al, 2016). Meanwhile, the drought stress-responsive conserved miRNAs were identified by small RNA sequencing. To further identify drought stress-responsive novel miRNAs in DXWR, data from the previous study with respect to sequencing, read filtering, and mapping to the reference genome were used in this study. Sequences that could not be mapped to any conserved miRNAs in miRBase (Release 21, http://www.mirbase.org) were used to predict potential novel miRNAs by the Mireap software (http://sourceforge.net/projects/mireap/) (Li et al, 2012). The criteria were as follows: length range of miRNA sequence should be 18–25 nucleotides (nt); length range of miRNA reference sequence should be 20–23 nt; maximal copy number of reference miRNAs should be 20; maximal free energy allowed for an miRNA precursor should be -18 kcal/mol; maximal space between miRNA and miRNA* should be 300; minimal base pairs between miRNA and miRNA* should be 16, with no more than four bulges; and maximal asymmetry of miRNA:: miRNA* duplex should be 4 bases.

Novel miRNA expression profile and differential expression analysis

To determine the miRNA expression profile, the miRNA count was normalized to the same order of magnitude according to the following formula: Normalized expression = Actual miRNA count / Total count of clean reads × 1 000 000. If the normalized read count of a given miRNA is zero, the expression value is set to 0.01 for further analysis. The differential expression of each miRNA was evaluated by the fold change of the normalized expression. The $\log_2$(A/B) (A and B represented the normalized expression of miRNA in DXWR with and without drought stress, respectively) was calculated. *P*-value was calculated and further corrected by Bonferroni correction (Li et al, 2017). The differentially expressed novel miRNAs were selected with $|\log_2(A/B)| \geq 1$ and $P < 0.01$.

Target gene prediction and enrichment analysis

The putative target genes for the novel miRNAs were predicted using the plant-targeted gene prediction software psRNATarget (http://plantgrn.noble.org/psRNATarget/) (Lian et al, 2016). *Oryza sativa* (rice) transcript MSU Rice Genome Annotation version 7 was used as the cDNA library for the target search as described previously (Lian et al, 2016). The GO enrichment analyses of target genes were performed using the GOseq R package (Lu et al, 2016). The KEGG database was used to search for high-level functions in biological systems (http://www.genome.jp/kegg/). KOBAS software was used to test the statistical enrichment of target genes in the KEGG pathways (Lu et al, 2016).

Drought treatment, quantitative RT-PCR (qRT-PCR) and stem-loop qRT-PCR

Drought treatment was performed following the methods of Zhang et al (2016). Total RNA was extracted from the samples using TRIzol reagent (Takara, Dalian, China) and treated with RNase-free DNase I (Promega, Madison, USA). miRNA cDNA synthesis was carried out using the TaqMan microRNA Reverse