Journal of Plant Growth Regulation (2022) 41:1099–1108
https://doi.org/10.1007/s00344-021-10366-7

miRNA-Based Drought Regulation in the Important Medicinal Plant *Dendrobium huoshanense*

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Received: 31 August 2020 / Accepted: 7 April 2021 / Published online: 19 April 2021

Abstract

Dendrobium is widely used in traditional Chinese medicine, and *Dendrobium* species show high diversity in potential adaptation to drought stress. Although miRNAs play crucial roles in regulating drought responses in plants, their functions in the drought response in *Dendrobium* remain unclear. To investigate the roles of miRNAs and their targets during drought stress, a small-RNA library from drought-treated *Dendrobium huoshanense* was constructed and sequenced. A total of 211 miRNAs were identified, comprising 115 known miRNAs and 96 novel miRNAs. Among them, 36 conserved and 33 novel miRNAs were characterized as differentially expressed miRNAs between drought and control samples. Target prediction, Gene Ontology (GO)-based functional classification, and Kyoto Encyclopedia of Genes and Genomes (KEGG)-based functional enrichment showed that drought-regulated miRNAs might play roles in the response to drought stress by targeting a series of stress-related genes. Importantly, integrated analysis of the miRNA transcriptome and qRT-PCR detection were performed to identify miR156, miR157d, and miR160a-5p and their targets (*auxin response factor*, *cytokinin receptor*, *two-component response regulator*, and *DELLA protein gene*). The results indicated that drought-regulated miRNAs might regulate their targets to respond to hormone signaling, thereby regulating the morphological and physiological responses of the plant to drought stress. Our results provide new insights into the complex regulatory network of medicinal plant responses to drought stress involving plant hormone signal transduction.

Keywords *Dendrobium huoshanense* · Drought · MicroRNA · Hormone signal transduction

Introduction

Drought is one of the major abiotic factors and destructive environmental stresses that restricts the growth, development and yield of plants; in the meantime, some signaling pathways in plants would been activated during the drought stress response (Ashraf 2010). Plants have developed a variety of defense strategies against drought at the morphological, physiological, biochemical, cellular, and molecular levels (Farooq et al. 2012). At the morphological level, to avoid desiccation, plants lower their growth rates, deepen their rooting systems, and modify their root to shoot ratios; at the physiological level, and they induce stomatal closure and antioxidant accumulation (Tátrai et al. 2016). At the molecular level, regulation of gene expression (enzymes and drought-related proteins) and epigenetic alterations (methylation, histone modifications and posttranscriptional alterations) are important mechanisms in the plant response to drought (Bhargava and Sawant 2013). Research to date shows that the plant drought stress responses,

Handling Editor: Rhonda Peavy.

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tolerance mechanisms and the genetic control of tolerance are complex.

MicroRNA (miRNA)-mediated regulatory mechanisms play crucial roles in plants, including regulating several plant developmental processes, such as leaf development, flowering time, and organ polarity, and responses to biotic and abiotic stresses (Chen 2005; Jeong and Green 2013). Many miRNAs have been found to participate in the response to drought stress in plants via signal transduction pathways, such as auxin signaling, and via ABA-mediated regulation, osmoprotectant biosynthesis, and scavenging of antioxidants (Candar-Cakir et al. 2016; Ding et al. 2013). Drought-related miRNAs and their targets have been identified in several plants, such as *Arabidopsis*, cotton, and tomato (Candar-Cakir et al. 2016; Liu et al. 2008; Xie et al. 2015b). For example, in a study employing small-RNA and degradome deep sequencing in tomato, 699-conserved miRNAs were identified, and some of those miRNAs associated with the drought response involving plant hormone signal transduction and drought-tolerant tomato breeding (Candar-Cakir et al. 2016). Furthermore, an analysis entailing citationRank-based literature mining in cotton led to the identification of a total of 337 miRNAs, and miRNAs associated with the expression of important genes related to drought stress were found (Xie et al. 2015b). However, most research on drought-related miRNAs has been focused on crop plants, and the potential functions of these miRNAs in medicinal plants are unknown.

Recently, some important drought-responsive miRNAs were identified in plants. miR169, miR398, and miR5505 were candidates with potential in developing drought tolerance in six *Oryza sativa* genotypes based on the genome-wide analysis (Xia et al. 2020). miR169 was found commonly present under drought stress but with different outputs depending on the species: downregulated in *A. thaliana* (Li et al. 2016) and *N. tabacum* (Yin et al. 2014) and upregulated in *O. sativa* (Xia et al. 2020). In *N. tabacum*, miR160 regulates the expression of auxin response factor genes; auxins are vital phytohormones for plant growth and development also involved in tolerance to drought stress (Li et al. 2016). miR156 and miR172 are other transcription factor-related regulators commonly involved in moderation of abiotic stresses (Pagano et al. 2021). Moreover, three conserved miRNAs (miR156, miR159, and miR319) were identified and played important roles in the seedlings of a drought-tolerant inbred *Z. mays* and *N. tabacum* (Li et al. 2013).

Dendrobium huoshanense is a famous perennial medical herb of the family Orchidaceae and is extensively used in China to remedy symptoms such as chronic superficial gastritis and throat phlegmonosis, which enhance immunity and achieve other benefits (Bao et al. 2001; Liu et al. 2018). *D. huoshanense* contains a variety of chemical components, such as polysaccharides and flavonoids, which have numerous pharmacological activities, including antioxidant, immunomodulation, antifatigue, digestion-promoting, salivary secretion-stimulating, hypoglycemic, antihypertension, and antitumor activities (Li et al. 2015). Although several studies have revealed the adverse impacts of drought stress on medicinal plants, such as negative effects on plant growth, development and yield of natural products, the effects of drought stress on the quality of medicinal plants are multilayered and very complex, and no study focused on the genome-wide identification of drought-responsive miRNAs in *D. huoshanense* has been conducted (Selmar and Kleinwächter 2013).

The current study attempts to identify expression pattern of miRNAs in response to drought stress in *D. huoshanense* through high-throughput sequencing and identifies the miRNA predicted target gene functions. We sequenced the genome of *D. huoshanense*. The total length of its assembly was 1.29 Gb and 20,879 protein-coding genes were identified. In this study, characteristics of small regulatory microRNAs (miRNAs) in the response to drought stress in *D. huoshanense* were investigated. A total of 115 conserved miRNAs and 96 novel miRNAs were identified from different libraries, of which at least 8 miRNAs were drought specific. Evidence from miRNA-target prediction, Gene Ontology (GO) term classification, Kyoto Encyclopedia of Genes and Genomes (KEGG)-based pathway analysis, and quantitative real-time PCR (qRT-PCR) results further suggested that these identified miRNAs may contribute to the response to drought stress in *D. huoshanense*. Together, these findings could increase our understanding of the complex regulatory network that underlies the draught tolerance of *D. huoshanense*.

Materials and Methods

Plant Material and RNA Extraction

Specimens of *D. huoshanense* were collected from a wild population in northern Yunnan Province, China, and available from the College of Biological and Pharmaceutical Engineering, West Anhui University, Lu'an, China. *D. huoshanense* plants were maintained in a greenhouse as previously reported (Yang et al. 2014; Zou et al. 2018).

Seeds of *D. huoshanense* were surface sterilized with 70% ethanol and $HgCl_2$ and then transferred to solid MS medium supplemented with 0.5 mg/L 6-benzylaminopurine and 0.5 mg/L 1-naphthaleneacetic acid (NAA). The plants were grown at 27 °C under a relative humidity of 50%. After 9 months of cultivation, 10-month-old individuals were chosen and transferred to plastic pots (20.0 cm in diameter) filled with a substrate mix of composted pine bark and small stones. The plants were maintained at 27/22 °C

(day/night), a photoperiod of 12/12 hr (day/night), under a relative humidity of 50/70% (day/night) and were watered every two days. After 2 months, strong individuals were selected and divided into two groups. Plants without watering for 7 days, then leaf water content was measured with the fourth mature leaves and the effects of drought stress were divided into mild, moderate, and severe drought stress with leaf water content of > 78%, ~ 75%, and < 72%, moderate drought stress-treated *D. huoshanense* was chosen as the drought-treated group. Plants watering every two days without undergoing drought or waterlogging as the control group. The fourth mature leaves from the apex of each individual were harvested and placed in liquid nitrogen for later RNA extraction.

Small-RNA Library Construction and Sequencing

Total RNA of *D. huoshanense* was extracted as previously described (Liu et al. 2018). The RNA was treated with DNase I (Invitrogen) to remove any potential genomic DNA contamination. RNA degradation and contamination were monitored on 1% agarose gels. The quality and quantity of RNAs were measured with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA concentration was measured using a Qubit® RNA assay kit and a Qubit® 2.0 fluorometer (Life Technologies, Pleasanton, CA, USA). Small RNAs (15–30 nt) were extracted from total RNA on a 15% denaturing polyacrylamide gel and ligated to specific 5′ adaptor and 3′ adaptor samples. Two sRNA libraries (two independent biological replicates) from the same treatment were, respectively, used as samples, and an Illumina HiSeq 4000 platform was used for high-throughput sequencing.

Data Analysis and Identification of miRNA Genes

The raw reads were processed, and miRNA genes were identified as in our previous study with minor modifications (Shao et al. 2019). Briefly, high-quality small-RNA reads were obtained from the raw reads by filtering out poor-quality reads and removing adaptor sequences using the FASTX toolkit with the default settings (Blankenberg et al. 2010). Adaptor-trimmed unique sequences were aligned to the *D. huoshanense* genome with Bowtie (Langmead et al. 2009). After removal of known noncoding RNAs (rRNAs, tRNAs, snRNAs, and snoRNAs) by BLASTall, BLASTn, and Bowtie, the unannotated small RNAs were used for novel miRNA prediction by using mireap (Li et al. 2012). Then, for annotation of the remaining sequences, conserved miRNAs were mapped to the miRBase database (v22.1 on March 26th, 2020; http://www.mirbase.org/ftp.shtml). The raw abundances of the miRNAs were normalized according to transcripts per million (TPM) normalization: small RNAs were mapped back onto the reference genome, and read count for each small RNA was obtained from the mapping results (t Hoen et al. 2008). PsRobot (https://tools4mirs.org/software/target_prediction/psrobot/) and TargetFinder (http://plantgrn.noble.org/psRNATarget/) were used for the prediction of miRNAs and their targets according to plant-like target interactions and based on previously described methods.

Expression Analysis of miRNAs and Target Genes

The stem-loop quantitative real-time PCR method was used to quantitate miRNA expression in this study using 5S rRNA as the internal control for each sample (Candar-Cakir et al. 2016). In brief, stem-loop reverse transcription was carried out using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The expression of target genes was detected as mentioned above, and the Actin gene was used as the internal control for each sample. qRT-PCRs were carried out using the CFX96™ Real-Time PCR System (Bio-Rad) with SYBR® Premix Ex Taq™ II (TaKaRa). The fold changes were calculated using $2^{-\Delta\Delta CT}$ values, and six independent experiments were performed. All data are presented as the mean ± SE of six replicates.

Statistical Analysis

Data were analyzed by one-way analysis of variance, followed by Dunnett's multiple comparison and Tukey's test using the SPSS v23.0 software. All results were expressed as the mean ± standard error of the mean (SD). $p < 0.05$ was considered statistically significant.

Results

Identification of miRNAs in D. huoshanense

A total of 84,908,472 raw reads were obtained from the small-RNA libraries generated from the drought treatment (51,851,851) and the control treatment (33,056,621), from which a total of 58,588,717 clean reads were obtained. After alignment against the *D. huoshanense* genome, 25,049,100 and 26,455,704 clean reads from the drought and control samples, respectively, were mapped to the plant genome. A total of 211 miRNAs were identified from the different samples of *D. huoshanense* (Table S1). miRNAs are well known to be highly conserved across plant species. A total of 115 known plant miRNAs were identified in *D. huoshanense* after the alignment of all clean reads from the two libraries against all known plant miRNAs in miRBase (Table S1). We identified 96 putative novel miRNA gene loci in *D. huoshanense* based on the methods described in a previous

study (Fig. 1a and Table S1) (Mutum et al. 2016). According to mature sequence length, 67.3% of all miRNAs were of canonical length (20–23 bp), and the largest percentage of miRNAs, 37.91%, were in the 21-nt class, followed by the 20-nt class (15.64%) (Fig. 1b). The distribution of 5' terminal bases differed among miRNAs of different lengths; those 20–23 bp in length had a strong preference for "U", in contrast to miRNAs with lengths of 24–28 bp (Fig. 1c).

miRNA Expression in Response to Drought Treatment

Among the 211 identified miRNAs, 8 miRNAs were expressed exclusively in the drought samples, whereas 190 were expressed in both drought and control samples (Fig. 2a). The abundance of miRNAs was normalized according to transcripts per million (TPM) normalization, and we used an absolute value of the $\log_2$ ratio ≥ 1 ($p < 0.05$) as the threshold to determine significant differences in miRNA expression. A total of 69 miRNAs were found to have significantly different expressions between the drought and control samples: 38 miRNAs were upregulated while the remainder were downregulated in drought samples relative to control samples (Fig. 2a and Table S1). To gain more insight into the characteristics of miRNA expression, a heatmap of the differentially expressed miRNAs was constructed (Fig. 2c). Compared to their expression following control treatment, numerous miRNAs were upregulated during the drought response, such as miR156, miR167d_1, miR160-5p, and miR396a-5p, which have been identified as upregulated in several plant species under drought stress (Gentile et al. 2015). These significantly differentially expressed miRNAs may play crucial roles in the drought response in *D. huoshanense*.

Identification and Functional Prediction of miRNA Targets

Plant miRNAs directly affect their target genes by cleaving them or repressing their translation. To understand the biological functions triggered by miRNAs in the response to drought treatment in *D. huoshanense*, the target genes of these miRNAs were identified. After strict filtration with a series of miRNA-target features, a total of 852 unique coding genes were predicted to be targets of these miRNAs, and a total of 1458 miRNA-target pairs were identified (Table S2).

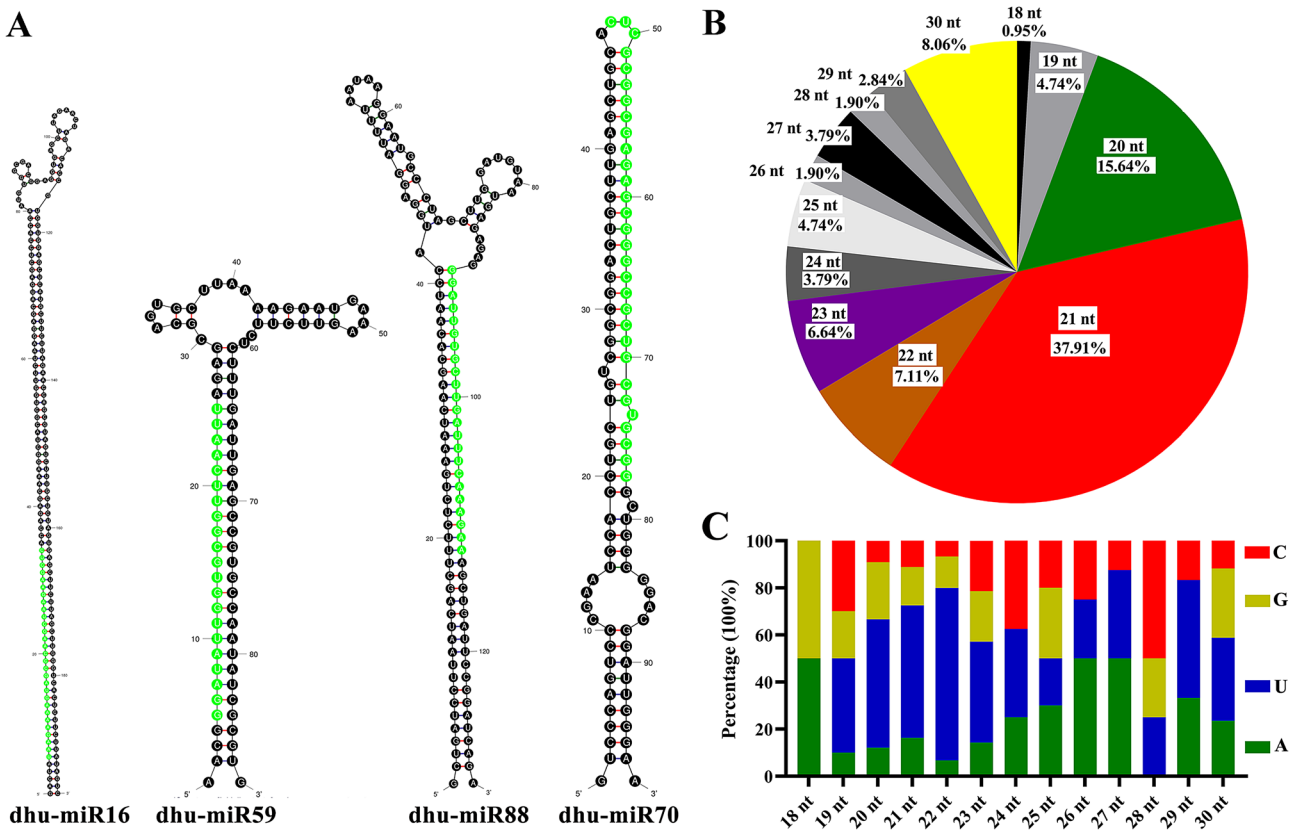

Fig. 1 Identification and characteristics of *D. huoshanense* miRNAs. **a** Examples of the secondary structure of locus-specific miRNA precursors. The green sequence represents mature miRNA. **b** Size distribution of miRNAs. **c** 5′-terminal nucleotides of miRNAs

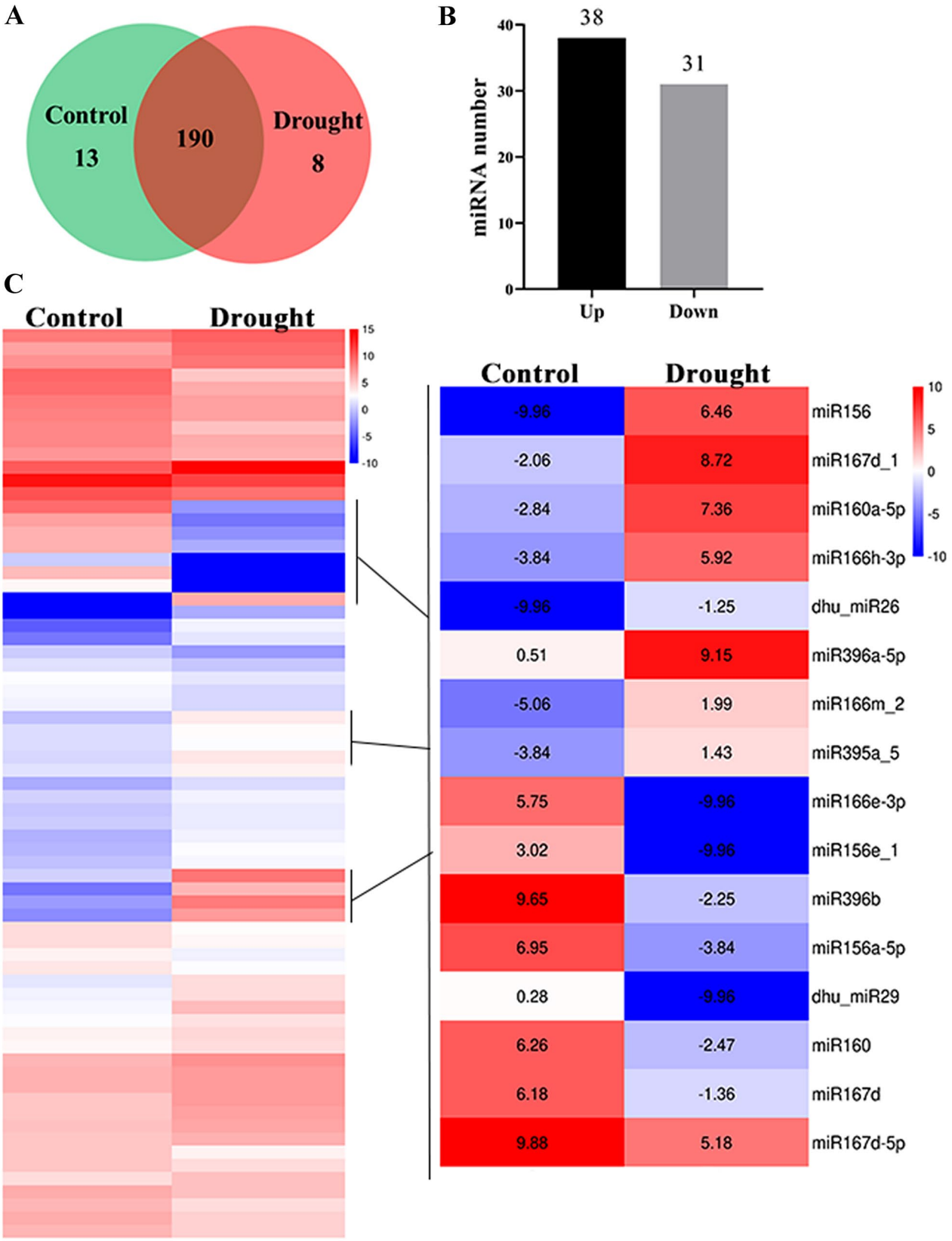

Fig. 2 Expression levels of miRNAs in the control and drought samples. **a** Venn diagram of the number of miRNAs from different samples. **b** The differentially expressed miRNAs. **c** Heatmaps of *D. huoshanense* miRNAs in the control and drought samples

Gene ontology (GO) functional analysis (Conesa et al. 2005) and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis were carried out to identify the potential function of each putative target gene. Based on sequence homology, the target genes were categorized into 30 functional groups ($p < 0.05$). "Regulation of transcription, DNA-templated," "Regulation of nucleic acid-templated transcription," "Regulation of RNA biosynthetic process," and "Regulation of RNA metabolic process" were the most enriched terms in the GO term categories (Fig. 3a). Their target genes were enriched in 12 KEGG pathways ($p < 0.05$) (Fig. 3b). Of these pathways, "plant hormone signal transduction" was the most enriched pathway term, with 24 target genes being annotated to this term. Plant hormone signaling machinery modulates plant growth, development, and defense, suggesting that miRNAs may regulate *D. huoshanense* responses to drought via the plant hormone signal transduction pathway (Wang et al. 2015).

miRNA Predicted Targeted Genes Involved in the Plant Hormone Signal Transduction Pathway

To further explore the interaction between drought-responsive miRNAs and their potential target genes involved in the plant hormone signal transduction pathway, a KEGG pathway map was constructed for the plant hormone signal transduction pathway (Fig. 4a). The auxin response factor (ARF), cytokinin receptor (CRE1), two-component response regulator (B-ARR), DELLA protein (DELLA), and brassinosteroid insensitive protein 1 (BRI1) genes, which play crucial roles in plant growth, shoot initiation, and induced germination, were targeted by drought responsive, differentially expressed miRNAs. To understand and visualize the regulation network between miRNAs and their possible targets involved in the plant hormone signal transduction pathway, regulatory network analysis was performed; the results are shown in Fig. 4b. A total of 11 miRNAs were predicted to target 27 genes involved in plant hormone signal transduction pathways (Table S3). The miR156 family had connections with 9 target genes, and the miR171 family had connections with 6 target genes, suggesting that these families likely function in the response to drought by targeting a number of different genes in *D. huoshanense*.

To validate the regulatory roles of miRNAs in plant hormone signal transduction during the drought response, we analyzed the expression profiles of the 11 miRNAs and their corresponding targets with qRT-PCR (Fig. 5a). The expression profiles of all 11 miRNAs were consistent with the sequencing data and showed significant differential expression between the drought and control samples (Fig. 5b). Among these 27 genes, 24 genes were detected with qRT-PCR in drought and control samples. Nine of these genes were significant differentially expressed, with 6 being upregulated while three being downregulated in the drought samples (Fig. 5a). Based on "Plant miRNAs could directly affect their target genes with cleavage," we searched the miRNAs and their target genes that showed opposing expression patterns. Eight miRNAs and eight target genes, representing 13 miRNA-target pairs, were found.

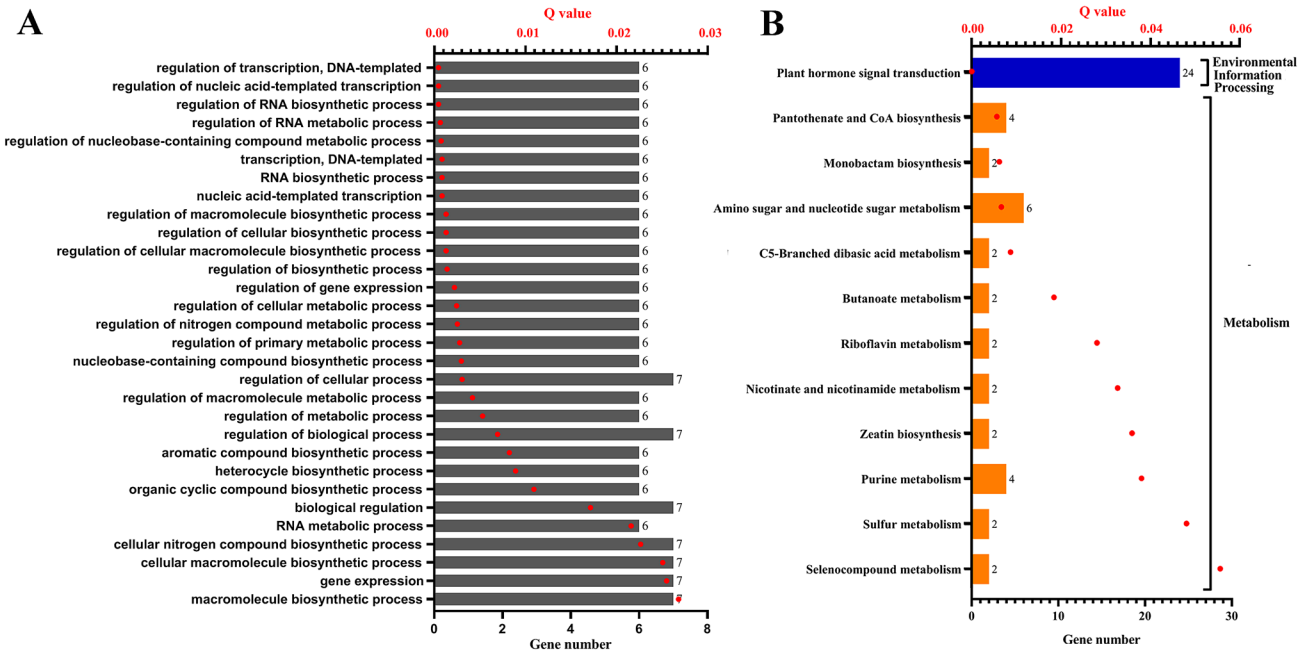

Fig. 3 Significantly enriched GO (**a**) and KEGG (**b**) terms for the target genes of differentially expressed miRNAs

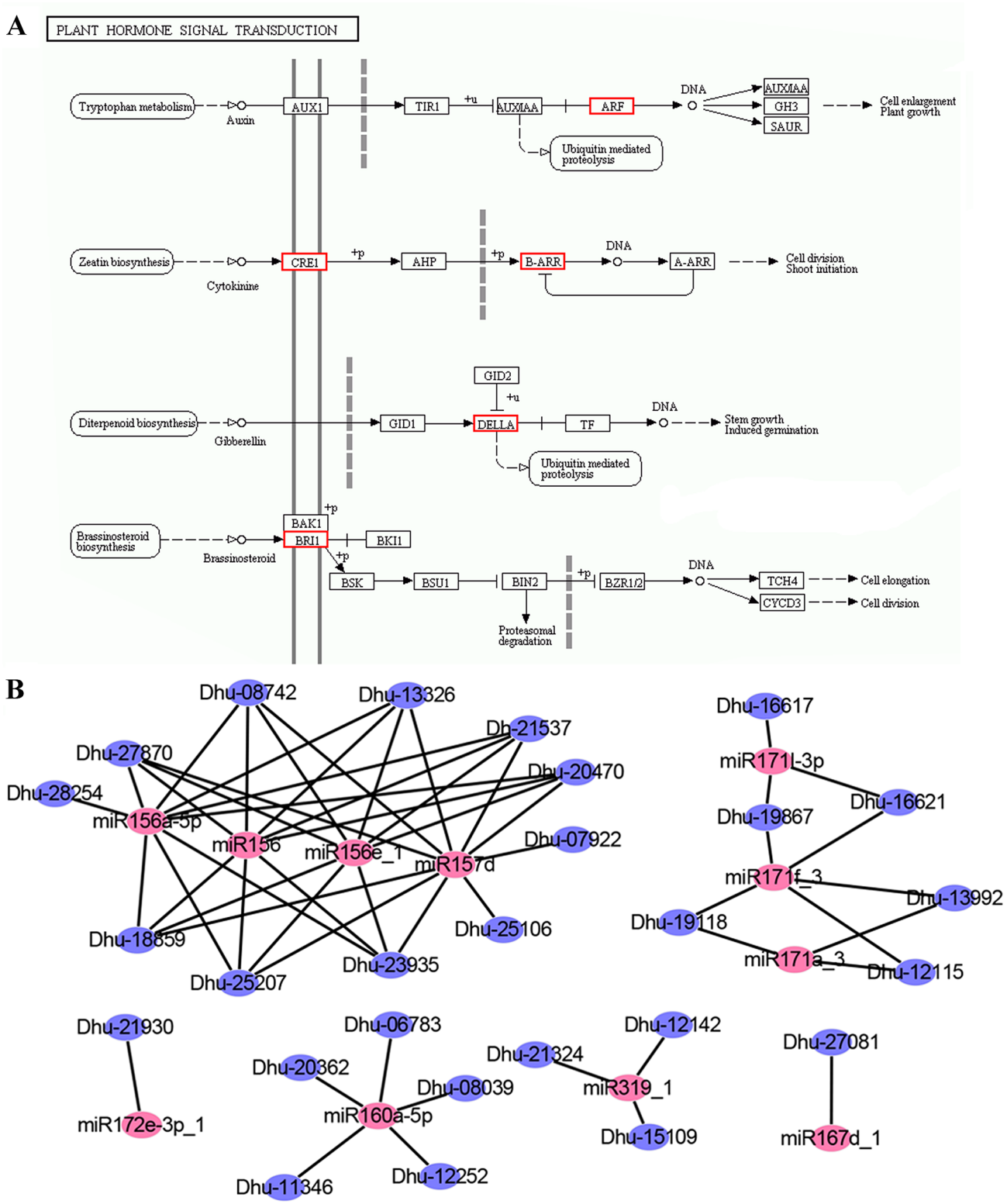

Fig. 4 Network analysis of drought-responsive miRNAs and their potential targets in hormone signaling pathways. **a** Target genes mapped for the plant hormone signal transduction pathway. **b** Network analysis of target genes involved in plant hormone signal transduction

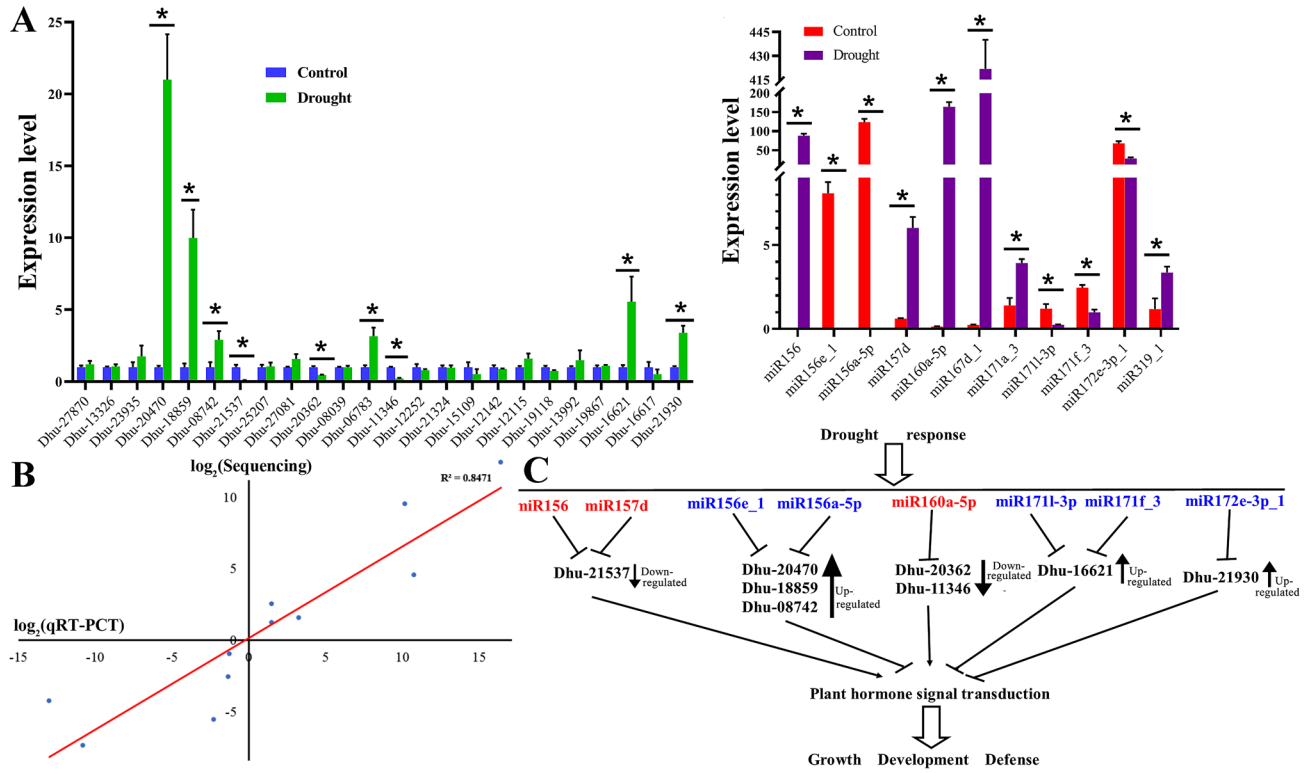

Fig. 5 The proposed model of the miRNA-mediated regulatory network associated with hormone signal transduction. **a** qRT-PCR analysis of expression profiles of miRNAs and their corresponding targets. **b** Correlation of qRT-PCR and sequencing data of miRNAs. **c** The proposed model of the miRNA-mediated regulatory network involved in hormone signal transduction. red, upregulation; blue, downregulation

In *Arabidopsis thaliana*, *Nicotiana tabacum*, *Triticum aestivum*, *Zea mays,* and *O. sativa*, expression of miR156 is known to be induced by different abiotic stresses, helping plants resist adverse environments through regulation of the squamosa promoter-binding protein-like target (Pagano et al. 2021); miR160-directed regulation of *Arabidopsis* auxin response factor is essential for the tolerance to various abiotic stresses (Li et al. 2016); Moreover, miR171 and miR172 family miRNAs could be specifically involved in the chilling stress response by regulating transcript targets influencing development (Chen et al. 2016). In our study, Genes encoding squamosa promoter-binding family protein (Dhu-21537) and auxin response factor family proteins (Dhu-20362 and Dhu-11346) were negatively regulated by drought-upregulated miRNAs (miR156, miR157d, and miR160a-5p), whereas genes encoding other squamosa promoter-binding family proteins (Dhu-20470, Dhu-18859, and Dhu-08742), a nodulation-signaling pathway protein (Dhu-16621), and a two-component response regulator protein (Dhu-21930) were negatively regulated by drought-upregulated miRNAs (miR156e_1, miR156a-5p, miR171I-3p, miR171f-3, and miR172e-3p_1). Based on information from our and previous studies, a schematic of the relationships between these miRNAs and plant hormone signal transduction is presented in Fig. 5c.

Discussion

Dendrobium is widely used in traditional Chinese medicine and contains many kinds of active ingredients. Some *Dendrobium* species, such as *D. huoshanense*, not only are adapted to mid- or high-land moist areas at elevations up to 800 m dpl but can also grow in dry lowland regions (Xiang et al. 2016). Knowledge of their strategies for adaptation is essential for their conservation and wide cultivation, and drought stress can affect the growth and secondary metabolites of *Dendrobium* (Metusala et al. 2017; Wu et al. 2016). Compared with *D. arcuatum*, *D. capra* shows more specialized anatomical features for adapting to drought and dry conditions (Metusala et al. 2017). miRNAs, as endogenous small noncoding regulatory RNAs, play important roles in abiotic and biotic stress responses by targeting plant mRNAs for cleavage or translational repression (Bartel 2004; Hajyzadeh et al. 2015). However, no genome-wide study on drought-responsive miRNAs in *Dendrobium* has been reported to date. In this research, miRNAs that exhibit

differential expression upon drought stress in *D. huoshanense* were determined.

Drought-responsive miRNAs have been identified in many plant species through microarray or deep-sequencing technologies, such as *Arabidopsis*, wheat, tobacco, potato, and barley (Candar-Cakir et al. 2016; Gentile et al. 2015; Jeong and Green 2013; Xie et al. 2015a). Studies have shown that the expression of miRNAs is altered in the response to drought stress; miRNAs, as gene regulators, are expected to participate in gene regulation in plants (Gentile et al. 2015). Based on the obtained transcriptome datasets, 36 conserved and 33 novel miRNAs were characterized as differentially expressed miRNAs in drought and control samples. In our study, miR156 and miR394a_1 were upregulated under drought stress while miR168a-3p_1 was downregulated. These results are similar to results from *Arabidopsis*, whereas the expression of these three miRNAs showed opposite patterns in rice and maize, revealing that the expression level or drought responsiveness of a miRNA is species dependent (Liu et al. 2008; Wei et al. 2009; Zhou et al. 2010).

Both drought-upregulated and drought-downregulated miRNAs are potentially relevant for engineering plant drought tolerance (Gentile et al. 2015). Hormones play a central role in regulating plant growth, development, and defense (Wang et al. 2015). In this study, ARF, CRE1, B-ARR, DELLA, and BRI1-coding genes, as key genes in plant hormone signal transduction pathways, were targeted by drought responsive, differentially expressed miRNAs in *D. huoshanense*. The qRT-PCR results demonstrated that miRNAs may regulate the expression of their target genes involved in hormone signaling and thereby control the growth and development of *D. huoshanense* during the drought response.

In previous studies, miR156 contributes to plants to drought environment through regulation of the squamosa promoter-binding protein-like target (Pagano et al. 2021); miR160-directed regulation of *Arabidopsis* auxin response factor is essential for the tolerance to various abiotic stresses (Li et al. 2016); Moreover, miR171 and miR172 family miRNAs could be specifically involved in the chilling stress response by regulating transcript targets influencing development (Chen et al. 2016). Importantly, in our study, genes encoding squamosa promoter-binding family protein and auxin response factor family proteins were negatively regulated by miR156 and miR160a-5p, whereas genes encoding other squamosa promoter-binding family proteins, a nodulation-signaling pathway protein, and a two-component response regulator protein were negatively regulated by miR171I-3p, miR171f-3, and miR172e-3p_1, further confirmed that conserved roles of miRNAs from miR156, miR160, and miR171 families in different crop species (Pagano et al. 2021).

In conclusion, genome-wide investigation led to the identification of drought-responsive miRNAs in the medical plant, *D. huoshanense*, and these miRNAs might regulate plant growth and development by altering their targets in response to hormone signaling. These findings highlight the importance of detailed characterization of stress-responsive miRNAs in plants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00344-021-10366-7.

Acknowledgements This work was supported by Postdoctoral Research Project of West Anhui University (WXBSH2019003) and Anhui Province (2020B453), National Natural Science Foundation of China (31901383, 21705119 and C0031750), Nature Science Research Project of Anhui Province (1808085QB33), Natural Science Fund of Education Department of Anhui Province (KJ2017A408 and KJ2019A0628).

Author Contributions The authors have made the following declarations about their contributions: Conceived and designed the experiments: YC, BH, and YW. Performed the experiments: YW, JD, RC, CS, PW, and YW. Analyzed the data: YW, JD, RC, CS, PW, and YW. Wrote the paper: YC, BH, and YW.

Declarations

Conflict of interest The authors have no conflict of interest.

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