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Identification and characterization of HD-Zip genes reveals their roles in stresses responses and facultative crassulacean acid metabolism in *Dendrobium catenatum*

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ABSTRACT

Dendrobium catenatum possesses special survival strategy including facultative crassulacean acid metabolism (CAM) to cope with various environmental stresses. The homeodomain-leucine zipper (HD-Zip) proteins had been reported to play essential roles in adaption to the environment. However, the role of the *HD-Zip* gene family in stresses responses and drought-induced facultative CAM in *D. catenatum* was largely unknown. In this study, 35 *HD-Zip* genes were identified in *D. catenatum* (*DcHDZs*), and were phylogenetically grouped into four subfamilies, which was further supported by the analysis of their conserved motifs and structures. Expression profile analysis of *DcHD-Zip* genes based on public transcriptomic data and qRT-PCR showed that most *DcHD-Zip* genes showed biased expression in leaf and flower than in stem and root. Three *DcHD-Zip* genes denominated as *DcHDZ04*, *DcHDZ20* and *DcHDZ22* belong to HD-Zip I and II subfamilies, were induced significantly under high light, low light, salt and heat stresses. Moreover, we found that the plant hormone biosynthesis and signal transduction played a vital role in facultative CAM of *D. catenatum* under drought, especially ABA. Nine *DcHD-Zip* genes were identified as DEGs and showed close relationship with several genes involved in plant hormone biosynthesis and signal transduction, suggesting their important roles in drought-induced facultative CAM of *D. catenatum*. This systematic analysis provided a foundation for further functional characterization of *HD-Zip* genes and also revealed an important regulation mechanism of facultative CAM in *D. catenatum*.

1. Introduction

Because of a sessile lifestyle, plants have evolved a variety of mechanisms to overcome abiotic and biotic stresses, including the modulation of stress responsive gene expression patterns. Transcription factors (TFs) can regulate the expression of their downstream genes at the level of transcription by binding to specific DNA sequences, thereby influencing and controlling various biological processes (Riechmann et al., 2000). The homeodomain-leucine zipper (HD-Zip) protein is a plant-specific TF and the most abundant group of homeobox (HB) protein superfamily. HD-Zip proteins are mostly characterized by the presence of a homeodomain (HD) that is responsible for specific DNA binding and a leucine zipper (LZ) motif that acts as a mediator of proteins dimerization (Sessa et al., 1998; Johannesson et al., 2001). The MEKHLA domain involved in light signaling and START domain with putative lipid-binding capability was also found in some HD-Zip proteins (Elhiti and Stasolla, 2009). Based on their structural features and physiological functions, HD-Zip proteins can be divided into four subfamilies, designed as HD-Zip I, II, III, and IV (Sessa et al., 1998; Aso et al., 1999).

Members of HD-Zip gene family have been identified in diverse plant species, such as *Arabidopsis* (Mukherjee et al., 2009), poplar (Hu et al., 2012), peach (Zhang et al., 2014a), grape (Jiang et al., 2015), *Citrus sinensis* (Ge et al., 2015) and physic nut (Tang et al., 2019). Studies indicate that HD-Zip proteins are involved in and play essential roles in plant growth, development, resistance and adaption to environment. In *Arabidopsis*, the ectopic expression of *ATHB12,* a HD-Zip I member, led to larger leaves with enlarged cells, strongly suggesting its positive effect

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Table 1
Summary of *DcHD-Zip* genes in *Dendrobium catenatum* genome.

Gene_name	Gene_ID	Subfamily	Accession	Nucleotide length (bp)	AA	pI	Mw (kD)	Predicted subcellular localization
DcHDZ01	Dca002090	I	XM_020843127.2	870	289	5.67	32.74	Nuclear
DcHDZ02	Dca004292	I	XM_020839532.2	882	293	5.64	32.72	Nuclear
DcHDZ03	Dca005144	I	XM_020847391.2	876	291	4.76	32.53	Nuclear
DcHDZ04	Dca006486	I	XM_020833900.2	663	220	5.05	25.51	Nuclear
DcHDZ05	Dca007297	I	XM_020837875.2	645	214	6.37	24.02	Nuclear
DcHDZ06	Dca007439	I	XM_020818167.2	1002	333	9.24	37.89	NA
DcHDZ07	Dca011557	I	XM_020816932.2	744	247	4.81	27.72	Nuclear
DcHDZ08	Dca013481	I	XM_020850390.2	630	209	4.9	23.19	Nuclear
DcHDZ09	Dca013741	I	XM_020823303.2	822	273	4.8	31.01	Nuclear
DcHDZ10	Dca017064	I	XM_020844608.2	819	272	5.76	31.12	Nuclear
DcHDZ11	Dca018495	I	XM_020818996.2	834	277	6.32	31.80	Nuclear
DcHDZ12	Dca023965	I	XM_020824131.2	909	302	4.98	34.35	Nuclear
DcHDZ13	Dca001930	II	XM_020837390.2	864	287	8.79	32.13	Nuclear
DcHDZ14	Dca003838	II	XM_020839462.2	612	203	8.38	23.74	Nuclear
DcHDZ15	Dca004571	II	XM_020818836.2	876	291	8.08	32.10	Nuclear
DcHDZ16	Dca005739	II	XM_020841153.2	1221	406	9.22	4.43	Nuclear
DcHDZ17	Dca006275	II	XM_020841472.1	621	206	8.81	24.24	Nuclear
DcHDZ18	Dca011941	II	XM_020841999.2	1002	333	8.7	36.74	Nuclear
DcHDZ19	Dca013653	II	XM_028691773.1	378	125	7.64	14.49	Nuclear
DcHDZ20	Dca015550	II	XM_020824734.2	705	234	9.24	25.84	Nuclear
DcHDZ21	Dca017808	II	XM_028691773.1	594	197	7.7	22.16	Nuclear
DcHDZ22	Dca019434	II	XM_020841748.2	783	260	8.94	29.08	Nuclear
DcHDZ23	Dca021377	II	XM_020836328.2	822	273	6.27	30.08	Nuclear
DcHDZ24	Dca024777	II	XM_020819633.2	831	276	5.43	30.96	Nuclear
DcHDZ25	Dca002633	III	XM_020841243.2	2538	845	5.93	92.77	Nuclear
DcHDZ26	Dca011824	III	XM_020843265.2	2529	842	5.91	92.14	Nuclear
DcHDZ27	Dca020751	III	XM_020826964.2	2604	867	6.03	95.63	Nuclear
DcHDZ28	Dca001533	IV	XM_020837121.2	2316	771	5.63	84.17	Nuclear
DcHDZ29	Dca007458	IV	XM_020828735.2	1824	607	5.26	66.47	Nuclear
DcHDZ30	Dca008733	IV	XM_028698561.1	2376	791	5.87	86.07	Nuclear
DcHDZ31	Dca009861	IV	XM_020844117.2	2337	778	5.66	84.29	Nuclear
DcHDZ32	Dca010357	IV	XM_020819989.2	2535	844	5.6	91.56	Nuclear
DcHDZ33	Dca014199	IV	XM_020821751.2	2445	814	5.81	88.36	Nuclear
DcHDZ34	Dca024155	IV	XM_020847273.2	2301	766	5.62	83.61	Nuclear
DcHDZ35	Dca026330	IV	XM_020843459.2	2118	705	5.94	78.65	Nuclear

on leaf growth (Hur et al., 2015). In *Cucumis sativus*, HD-Zip I member *CsGL1* was identified to function in trichome formation (Li et al., 2015). Three *Arabidopsis* HD-Zip I subfamily genes, including *ATHB6*, *ATBH7* and *ATBH12*, were highly induced by ABA and water stress (Söderman et al., 1996, 1999; Henriksson et al., 2005). The HD-Zip II genes were mainly implicated in phytochrome-mediated organ development, such as leaf morphogenesis (Ciarbelli et al., 2008), and some also respond to light quality changes, shade avoidance, and auxin (Morelli and Ruberti, 2002; Sawa et al., 2002). Steindler et al. (1999) indicated that the involvement of HAT4, a member of HD-Zip II subfamily, in shade avoidance responses has been corroborated by alterations in development observed in seedlings with increased or reduced *HAT4* expression. The HD-Zip III genes were key developmental regulators of apical embryo and shoot radial patterning, shoot meristem formation, lateral organ polarity development, and auxin transport (Baima et al., 2001; McConnell et al., 2001; Emery et al., 2003; Ohashi-Ito and Fukuda, 2003; Prigge et al., 2005). Zhu et al. (2013) showed that *PtrHB7*, a class III HD-Zip gene, played a critical role in regulation of vascular cambium differentiation in Populus. Genetic analysis showed that HD-Zip IV proteins participated in epidermal processes, trichome formation, root development, and anthocyanin accumulation (Javelle et al., 2011). Although *HD-Zip* genes have been studied in several plant species, there is little information about *HD-Zip* genes in genus Dendrobium.

Dendrobium catenatum (Orchidaceae), a synonymous name of *Dendrobium officinale*, is a member of epiphytic orchids which takes root on the surface of tree bark or rocks (Atwood, 1986). Due to the special living environment, *D. catenatum* has evolved novel features and sophisticated defense mechanisms that allow it to exploit its environment and against serious abiotic stresses, including abundant polysaccharides and facultative crassulaceaen acid (CAM) metabolism. *D. catenatum* could serve as a model to shed light on the mechanism of stress response in epiphytic orchids. Furthermore, *D.catenatum* is a well known traditional Chinese medicinal herb with an extensive range of pharmacological properties and ornamental values. Stems of *D. catenatum* contain a large number of polysaccharides that exert proven function on anti-inflammatory, immune-enhancing, antioxidant, and anti-glycation activities (Hsieh et al., 2008; Ng et al., 2012). Therefore, it is not only of great scientific significance, but also of great practical value to study the mechanism of stress response in *D. catenatum*.

Studies indicated that the abundant polysaccharides contribute to stresses tolerance of *D. catenatum* (Yan et al., 2015; Zhang et al., 2016a). Yu et al. (2017) reported that transgenic *Arabidopsis thaliana* seedling overexpressing *UDP glucose 4-epimerase* gene from *D. officinale* accumulated 34.84–44.78 % more water-soluble polysaccharide, and showed an enhanced tolerance to salt and osmotic stresses. He et al. (2017) reported that the contents of mannose-containing polysaccharides were increased by an enhanced expression level of *GDP-mannose pyrophosphorylase* (*GMP*) from *D. officinale*, which led to an enhanced salt stress tolerance in transgenic *Arabidopsis*. Although *D. catenatum* is considered as drought-resistant material (Su and Zhang, 2003), it is hypersensitive to low temperature. A comprehensive regulatory mechanism including cold signal transduction, transcriptional regulation, and gene expression contributes to cold acclimation in *D. catenatum* (Wu et al., 2016). A recent study showed that several transcription factors, such as WRKY, NF-YC, bZIP, bHLH and Trihelix, and genes from the glutathione metabolism pathway were involved in cadmium (Cd) stress response of *D. officinale* (Jiang et al., 2020). Moreover, *D. catenatum* possesses the facultative crassulacean acid metabolism (CAM) pathway which is a water-conserving photosynthetic pathway. The facultative CAM is induced in response to environmental stresses, such as drought (Chu et al., 1990; Brilhaus et al., 2016). CAM is linked to carbon fixation metabolism, circadian clock regulation, stomatal movement, sugar metabolism and transport pathways (Ming et al., 2015; Zhang et al., 2016b; Brilhaus et al., 2016; Yang et al., 2017).

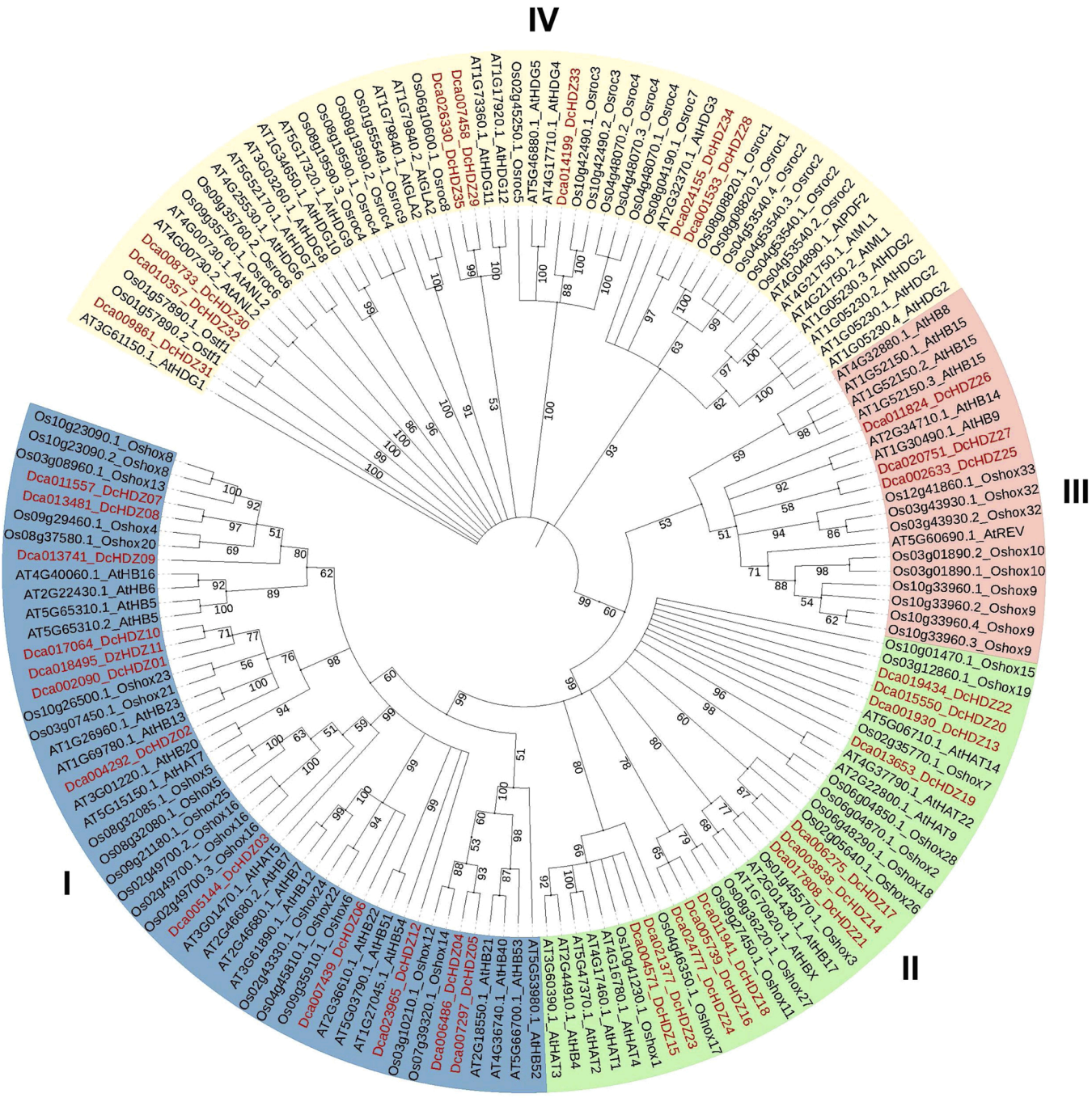

Fig. 1. Phylogenetic tree of *HD-Zip* genes from *D. catenatum, Arabidopsis* and rice. The phylogenetic tree was constructed using the ML method with 1000 bootstrap replications. The four subfamilies were distinguished in different colors. The identified *DcHD-Zip* genes were highlighted by red letters.

Zhang et al. (2019) indicated that alternative splicing events might be involved in CAM metabolism. To date, the regulation mechanism underlying the facultative CAM are largely unclear. Numerous studies have recently demonstrated that *HD-Zip* genes were involved in regulation of plants' responses to abiotic stresses (Zhao et al., 2014; Annapurna et al., 2016). However, responses to abiotic stresses of *HD-Zip* genes, especially in drought-induced facultative CAM were unclear in *D. catenatum*. In this study, we performed a genome-wide analysis of *DcHD-Zip* genes in *D. catenatum* genome, and investigated their characteristics including their classification, conserved motifs, gene structures, *cis*-elements and responses to abiotic stress. Moreover, we used public RNA-Seq data to identify key genes and pathways involved in the facultative CAM, and identified nine *DcHD-Zip* genes showing different expression levels in the facultative CAM of *D. catenatum*.

2. Materials and methods

2.1. Identification of DcHD-Zip genes

The genome sequences of the *D. catenatum* were downloaded from NCBI under the accession codes JSDN00000000 (Zhang et al., 2016a). The *HD-Zip* genes sequences of *A. thaliana* and rice were retrieved from TAIR (https://www.arabidopsis.org/) and Phytozome (http://www.phytozome.net/). We used HD-Zip proteins sequences from *Arabidopsis* and rice as query sequences to search the *D. catenatum* protein database for candidate sequences by using the BLASTP program. The hmmsearch

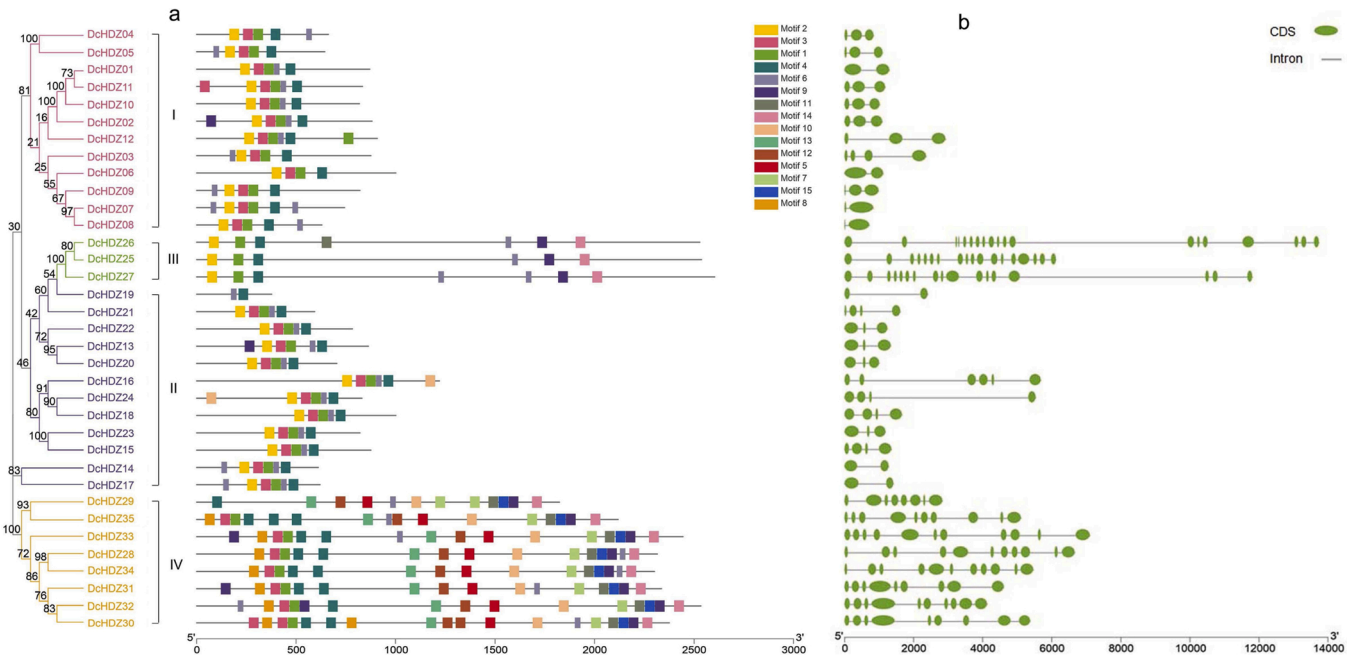

Fig. 2. Architecture of conserved motifs and gene structures of *DcHD-Zip* genes. a, The motif composition and distribution of *DcHD-Zip* genes. b, Gene structures of *DcHD-Zip* genes. Exons and introns were represented by green ellipses and grey lines.

Table 2
Functionally annotated stress-related and hormone-related *cis*-elements identified in the promoters of more than 10 *HD-Zip* genes in *Dendrobium catenatum* genome.

Cis-element	Number of genes	Functions of *cis*-elements
CAAT-box	34	common cis-acting element in promoter and enhancer regions
TATA-box	34	core promoter element around -30 of transcription start
Box 4	31	part of a conserved DNA module involved in light responsiveness
G-Box	28	cis-acting regulatory element involved in light responsiveness
ABRE	26	cis-acting element involved in the abscisic acid responsiveness
TGACG-motif	26	cis-acting regulatory element involved in the MeJA-responsiveness
CGTCA-motif	25	cis-acting regulatory element involved in the MeJA-responsiveness
ARE	23	cis-acting regulatory element essential for the anaerobic induction
GT1-motif	23	light responsive element
GATA-motif	20	part of a light responsive element
CAT-box	19	cis-acting regulatory element related to meristem expression
TCT-motif	18	part of a light responsive element
MBS	16	MYB binding site involved in drought-inducibility
O2-site	16	cis-acting regulatory element involved in zein metabolism regulation
TCA-element	16	cis-acting element involved in salicylic acid responsiveness
TCCC-motif	14	part of a light responsive element
TC-rich repeats	14	cis-acting element involved in defense and stress responsiveness
CCAAT-box	13	MYBHv1 binding site
LTR	13	cis-acting element involved in low-temperature responsiveness
TGA-element	13	auxin-responsive element
MRE	12	MYB binding site involved in light responsiveness
TATC-box	11	cis-acting element involved in gibberellin-responsiveness

program of the HMMER software (version 3.2.1) (http://hmmer.org/download.html) was also applied to the identification of homeobox (PF00046) and the leucine zipper domain (PF02183) in Pfam 32.0 database (http://pfam.xfam.org/). The results of HMMER and BLASTP searches were combined and used for further analysis. The candidates were further screened using SMART (http://smart.embl-heidelberg.de/) and NCBI Conserved Domain-search (https://www. ncbi.nlm.nih.gov/cdd).

2.2. Phylogenetic analysis

To classify and investigate phylogenetic relationships of *DcHD-Zips*, the all predicted *DcHD-Zips* together with those of *Arabidopsis* and rice were aligned with CLUSTAL. Then, a Maximum-Likelihood (ML) phylogenetic tree was constructed by MEGA X (version 10.1.7) (Kumar et al., 2018), with the bootstrap values of 1000 replicates. The phylogenetic tree was imported to iTOL (https://itol.embl.de/) for visualization (Letunic and Bork, 2016).

2.3. Protein properties, conserved motifs, gene structures and cis-elements analyses of DcHD-Zip genes

To investigate protein properties of the *DcHD-Zips*, molecular weight (MW) and isoelectric point (PI) were computed using the online ExPASy-ProtParam tool (http://web.expasy.org/protparam/). The subcellular localization of *DcHD-Zips* were predicted by ProComp 9.0 (http://linux1.softberry.com). Based on the amino-acid sequences, MEME (http://meme.nbcr.net/meme/) (Bailey et al., 2006) was used to analyze the conserved motifs. And the results were visualized by using TBtools (Chen et al., 2020). The Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/) was used to analyze the exon and intron of the *DcHD-Zip* genes. The DNA sequences (2000 bp) upstream of the initiation codon for each candidate gene were extracted, and the *cis*-elements were predicted with PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

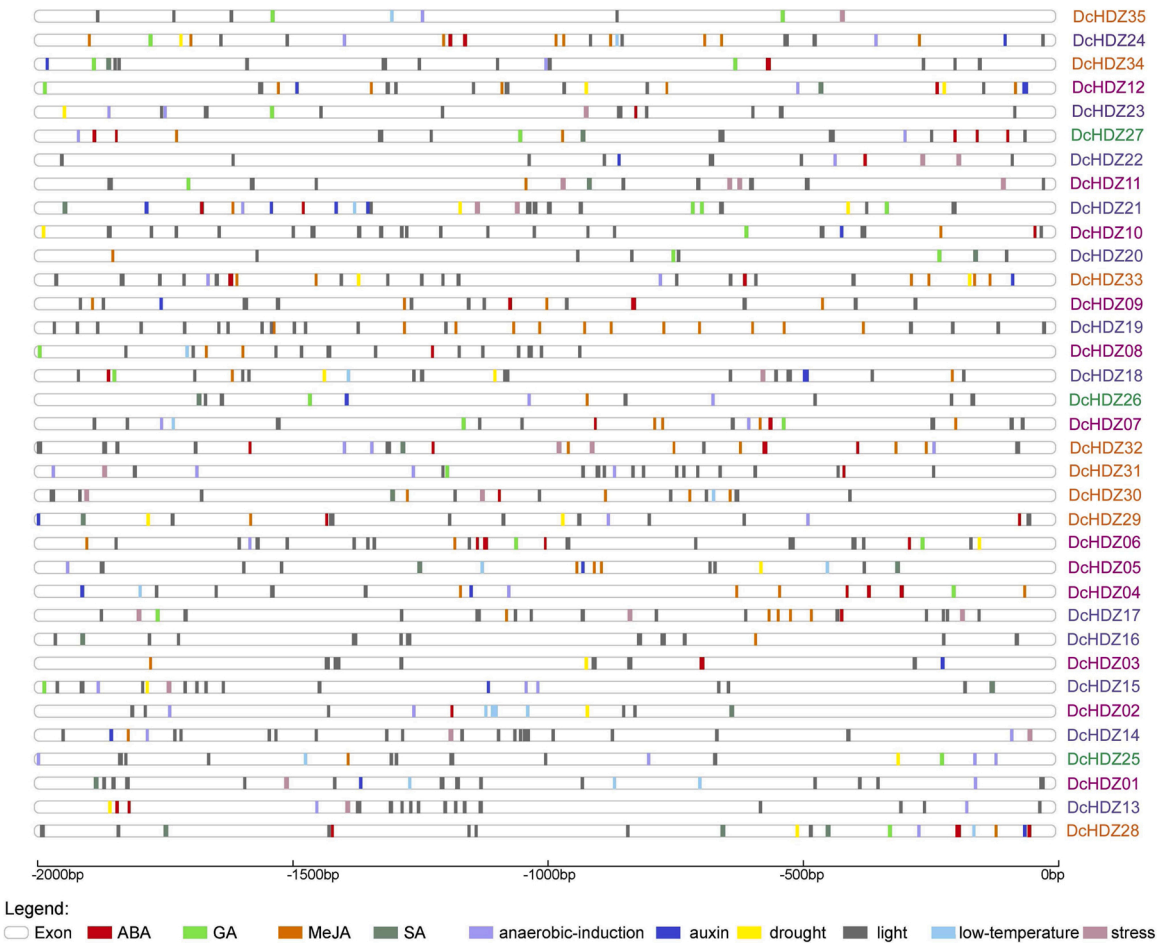

Fig. 3. *Cis*-elements in promoters of *DcHD-Zip* genes. The grey bar represented the upstream of the *DcHD-Zip* genes. Different colored wedges represented different *cis*-elements.

2.4. Expression analysis of HD-Zip genes under stresses using public RNA-seq data

The raw RNA-seq data of different tissues, and leaves under cold and methyl jasmonate (MeJA) treatments of *D. catenatum* were downloaded from the NCBI Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) under the BioProject number PRJNA283237, PRJNA314400 and GSE124762. Under cold treatment, *D. catenatum* were placed in chambers set at 0℃ for 20 h, and then leaves were collected for transcriptome analyses (Wu et al., 2016). Under MeJA treatment, the leaves of 6-month-old *D. catenatum* were sprayed with 100 μM MeJA for 24 h and then were frozen at -80℃ for RNA-seq sequencing (Chen et al., 2019). Hitsat2 (version 2.1.0) (Kim et al., 2015) was used for mapping reads to the *D. catenatum* reference genome. The Stringtie (version 2.0.6) (Pertea et al., 2015) was used to analyze gene expression level, and then the Fragments Per Kilobase Million (FPKM) value was used to normalize gene expression level.

The raw RNA-seq data of *D. catenatum* leaf under drought during the day and night were downloaded from the NCBI SRA database under the BioProject number PRJNA432825 (Wan et al., 2018). Briefly, *D. catenatum* was treated with drought stress, and the mature leaf (fourth and fifth leaves) samples were collected when volumetric water content of the base material were ~30–35 % (moist) and 0 % (dry) at both 09:00 am (Moist condition in the Daytime, MD and Dry condition in the Daytime, DD) and 21:00 pm (Moist condition at Night, MN and Dry condition at Night, DN). *D. catenatum* is considered as drought-resistant species (Zhang et al., 2014b), thus 30–35 % volumetric water content of the base material could be a suitable condition. The RNA-Seq data summary and assessment were listed in Wan et al. (2018). The clean reads were *de novo* assembled using Trinity software (Grabherr et al., 2011) and mapped to the *D. catenatum* reference genome (Zhang et al., 2016a) using Hitsat2, and the resulting assemblies were merged together to give rise to the final transcriptome assembly using CD-HIT-EST v4.6 (Fu et al., 2012). Differentially expressed genes (DEGs) were defined with a threshold of fold change $\geq$2 and false discovery rate (FDR) $\leq$ 0.01. Enrichment analyses of DEG sets in the KEGG pathways (FDR $<$ 0.05) were performed using DAVID (https://david-d.ncifcrf.gov/) (Huang et al., 2009). The heatmaps of gene expression and the correlation coefficient calculation were performed by R software. The relationships of genes were visualized using Cytoscape (version 3.3.0).

2.5. Plant material and stress treatments

D. catenatum 3-years old plantlets were used in this study. For light treatments, the plantlets were treated under dark (designed as LL), high light (250 μmol photons m^2s^{-1}, designed as HL) and 12 h light (80 μmol photons m^2s^{-1}) /12 h dark photoperiod (control) for 24 and 48 h in greenhouse. For heat treatments, the plantlets were treated at 35℃ for 4, 24 and 48 h, and 25℃ was used as control in a growth chamber. Under salt stress, plantlets were treated with 0.5 M NaCl and sterile water (control) for 24 and 48 h in greenhouse. After treatments, mature leaves were collected and frozen immediately in liquid nitrogen, and stored at -80℃ for RNA extraction. Three biological replicate samples were contained in each treatment.

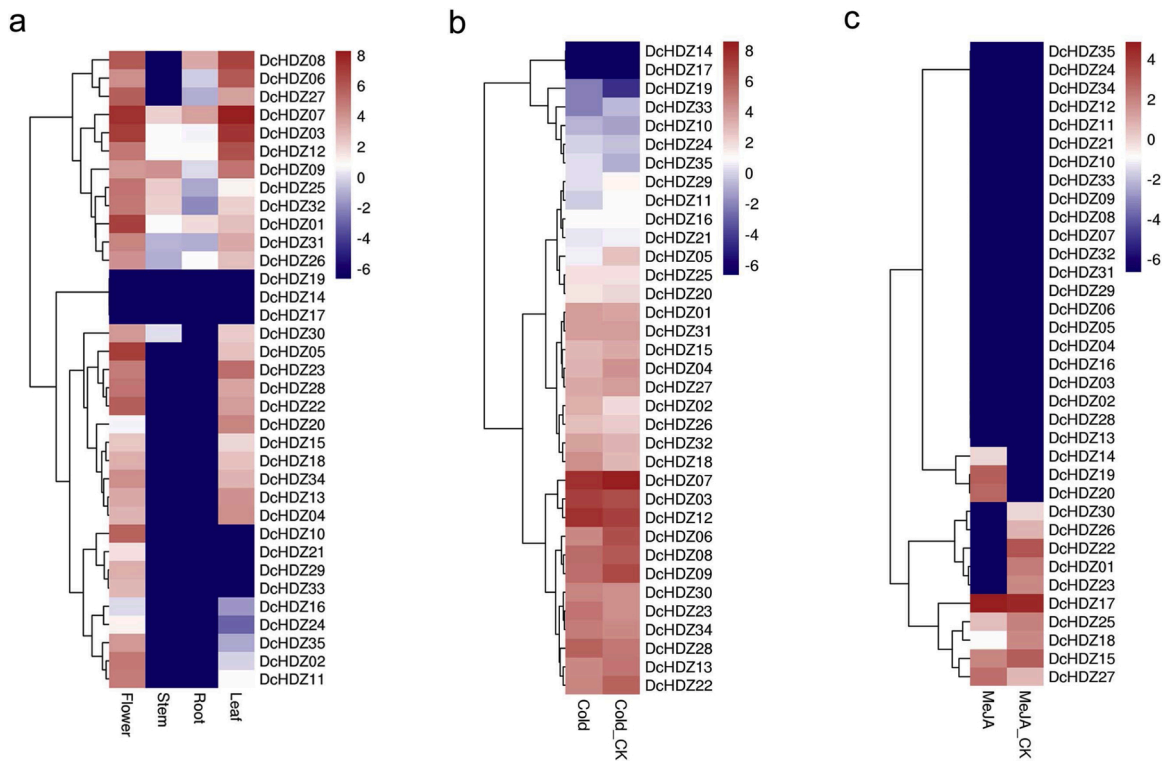

Fig. 4. Expression profiles of *DcHD-Zip* genes in different tissues and different treatments. a, Tissues. b, Cold. c, MeJA. The $\log_2$ (FPKM values) of genes were showed by different colored rectangles. Red indicates high expression level. Blue indicates low expression level. CK means control.

2.6. Real-Time PCR experiment

Total RNAs were extracted using RNAprep Pure kit (DP441, Tiangen, Beijing, China), and were used as template to synthesize the first-strand cDNA by using the FastKing RT kit (Tiangen). The primers were designed based on *DcHD-Zip* genes sequences using Premer premier 5.0 software (Table S1). The *DcActin* gene was selected as an internal standard. qRT-PCR was performed on ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). qRT-PCR assay was performed as described Yao et al. (2016). The $2^{-\Delta\Delta CT}$ method was used to analyze relative transcript abundances. Student's *t*-test test was employed using SPSS software (version 18.0) to calculated levels of significance. $P < 0.05$ was adopted as the criterion for statistical significance.

3. Results

3.1. Identification of HD-Zip gene family members in Dendrobium catenatum

After strict screening, a total of 35 *HD-Zip* genes were identified in the *D. catenatum* genome, and they were designated as *DcHDZ01-DcHDZ35* (Table 1). According to PFam and SMART database searches, 12 and 12 *HD-Zip* genes were classified into I and II subfamilies containing both HD and LZ domains. Three genes were assigned to the HD-Zip III subfamily, and the remaining eight genes were classified into the HD-Zip IV subfamily. The full-length coding sequences of these *DcHD-Zip* genes in *D. catenatum* genome ranged from 2,604 bp (*DcHDZ27*) to 378 bp (*DcHDZ19*) with an average of 1,284 bp. The isoelectric points varied widely from 4.76 (*DcHDZ03*) to 9.24 (*DcHDZ06*). The predicted subcellular localizations of these proteins revealed that 34 of 35 DcHD-Zip proteins may be located in the nuclear.

3.2. Phylogenetic analysis of DcHD-Zip proteins

To further explore the classification and evolutionary characteristics of these *DcHD-Zip* genes, multiple sequence alignment of *DcHD-Zip* protein sequences with their homologs from *Arabidopsis* and rice was carried out. A unrooted phylogenetic tree showed that all *HD-Zip* genes were divided into four groups, named the I, II, III and IV (Fig. 1), as mentioned-above. In *D. catenatum,* the I and II subfamilies had the most members (12), followed by the IV subfamily (8) and III subfamily (3), in line with the predication result showed in Table 1. We found that class IV genes diverged early from class I, II and III genes. The class III genes were close to class II.

3.3. Conserved motifs, gene structures and cis-elements analysis

A total of 15 motifs ranging from 21 to 49 amino acids were identified by MEME analysis as shown in Figs. 2a and S1. Almost all members in the same subfamily shared common motif compositions with each other. Motif 4 and 6 were the most conserved and were found in all *DcHD-Zip* genes. Motif 9 and 14 were absent in *DcHD-Zip* I and II subfamilies genes and appeared in III and IV subfamilies genes. The other eight motifs (5, 7, 8, 10, 11, 12, 13 and 15) were identified only in *DcHD-Zip* IV subfamily genes. All the *DcHD-Zip* III subfamily members contained motif 1, 2, 4, 6, 9 and 14. Almost the *DcHD-Zip* IV subfamily members contained motif 1–15, except for motif 2. The exon-intron structure analysis showed that the *DcHD-Zip* I and II subfamilies members had two ~ four and two ~ five exons, respectively (Fig. 2b). The *DcHD-Zip* III subfamily genes had the most exons and more than ten exons. The motif composition and exnon/intron numbers of genes in the same subfamily were closer than that of genes between different subfamilies.

The sequences of the 2.0 kb region upstream of the translation initiation site of these *DcHD-Zip* genes were analyszed to investigate putative stress-related and hormone-related elements. The functionally annotated of those *cis*-elements identified in the promoters of more than

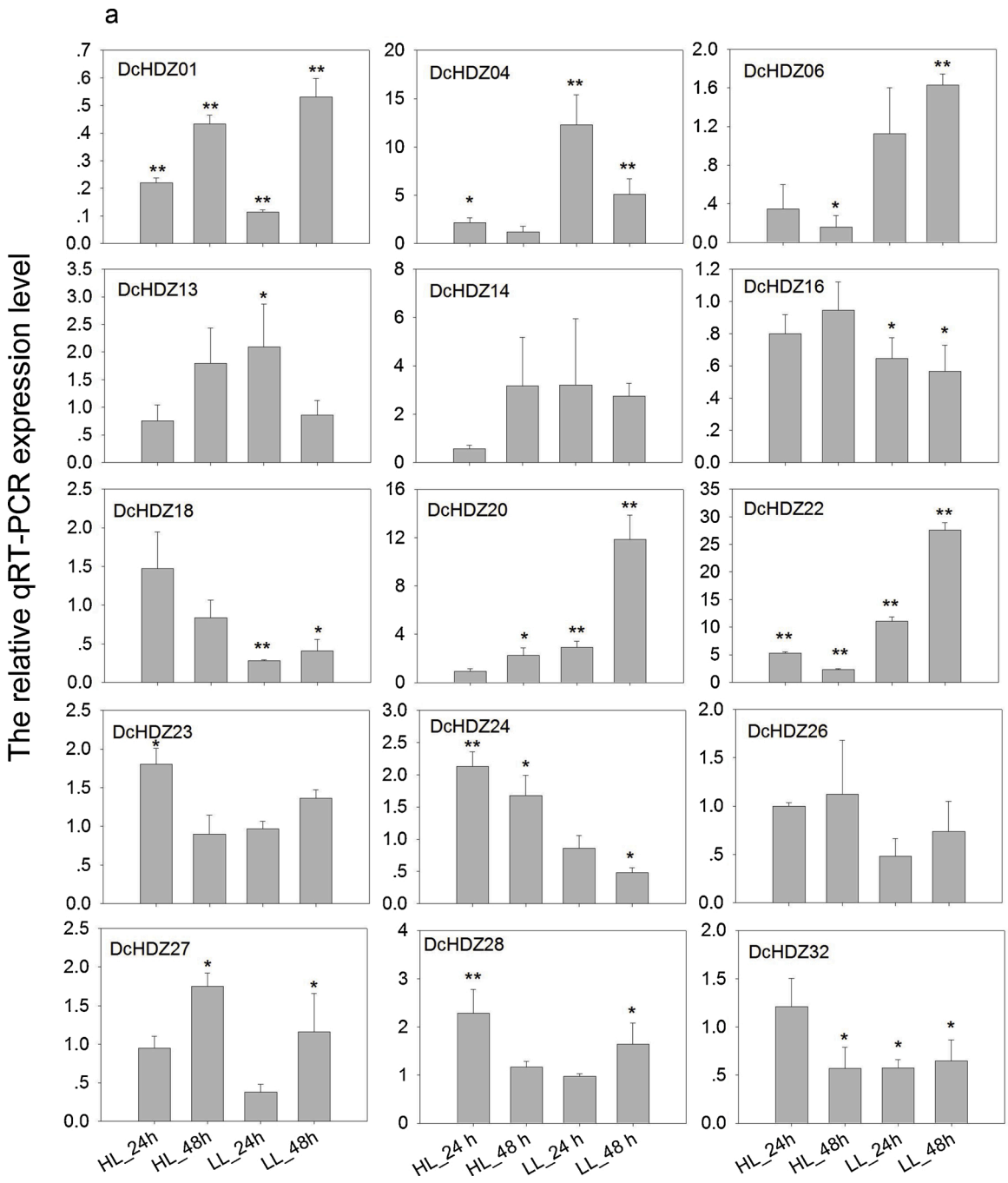

Fig. 5. qRT-PCR analysis of the expression pattern of 15 selected *DcHD-Zip* genes under different abiotic stresses treatments. a, High and low light. b, Heat. c, Salt. The relative qRT-PCR expression levels were calculated with $2^{-\Delta\Delta CT}$ and the *DcActin* gene was used as endogenous reference gene. Bars marked with asterisks indicate significant differences (Student's *t*-test) to corresponding control samples for the time points under treatments (* $p < 0.05$, ** $p < 0.01$).

10 HD-Zips were listed in Table 2. The Box 4 and G-Box, both which are involved in light responsiveness, were present in 31 and 28 *DcHD-Zip* promoters, respectively. Twenty-six ABRE that is involved in abscisic acid (ABA) responsiveness were identified in *DcHD-Zip* promoters. MeJA response elements (TGACG-motif and CGTCA-motif) were found to be present in 26 and 25 *DcHD-Zip* promoters, respectively. There were 13 *DcHD-Zip* promoters contained low-temperature-responsive element (LTR) that is involved in response to cold stress. Interestingly, we found that the most identified *cis*-elements in *DcHD-Zip* promoters were involved in light responsiveness containing 25 categories *cis*-elements (Fig. 3), such as Box 4 (31), G-Box (28), GT1-motif (28), GATA-motif (28), TCT-motif (23), TCCC-motif (14) and MRE (12) (Table 2). Furthermore, all identified *DcHD-Zip* genes contained at least one *cis*-elements related to light responsiveness.

3.4. Distinct expression profiles of DcHD-Zip genes in different tissues

To analyze the expression profiles of these *DcHD-Zip* genes, we investigated their transcripts abundance patterns across multiple tissues including flower, leaf, stem and root based on RNA-seq data available in public databases. The heat map showed that almost all *DcHD-Zips* had tissue-specific expression patterns (Fig. 4a). Most of them showed higher

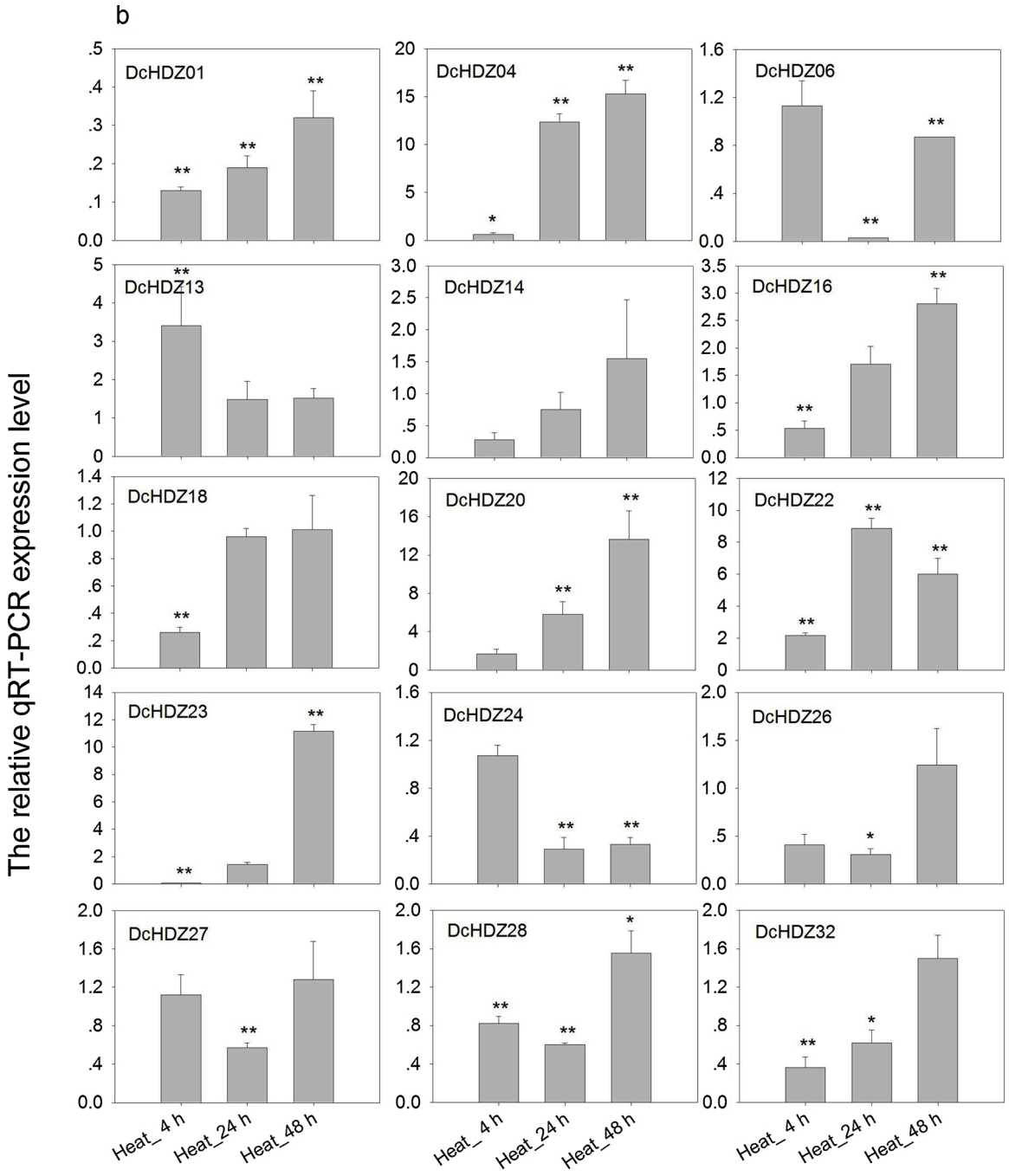

Fig. 5. (*continued*).

expression level in flower and leaf than in stem and root, and eight *DcHD-Zips* (*DcHDZ02*, *10*, *11*, *21*, *24*, *29*, *33* and *35*) were specifically expressed in flower. The *DcHDZ07* gene, belongs to I subfamily showed constitutive expression with high expression levels in the four tested tissues. However, most of identified *DcHD-Zips* were lowly expressed in root and stem. Of them, three *DcHD-Zips* genes (*DcHDZ14*, *17* and *19*) were no detected in the four tissues under normal growth condition.

3.5. Distinct expression profiles of DcHD-Zip genes under cold and MeJA treatments

We analyzed expression levels of 35 *DcHD-Zip* genes in leaves under cold treatment (0℃ for 20 h) (Fig. 4b). There were 16 up-regulated and 17 down-regulated *DcHD-Zip* genes under cold treatment, respectively (Fig. 4b). Of those up-regulated genes, *DcHDZ02*, *12*, *18*, *23* and *28* were highlighted by the more than 1.5 fold change in the FPKM value of them under cold condition compared to control. We also characterized the expression profiles of those *DcHD-Zip* genes under MeJA treatment (100 μM MeJA for 24 h). The result showed that most of *DcHD-Zip* genes were lowly expressed in MeJA treatment and control (Fig. 4c). Four *DcHD-Zip* genes including *DcHDZ14*, *19*, *20* and *27* were up-regulated under MeJA treatment, and eight *DcHD-Zips* (*DcHDZ01, 15, 18, 22, 23, 25, 26* and *30*) were suppressed by MeJA.

3.6. qRT-PCR verified the expression of DcHD-Zip genes in response to light, heat and NaCl treatments

To gain insight into potential functions, qRT-PCR was used to assess

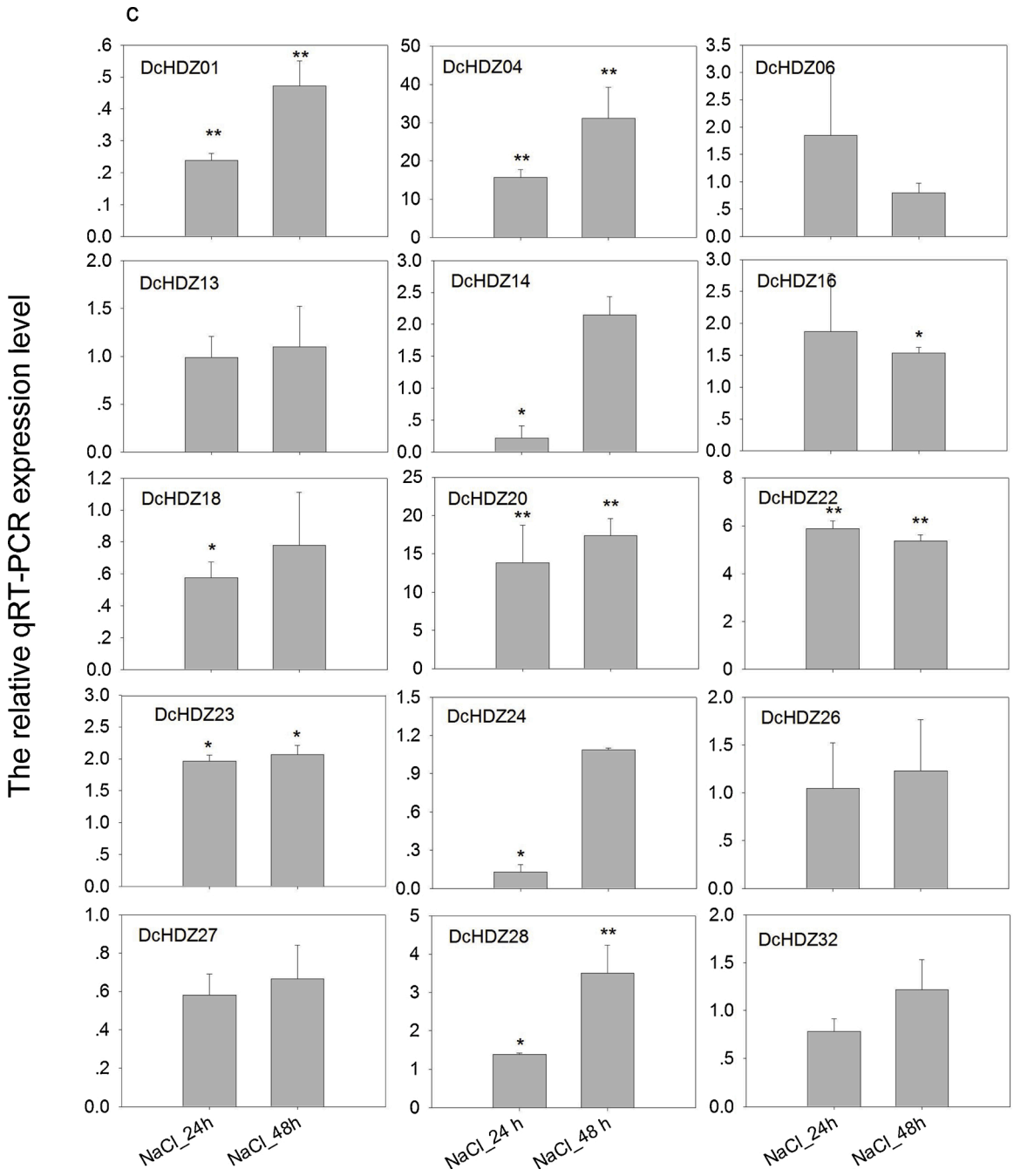

Fig. 5. (*continued*).

the high light (HL), dark (LL), heat (35℃) and NaCl treatments on the expression of 15 selected *DcHD-Zip* genes in *D. catenatum* (Fig. 5). Because the most identified *cis*-elements in *DcHD-Zip* promoters were involved in light responsiveness. Furthermore, *HD-Zip* II genes have been reported to mainly implicate in light responsiveness (Ciarbelli et al., 2008; Morelli and Ruberti, 2002; Turchi et al., 2013). Thus, eight *HD-Zip* genes belong to II subfamily were choosen for qRT-PCR. Additionally, based on the public RNA-Seq data analysis, seven representative *HD-Zip* I, III and IV subfamilies genes showing different expression level under stresses were also selected. As shown in Fig. 5a, expression levels of eight and six *DcHD-Zip* genes were up-regulated in leaves after HL and LL treatments for 24 h, respectively. With the extension of time to 48 h, the numbers of up-regulated *DcHD-Zip* genes increased to nine and eight. The three *DcHD-Zip* genes including *DcHDZ04*, *20* and *22* showed higher expression levels under both light treatments compared to control. Under heat stress, ten genes were up-regulated, especially in 48 h. It is note that the expression levels of *DcHDZ04*, *20*, *22* and *23* significantly increased, while *DcHDZ01* decreased (Fig. 5b). Expression levels of eight and 11 *DcHD-Zip* genes were up-regulated after NaCl treatment for 24 h and 48 h, respectively (Fig. 5c). Salt stress led to significant up-regulation of *DcHDZ04*, *20* and *22* at 24 h and 48 h, and suppressed the expression of *DcHDZ01*, *18* and *27*.

3.7. The role of DcHD-Zip genes in drought-induced facultative CAM of *D. catenatum*

In order to explore the roles of *DcHDZs* in facultative CAM pathway upon water-deficit stress in *D. catenatum*, a comprehensive trancriptomic analysis were performed. A total of 5,478 DEGs were identified (fold change $\geq$2 and FDR $\leq$ 0.01) (Table S2) and compared in pairs as

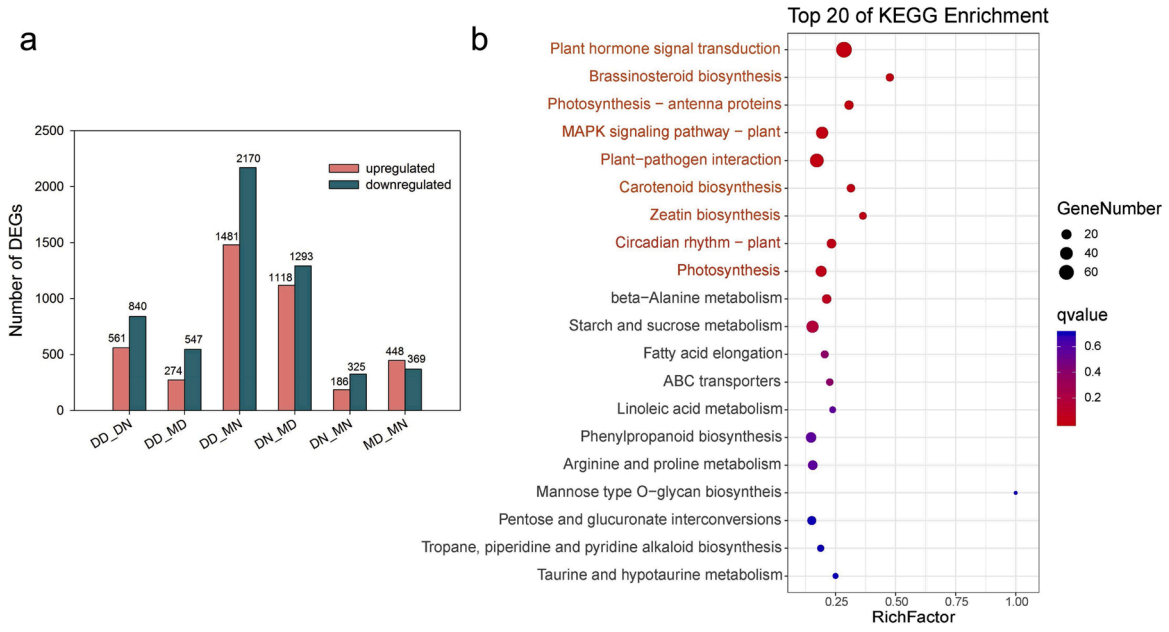

Fig. 6. Transcriptomic analysis of *D. catenatum* leaves under drought in the daytime and at night. a, The numbers of up-regulated and down-regulated DEGs. b, KEGG analysis of DEGs. Significantly enriched KEGG pathways were highlighted in red (FDR < 0.05).

shown in Figs. 6a and S2, indicated that a marked change on gene expression profiles happened. Several characteristic genes of CAM showed different expression levels in the daytime and at night under drought, including two *phosphoenolpyruvate carboxylases* (*PEPCs*) and one *phosphoenolpyruvate carboxylase kinase* (*PPCK*) (Table S2). KEGG enrichment analysis indicated that plant hormone signal transduction (ko04075) was significantly enriched in all DEGs (FDR < 0.05) (Fig. 6b), and was also enriched in DEGs of all pairwise comparisons between day and night (Table S3).

An overview of gene expression profile involved in plant hormone signal transduction was presented in Fig. 7. Almost all phytohormones including ABA, ethylene (ETH), cytokinine (CTK), auxin (IAA), gibberellin (GA), brassinosteroid (BR), jasmonic acid (JA) and salicylic acid (SA), might participate the regulation of drought-induced facultative CAM in *D. catenatum*. It is notable that *2C-type protein phosphatases* (*PP2C*) and *SNF1-related protein kinases2* (*SnRK2*) involved in ABA signaling and three *9-cis-epoxycarotenoid dioxygenase* (*NCED*) involved in ABA biosynthesis were highly expressed in the daytime and lowly expressed at night under drought, contrary to the *ABA 8'-hydroxylase* (*CYP707A3*) involved in ABA degradation (Figs. 7 and S3; Table S4). In ethylene signal transduction pathway, one *ethylene receptor* (*ETR*), four *ethylene-insensitive* (*EIN*), two *ethylene-responsive transcription factor* (*ERF*) and one *EIN3-binding F-box protein* (*EBF*) were highly expressed at night in DN and MN. Three *brassinazole-resistant* (*BZR*) and one *brassinosteroid insensitive 1* (*BRI1*) *kinase inhibitor* 1 (*BKI1*) involved in BR signal transduction also displayed higher expression levels at night in DN and MN, contrasting to the expression profile of two *BRI*. At night, several genes, including *cytochrome P450 family 90 subfamily A* (*CPD*), *brassinosteroid-6-oxidase 1* (*BR6OX1*), *3-epi-6-deoxocathasterone 23-monooxygenase* (*CYP90D1*) and *cytochrome P450 family 724 subfamily B polypeptide 1* (*D11*) involved in BR biosynthesis showed higher expression levels than that in the daytime (Fig. S3; Table S4). In CTK and JA signal transduction, many DEGs showed higher expression levels in MD and MN compared DD and DN (Fig. 7), which in line with most of DEGs involved in CTK biosynthesis (Fig. S3). In GA signal transduction, two *gibberellin receptor* (*GID*) and four *DELLA* were preferential expressed in DN and MN, which is consistent with the expression profile of three *POZ domain and ankyrin repeat-containing protein* (*NPR1*) involved in SA signal pathway (Fig. 7).

Nine *DcHD-Zip* genes were annotated as DEGs and their expression profiles were showed in Fig. 8a. Of them, *DcHDZ12* belonged to profile 2 and the *DcHDZ13* and *DcHDZ22* belonged to profile 7 according to their expression patterns (Figs. S4 and S5). In profile 2, DEGs were highly expressed in the daytime (DD and MD), and were lowly expressed at night (DN and MN), in contrast to that of profile 7 (*P*-value < 0.001). The Pearson correlation analysis were carried out to predict the relationship between the three *DcHD-Zip* genes mentioned above and other DEGs, considering positive (≥0.9) and negative (≤ − 0.9) relationship (Table S5). In our result, 23 genes showed a negative correlation with the *DcHDZ12*, while 54 genes were detected as positive. There were 14 negative and 22 positive correlation genes with the *DcHDZ13*. For *DcHDZ22*, three genes showed a negative correlation including *DDB1-and CUL4-associated factor 8*, *extradiol ring-cleavage dioxygenase* and *nuclear transcription factor Y*, while 51 genes were detected as positive (Fig. 8 and Table S5). Several genes involved in plant hormone signal transduction had close relationship with the three *DcHD-Zip* genes. The *protein phosphatase 2C* (*PP2C*) showed a positive correlation with the *DcHDZ12* and *DcHDZ22. Auxin response factor 19* (*ARF19*) showed positive correlation with *DcHDZ13* and *DcHDZ22,* and negative correlation with *DcHDZ12*, respectively.

4. Discussion

Dendrobium catenatum, a well known traditional Chinese medicinal herb. The growth, development, stress-resistant and accumulation of pharmaceutical ingredient of *D. catenatum* were significantly affected by genetic and environmental factors. The homeodomain-leucine zipper (HD-Zip) protein is a plant-specific transcription factor and is involved in plant growth, development, resistance and adaption to the environment. However, detailed information concerning *DcHD-Zips* characters and functions, particularly their role in stresses responses and facultative CAM pathway of *D. catenatum*, remained unclear. In this study, a total of 35 *DcHD-Zip* genes were identified in *D. catenatum* genome. Compared with other plant species, the number of *HD-Zip* genes in *D. catenatum* is close to physic nut (32) (Tang et al., 2019), peach (33) (Zhang et al., 2014a), grape (31) (Jiang et al., 2015), which is less than that in *Arabidopsis* (47) (Mukherjee et al., 2009), potato (Li et al., 2019), maize (Lespinet et al., 2011) and poplar (63) (Hu et al., 2012) and is

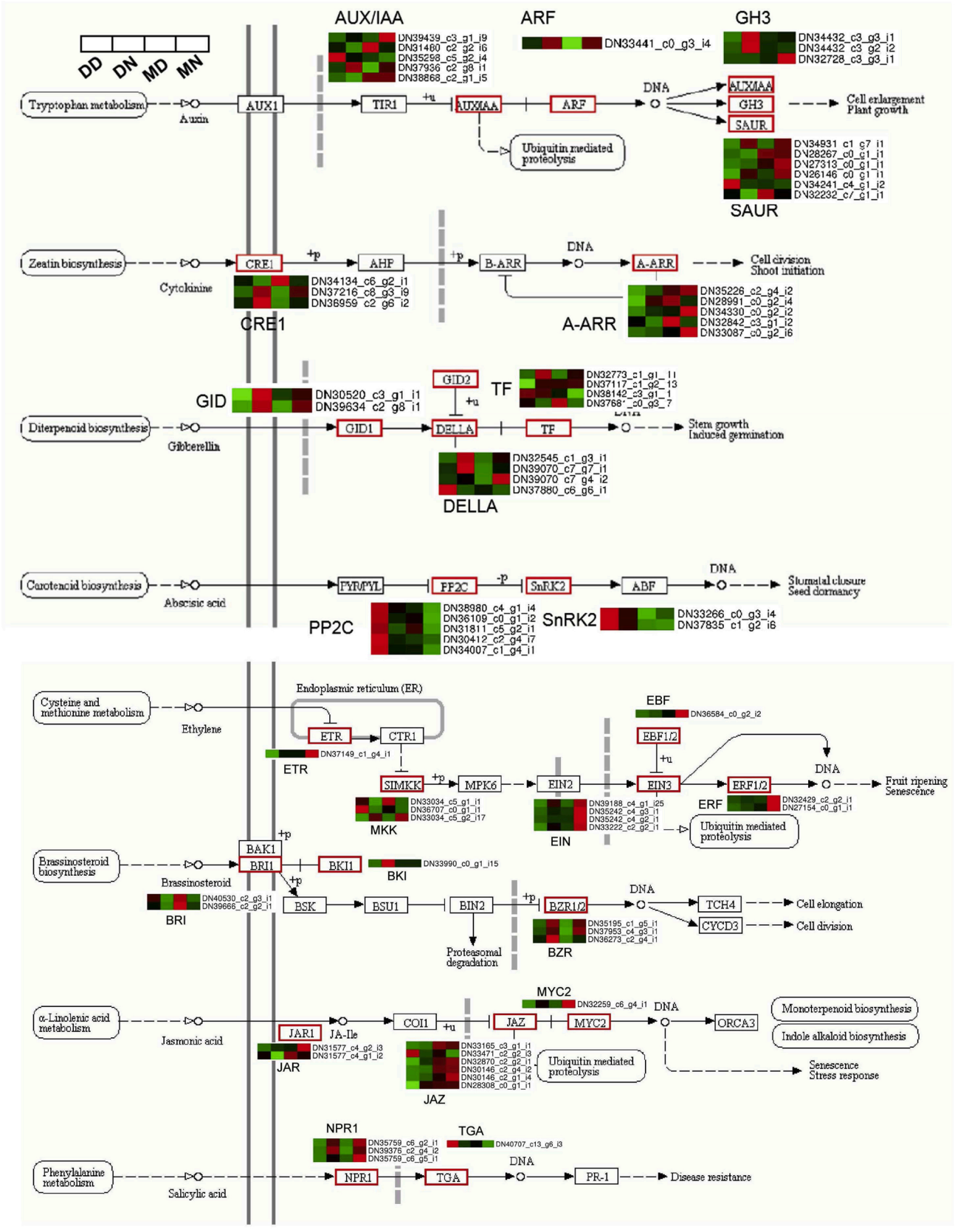

Fig. 7. Expression pattern of key genes involved in plant hormone signal transduction pathway. Full names and FPKM value of genes are showed in Table S4. The expression level of genes in different samples are indicated by heat map.

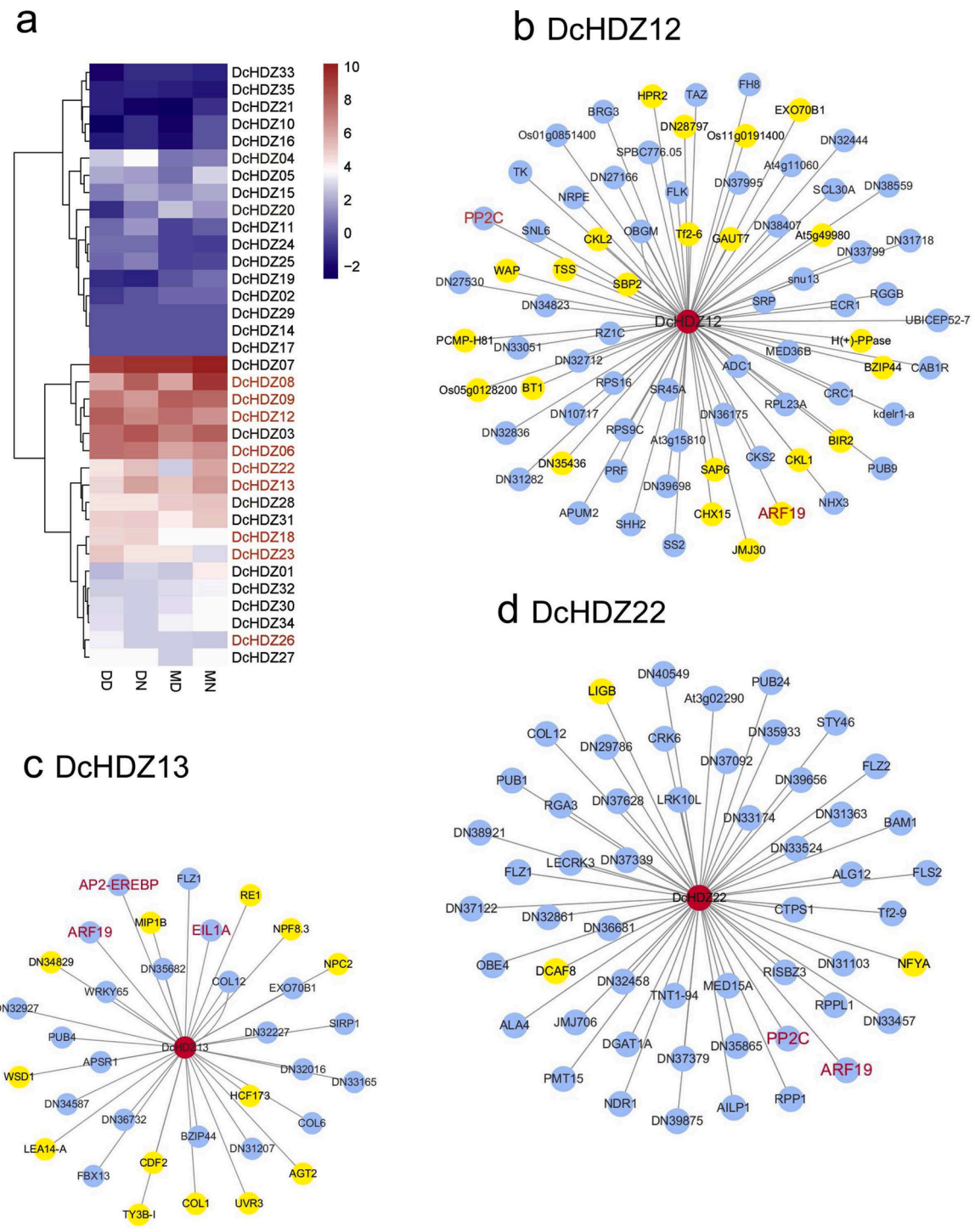

Fig. 8. The expression profiles and interaction network of *DcHD-Zip* gene in *D. catenatum* under drought in the daytime and at night. a, Expression profiles of *DcHD-Zip* genes. Red indicates high expression level. Blue indicates low expression level. The nine *HD-zip* genes with red letters were identified as DEGs. b, c and d, The interaction network of *DcHDZ12*, *13* and *22*, respectively. The blue cycles indicate genes showed a positive correlation with *DcHD-Zip* genes. The yellow cycles indicate genes showed a negative correlation with *DcHD-Zip* genes. The genes with red letters are involved in phytohormone biosynthesis and signal transduction pathway. The correlation coefficient and full names of genes are showed in Table S5.

more than that in *Citrus sinensis* (27) (Ge et al., 2015). Studies reported that partial *HD-Zip* genes originated from tandem duplication and segmental duplication in some species, especially segmental duplication (Henriksson et al., 2005; Wei et al., 2019). The phylogenetic tree combing the *Arabidopsis* and rice *HD-Zip* genes showed that 35 *DcHD-Zip* genes could be divided into four groups. And there were fewest members in group III, as in many other plants such as *Arabidopsis*, rice, maize and grape (Ariel et al., 2007; Jain et al., 2008; Zhao et al., 2011; Li et al., 2017). The conserved motifs and gene structures provided further support for the classification of *DcHD-Zip* genes in *D. catenatum*. Most DcHD-Zip proteins of the same group apparently had similar motifs constituents and exon-intron arrangements (Fig. 2b). Overall, the similarities in conserved motifs and gene structures in the same subfamily corroborate their classification and inferred evolutionary relationships.

A lot of evidences have demonstrated that *HD-Zip* genes participate in various aspects of growth, development and response to abiotic stresses (Henriksson et al., 2005; Ariel et al., 2007; Zhang et al., 2012; Turchi et al., 2013, 2015; Shen et al., 2018; Yue et al., 2018). In our study, most *DcHD-Zip* genes displayed distinct tissue-specific expression patterns. Thirty-one and 22 *DcHD-Zip* genes showed tissue specific expression in flower and leaf, respectively. However, only three *DcHD-Zip* genes belong to HD-Zip I subfamily were expressed in root. In tomato, HD-Zip class I, II, and III genes have a broad expression in all tissues, and HD-Zip class IV genes showed a higher expression in reproductive organs (Wei et al., 2019). These finding revealed that the functional divergence of different *DcHD-Zip* genes within and between subfamilies occurred during evolution processes.

In response to various abiotic stresses, *DcHD-Zip* genes displayed different expression pattern. We found that 16 and 17 *DcHD-Zip* genes were up-regulated and down-regulated under cold treatment, respectively (Fig. 4b). Four and eight *DcHD-Zip* genes were up-regulated and inhibited by MeJA (Fig. 4c). These results showed that the expression profiles of most of *DcHD-Zip* genes under control conditions were different between the cold treatment and MeJA treatment experiments (Fig. 4b and c). We infer that it may be attribute to the different cultivars or/and developmental stages of *D. catenatum* used in the two experiments. Under high light, low light, NaCl and heat stresses, three *DcHD-Zip* class I and II genes, namely as *DcHDZ04, 20* and *22*, showed high expression levels and increased during treatments, suggesting their important roles in response to those abiotic stresses. The ectopically expression and/or overexpression of the *Arabidopsis AtHB7* and *AtHB12* gene, two members of the HD-Zip class I genes, enhanced tolerance to drought or salinity stress in transgenic plants (Re et al., 2014), as well as their homologs in sunflower (*HaHB4*) (Gonzalez et al., 2019) and maize (*ZmHDZ4*) (Lespinet et al., 2011). Harris et al. (2011) demonstrated that the expression of *OsHOX19* that is a homolog of *DcHDZ20* and *22,* increased in response to severe drought in both drought-sensitive and -resistant rice cultivars. These results indicated that members of HD-Zip I and II subfamilies played a vital role in regulating plant response to abiotic stresses.

Our results showed that a closer relationship was found between DD and MD, than DD and DN, which indicated that circadian rhythm significantly affected the gene expression of *D. catenatum* leaves under drought. Enrichment analysis uncovered that plant hormone biosynthesis and signal transduction played an important role in drought-induced facultative CAM pathway, especially ABA. Four *NCEDs* involved in ABA biosynthesis and one *ABA 8′-hydroxylase* (*CYP707A3*) involved in ABA catabolism were highly and lowly expressed respectively in the daytime than at night under serious drought (Figs. 7 and S3). ABA diminished the aperture of the stomatal pore, and thereby contributed to the ability of the plant to conserve water during periods of drought (Leckie et al., 1998). Exogenous applications of ABA could partially substitute for water stress in induction of CAM in *Mesembryanthemum crystallinum* (Chu et al., 1990). Additionally, many genes related ABA, BR, ETH and IAA signaling were involved in the regulation of the facultative CAM pathway in *Talinum triangulare* (Brilhaus et al., 2016). Thus, the function and mechanism of other phytohormones in facultative CAM pathway need to be further clarified in the future studies. Notably, nine *DcHD-Zip* genes including four class I, four class II and one class III genes were differentially expressed under drought in the daytime and at night (Fig. 8). Of them, the *DcHDZ12* was highly expressed in the daytime and lowly expressed at night, contrary to the *DcHDZ13* and *22*. The expression levels of the three *DcHD-Zip* genes were affected by circadian rhythm, which suggests their important roles in facultative CAM pathway of *D. catenatum.* The Pearson correlation analysis found many DEGs had close relationship with the three *DcHD-Zip* genes, including several genes involved in plant hormone biosynthesis and signal transduction (Fig. 8 and Table S5). The *DcHDZ22* that is a homolog of *OsHOX19*, had close relationship with *PP2C* involved in ABA signaling. Both *OsHOX19* and *PP2C* expression were increased by the imposition of drought stress (Harris et al., 2011). Several other homologs, such as *AtHB6, AtHB7 and AtHB12* from *Arabidopsis*, *HaHB4* from sunflower, *CpHB2, CpHB6* and *CpHB7* from *Craterostigma plantagineum* (Himmelbach et al., 2002; Olsson et al., 2004; Manavella et al., 2006; Deng et al., 2002) are induced by drought and ABA. Moreover, the inductions of *AtHB6*, *AtHB7* and *AtHB12* are abolished in the ABA-insensitive mutants *abi1* and *abi2* in *Arabidopsis* (Himmelbach et al., 2002; Olsson et al., 2004), suggesting the important roles of HD-Zip genes in ABA-mediated adaptation to drought stress. In addition, an *auxin response factors 19* (*ARF19*) which could modulate early auxin response genes expression and is involved in ethylene responses (Li et al., 2006), had close relationship with the three *DcHD-Zip* genes. Sawa et al. (2002) indicated that the *HAT2* gene, a member of the HD-Zip gene family, affects auxin response in *Arabidopsis*.We inferred that the *DcHD-Zip* genes played important role in response to stresses and facultative CAM pathway of *D. catenatum* through phytohormone-mediated way.

5. Conclusions

The HD-Zip TF family plays various important roles in plant developmental and physiological processes in and biotic/abiotic stress responses. Thirty-five *DcHD-Zip* genes were identified in *D. catenatum*, and were assigned to four subfamilies according phylogenetic analysis, exon-intron structures and conserved motifs analysis. Their expression profiles clearly indicated that most *DcHD-Zip* genes displayed distinct tissue-specific expression pattern and members of HD-Zip I and II subfamilies played a vital role in regulating plant response to abiotic stresses according to the public RNA-Seq data and qRT-PCR analysis. Notably, our results revealed that plant hormone biosynthesis and signal transduction played a vital role in facultative CAM of *D. catenatum* under drought in which nine *DcHD-Zip* genes were expressed differentially. Together, these data provide useful information for *DcHD-Zip* genes and extend our knowledge of the regulation mechanism of facultative CAM.

Author contributions

HH and YHW conceived and designed the experiments; HH, WH, YT performed the experiments and analyzed the data; HH, YT and YHW wrote and revised the paper.

CRediT authorship contribution statement

Hui Huang: Conceptualization, Methodology, Software, Data curation, Writing - original draft. **Hui Wang:** Methodology, Software. **Yan Tong:** Methodology, Software, Data curation, Writing - review & editing. **Yu-Hua Wang:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2021.110058.

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