Enriched networks ‘nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes’ and ‘response to stimulus’ potentially conferred to drought adaptation of the epiphytic orchid *Dendrobium wangliangii*

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Abstract *Dendrobium wangliangii* is an endangered and epiphytic orchid with tolerance to seasonally extreme arid conditions and occurs exclusively in the hot-dry valley area of southwestern China. To reveal its molecular basis responsible for ecological adaptation, large-scale transcriptome sequencing was performed using Illumina sequencing with pooled mRNA extracted from whole plants and pseudobulbs during drought and rainy seasons. Based on the target transcript selection, the differentially expressed genes were related to 8 well-known drought-tolerant categories, and to morphological traits in resistance to water stress including pseudobulbs and roots. Further gene ontology enrichment analysis revealed that ‘nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes’ and ‘response to stimulus’ were the two most important aspects in resistance to drought stress

with respect to the whole plant. In addition, the difference in the number and category of differentially expressed genes in whole plant and stem suggested the involvement of genes specifically localized in the stem, such as GTP-binding protein, lipases, signaling related transcripts and those involved in the ATP metabolic process. The comprehensive analysis of the epiphytic orchid in response to water deprivation indicates that integral tactics lead to active adaptation as a basal defense response to drought stress by the endangered epiphyte, including the collaboration of metabolic processes, responses to a various stimulus and other candidate genes contribute to its extreme drought tolerance. Insights from this study can be further utilized to understand stress-responsive genes in other medicinally important species and to improve the drought tolerance of food crops.

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Introduction

Drought stress is one of the major abiotic factors seriously affecting plant survival. The global drought incidence is likely to reach beyond 20% at the end of this century (Singh and Laxmi 2015; Joshi et al. 2016). The broad adaptation of plant species across various extreme dry conditions suggests that plants have survived and evolved to endure drought stress with a series of morphological, physiological, and biochemical strategies (Basu et al. 2016). Drought stress varies in severity and duration causing both general and specific effects on plant growth and development. Therefore, drought tolerance is an outcome of comprehensive crosstalk of various physiological responses, such as multiple stress perception and signal transduction, various osmolytes accumulation, reduced transpiration, shoot growth inhibition, lateral root meristem growth, photosynthetic adaptation process, tillering and antioxidant system (Baldoni et al. 2015; Basu et al. 2016; Joshi et al. 2016). Taking advantage of modern omics approaches, many drought stress-responsive genes have been identified and characterized (Joshi et al. 2016).

A variety of desiccation-induced genes has been characterized, especially with metabolic or regulatory roles, such as those involved in osmoprotectants and abscisic acid (ABA) biosynthesis, reactive oxygen species (ROS)-related, ion homeostasis, detoxification, proteolysis of cellular substrates, late embryogenesis abundant (LEA) protein, heat shock protein (HSP), water channel and ion transporter (Osakabe et al. 2014; Joshi et al. 2016). Besides, the regulatory genes consist of transcription factors (TFs) (ABA-responsive element binding protein (AREB), ethylene-responsive transcription factors (ERFs), NAC (NAM (no apical meristem) and ATAF1/2 and CUC2 (cup-shaped cotyledon)), basic leucine zippers (bZIPs), WRKY and v-myb avian myeloblastosis viral oncogene homologs (MYBs)), those encoding ABA biosynthetic pathway, protein phosphatases (phosphoesterases and phospholipase) and signaling protein kinases (mitogen-activated protein kinases (MAPK), receptor protein kinases, ribosomal protein kinases, calcium-dependent protein kinases (CDPK), and transcription regulation protein kinases), which synchronize signal transduction and enable plants to adapt and survive under extreme stress conditions (Osakabe et al. 2014; Joshi et al. 2016; Sah et al. 2016). Additionally, response to various biotic and abiotic stimulus is identified as the pathway conferring drought adaptation (Xu et al. 2013; Bhardwaj et al. 2015; Li et al. 2015; Tian et al. 2015).

Dendrobium spp. are naturally distributed in warm and humid areas with abundant sunlight, which provides optimal conditions for growth and development. Surprisingly,

Dendrobium wangliangii (Orchidaceae) is endemic to the hot-dry valley area along the Jinsha River in Yunnan province, southwestern China (Hu et al. 2008). According to the criterion of the International Union for Conservation of Nature (IUCN), *D. wangliangii* is classified into ‘critically endangered species’ (Zhao et al. 2013). As a typical epiphyte species, this *Dendrobium* inhabit on the trunk or branches of *Quercus* species, especially *Quercus variabilis*, *Quercus spinosa*, and *Quercus yunnanensis* (Fig. S1; Hu et al. 2008). The annual precipitation in this region is distributed unevenly throughout the year (Table S1), and the potential evaporation is approximately three to six times higher than the precipitation. The orchid acclimates itself to harsh environments due to its outstanding tolerance to drought and torridity, especially under arid spring conditions. *D. wangliangii* also serves as a precious germplasm resource with high medicinal and ornamental values (Zhao et al. 2013). Epiphytes have to entirely rely on water stored within their tissues. A combination of high light intensity, high temperatures, and drought stress, always causes photosynthetic inhibition in plants (Demmig-Adams and Adams 1992). To survive in such a stress-prone environment, epiphytic orchids have evolved multitude of adaptations to water shortages such as succulent leaves, thick cuticles, water storage tanks, drought deciduousness, low stomatal conductance or crassulacean acid metabolism (Zotz and Tyree 1996). Despite the importance of *D. wangliangii*, currently, there is no detailed investigation to illustrate the underlying molecular mechanisms of drought tolerance.

Recent advances in next-generation sequencing technologies have revolutionized plant transcriptomics. This has enabled studies to characterize the transcriptomes of plants, particularly those without a reference genome (Xu et al. 2013). Transcriptome analysis not only provides an efficient tool to identify candidate genes for phenotypic variation and target traits (Müller et al. 2012), but gives deep insights into the regulation of transcribed regions and the expression patterns under drought stress (Zhang et al. 2014; Magalhães et al. 2016). In this study, our aim was to decipher putative genes and molecular functions involved in the adaptation of *D. wangliangii* to a drought-prone environment via transcriptomic analysis. The results indicated that diverse strategies conferred the adaption to dry-hot valley environment for the unique orchid, particularly in GO terms of ‘nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes’ and ‘response to stimulus’. In addition, DEGs specifically localized in the stem, such as GTP-binding protein (GPA1) involved in maintaining water status, lipases, signaling related transcripts as well as genes involved in the ATP metabolic process. We believe that these factors could possibly contribute to the drought stress adaptation of this plant and

may help us better understand the adaptive mechanisms of *D. wangliangii* in response to extreme drought, thereby improving water-deficit stress tolerance in crops through breeding and transgenic strategies.

Materials and methods

Plant material and RNA extraction

Dendrobium wangliangii was collected from a wild population in northern Yunnan Province, China. The exact location of *D. wangliangii* is not provided here to prevent this endangered species from the illegal collection. The basic climate data (2009–2013) of the location has been provided by local meteorologic bureau (Table S1). We randomly selected 15 plants and organs (including leaves, stems and roots) and harvested them from the living plants at 14:00 p.m. (the hottest time for a day), on April 25, 2013 (the extreme dry season around the year) with consistent growth potential (about 8 years old) over 100 meters away from each other. Then, each plant was labeled with the symbol. During the rainy season, the organs (also including leaves, stems, and roots) were collected from the same labeled orchids on August 25, 2013 (the end of the rainy season). The samples were immediately frozen in liquid nitrogen and stored at -80°C until use. To obtain an overview of the transcriptomic alteration, whole plants including leaves, stems, and roots were collected. The samples were grouped under two categories: ‘dwdp’ (whole plants collected in the dry season) and ‘dwdp2’ (whole plants collected in the rainy season). Generally, the orchid pseudobulbs (stems) are considered as an adaptive trait to facilitate a slow reduction in the water content and a decline in water potential under drought stress (Stancato et al. 2001; He et al. 2013). Therefore, stems of *D. wangliangii* were harvested and labeled: ‘dwds’ (stems collected in the dry season) and ‘dwds2’ (stems collected in the rainy season). Each sample included 15 individuals. Total RNA was extracted using Trizol reagent (Invitrogen, California, USA) following the manufacturer’s instructions. The quality of RNA was assessed by electrophoresis and ultraviolet spectrophotometry.

cDNA library preparation and Illumina sequencing

Four cDNA libraries were constructed from pooled RNA samples, respectively. From each pooled sample, 10 mg of total RNA was used for cDNA library construction following the protocol supplied with the TruseqTM RNA sample prep kit (Illumina, San Diego, USA). Briefly, total RNA was treated with DNase I prior to library

construction, and poly (A) mRNA was isolated using magnetic oligo (dT) beads (Invitrogen). The mRNA was first fragmented by treatment with divalent cations and heat and then transcribed into first-strand cDNA using reverse transcriptase and random hexamer primers, followed by second strand cDNA synthesis using DNA polymerase I and RNase H. The double-stranded cDNA was further end repaired, and indexed adapters were ligated. The reads were sequenced by paired-end. Adaptor-ligated fragments were separated by agarose gel electrophoresis. Suitable fragments were selected and enriched by PCR to create the final cDNA library. The cDNA library was sequenced on an Illumina HiSeq2000 sequencing platform.

Sequence data processing and de novo assembly

Image de-convolution and quality value calculations were performed using Illumina HCS 1.1 software. The raw paired-end reads were trimmed and quality controlled by SeqPrep (Version: 1.1-2) (<https://github.com/jstjohn/SeqPrep>) and Sickle (Version 0.4) (<https://github.com/najoshi/sickle>) with default parameters. In detail, the raw sequencing reads were processed to discard the dirty reads, which included reads with adaptors, reads with more than 10% $Q < 20$ bases (those with a base quality less than 20), and low-quality sequences (reads with ambiguous bases ‘N’). Clean and high-quality reads from the samples were then assembled separately and into one assembly combining all the libraries using the short read assembly program Trinity (Version 2.1.1) (<http://trinityrnaseq.sourceforge.net/>) with default settings to generate contigs.

Functional transcript annotation and classification

Before functional annotation, the transcripts were analyzed using Trinity software (http://trinityrnaseq.sourceforge.net/analysis/extractproteins_from_trinity_transcripts.html) to predict the open reading frames (ORFs). Functional annotation of the transcripts was then performed by homology searches against major public databases. All transcripts were compared with sequences in NCBI non-redundant protein sequences/NCBI non-redundant nucleotide sequences (Nr/Nt), Clusters of Orthologous Groups of proteins (KOG/COG), Protein family (Pfam), Swiss-Prot, String, KEGG Orthology database (KO) and GO with an E-value of less than 10^{-5} using BlastP (Version 2.2.25) (Camacho et al. 2009). BLAST2GO (Version 2.5.0) (<http://www.blast2go.com/b2ghome>) was used to obtain GO annotations of unique assembled transcripts for defining biological processes, molecular functions and cellular components (Conesa et al. 2005).

Analysis of differentially expressed genes (DEGs)

To identify DEGs (differential expression genes) between four separate libraries (dwdp2 vs. dwdp, dwds2 vs. dwds), the expression level of each transcript was calculated according to the reads per kilobase of exon per million mapped reads (RPKM) method (Mortazavi et al. 2008). RSEM (Version 1.3.0) (<http://deweylab.biostat.wisc.edu/rsem/>) was used to map the reads into the consensus sequence to quantify gene abundances (Li and Dewey 2011). R statistical package software EdgeR (Version 3.2.4) (Empirical analysis of Digital Gene Expression in R, <http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>) was utilized for differential expression analysis (Robinson et al. 2010). The *p* value in multiple tests was determined by the value for the false discovery rate (FDR). We used $FDR \leq 0.001$ and $|\log_2 \text{fold change (FC)}| \geq 2$ as the threshold to determine the significance of differences in gene expression. Venn diagram was constructed with the up- and down-regulated transcripts in dwdp2 versus dwdp and dwds2 versus dwds using PowerPoint 2007. The obtained DEGs were used to generate a heat map, and the DEGs were visualized using Adobe.

DEGs enrichment pathway analysis

Functional-enrichment GO analysis was performed to identify significantly enriched DEGs in GO terms and metabolic pathways compared with the whole transcriptome. Firstly, *p* value < 1 and corrected-*p* value ≤ 1 was used as the threshold for GO enrichment pathway of DEGs. Base on above analysis, *p* value < 1 and corrected-*p* value < 1 was used for identifying significant enrichment pathways of DEGs. GO functional enrichment pathway analyses were carried out using Goatools (<https://github.com/tanghaibao/Goatools>) (Xie et al. 2011). For GO-network generation, we first obtained the DEGs (foreground genes) from dsdw2 versus dsdw and dpdp2 versus dpdp, based upon analysis of differentially expressed genes. Together with all the assembled transcripts (background genes), each unigene was compared to the GO database, obtaining GO annotation (3 ontologies) for each gene. To carry out enrichment analysis, the lists of foreground and background genes and their corresponding GO numbers were input into topGO software (Alexa and Rahnenfuhrer 2016) at the same time, and then the Q-value of each GO term was calculated. Q-value < 0.5 was set as the threshold for significant enrichment. According to the paths to root (connection of known terms) of the union of GO enrichment analyses for two comparison groups, directed acyclic graphs (DAGs) were constructed to illustrate their network relationships, and they were visualized using Cytoscape 3.2.1. The Figure shows the combination of the

two comparison groups (dsdw2 vs. dsdw and dpdp2 vs. dpdp), and each GO annotation has a corresponding Q-value. GO terms with significant enrichment (Q-value < 0.5) in dwds versus dwds2 are presented in green and in blue for dwdp versus dwdp2. The GO terms that were not significantly enriched in both groups are displayed in grey.

Results

Sequencing and assembly

A total of 51,626,692, 48,968,212, 5,386,582,500 and 5,660,184,026 raw reads were generated from the four respective libraries: dwdp (whole plants collected in the dry season), dwds (stems collected in the dry season), dwdp2 (whole plants collected in the rainy season) and dwds2 (stems collected in the rainy season). After cleaning and quality checks, 46,827,948, 45,125,436, 5,125,783,176 and 5,385,806,002 clean reads were obtained for each sample (Table 1), with a total of 19.250 G. The four libraries were totally assembled into 71,934 non-redundant transcripts (SRA accession number in NCBI: PRJNA374407), and 57,940, 40,155, 54,683 and 41,463 for each sample respectively. All transcripts were more than 351 bp in length; the longest gene was 15,501 bp, and the mean length was 835.58 bp. The N50 and N90 were 1016 bp and 410 bp, respectively. The length distribution of the assembled transcripts is presented in Fig. S2.

Functional annotation of the assembled transcripts of *D. wangliangii*

Among the total 71,934 transcripts identified, 50,502 unique sequences were assigned to one or more ontologies in GO. As illustrated in Table S2, the main biological processes involved cellular, metabolic and single-organism processes, biological regulation, response to stimulus, localization, regulation of biological processes, establishment of localization, and cellular component organization of biogenesis. Moreover, binding, catalytic activity, and

Table 1 Statistical summary of the results of the Illumina sequencing and transcript assembly of *D. wangliangii*

	Dwdp	dwds	dwdp2	dwds2
Raw reads	51,626,692	48,968,212	56,041,426	56,041,426
Clean reads	46,827,948	45,125,436	48,959,130	51,444,364
Clear bases (G)	4.678	4.518	4.902	5.152
Unigenes	57,940	40,155	54,683	41,463

transporter activity were the three largest groups in the molecular function categories.

Gene expression regulated under drought stress

The expression of read-mapped genes was analyzed based on FPKM value. Within the identified transcripts, 5,462 differentially expressed genes (DEGs) from dwdp2 versus dwdp were detected, including 4,323 up-regulated and 1039 down-regulated genes. A total of 4041 DEGs from dwds2 versus dwds were detected, including 2812 up-regulated and 1229 down-regulated genes. As depicted in Fig. 1, 525 transcripts were shared in both up-regulated genes from dwdp2 versus dwdp and dwds2 versus dwds. A total of 258 shared genes was observed in the down-regulation from dwds2 versus dwds and dwdp2 versus dwdp. Heat maps and the corresponding GO biological processes of all DEGs are shown in Figs. 2 and 3. Specifically, the majority of DEGs associated with metabolic processes, response to stimulus, localization, cellular component organization or biogenesis, biological regulation, and regulation of biological processes were shared in both up- and down-regulated categories for dwdp2 versus dwdp and dwds2 versus dwds. The developmental process showed enrichment in the up-regulated groups of dwdp2 versus dwdp and in the down-regulated groups of dwds2 versus

dwds. Interestingly, the signaling category consisting of 56 transcripts was specific in the up-regulated categories of dwds2 versus dwds.

Identification of drought-tolerant genes in response to water shortage

Here, we enumerated a set of genes associated with drought tolerance (Le et al. 2012; Padmalatha et al. 2012; Xu et al. 2013; Osakabe et al. 2014; Bhardwaj et al. 2015; Li et al. 2015; Tian et al. 2015; Joshi et al. 2016; Basu et al. 2016), including 844 candidates in dwdp2 versus dwdp and 539 candidates in dwds2 versus dwds, especially in 8 major categories such as direct signal transduction, hormones, transporters, Transcription factors (TFs), ROS scavenging, osmoprotectant biosynthesis-related proteins, lipid metabolism and dehydration-responsive genes (Table S3,4). As Fig. S3 indicated, 140 transcripts were shared among the two comparisons, while 704 genes were specific to dwdp2 versus dwdp, and 379 transcripts were specific to dwds2 versus dwds.

In the current study, several classes of protein kinases and phosphatases were up-regulated under drought stress (Table S3,4), primarily related to the MAPK cascade and Ca^{2+} signaling. Furthermore, phosphatases were highly up-regulated in both comparisons of dwdp2 versus dwdp and dwds2 versus dwds (Table S3,4), e.g., all phosphatases (19 transcripts) except one serine/threonine-protein phosphatase 5 exhibited up-regulation in the whole plant (Table S3). Among the 24 identified Ca^{2+} sensor-related genes, 95.8% (23 transcripts) showed enhanced expression (Table S3).

Regarding DEGs encoding TFs in *D. wangiianii*, 97 (91 up-regulated) and 63 (43 up-regulated) transcripts were identified in the whole plant and stem (Fig. 4; Table S3,4). Specifically, the differentially expressed TFs in the whole plant, especially ERFs (30 vs. 12), including several classes such as ERFs, aintegumenta (ANT), cytokinin-responsive protein (CRFs) and WRINKLED transcription factors (WRIs), and zinc finger protein transcription factors (ZEP) (12 vs. 4), showed a dominance in number compared with the stem (Fig. 4). Likewise, three members of ZEP (C_2H_2 , C_3H and Zinc finger protein (ZAT)) were identified in the whole plant of *D. wangiianii*. Compared to ERFs and ZEPs, Mcml Agamous Deficiens Srf-box (MADX-box) (4 transcripts) and heat shock protein TFs (HSF) (4 transcripts) were abundant in the stem (Fig. 4; Table S3,4). Surprisingly, consistent with all six down-regulated small heat shock proteins (sHSPs), each HSP TF in the stem and 66.7% in the whole plant showed significant down-regulation (Table S3,4). For MYB, all 15 MYBs loci were up-regulated in the whole plant while 7 out of 15 MYBs were down-regulated in the stem (Table S3,4). In addition, 12

Fig. 1 Venn diagram showing the gene expression statistics of the contigs of four groups: the brown color represents up-regulated transcripts in dwdp2 versus dwdp (4323); the purple color represents up-regulated transcripts in dwds2 versus dwds (2821); the blue color represents down-regulated transcripts in dwdp2 versus dwdp (1139); the green color represents down-regulated transcripts in dwds2 versus dwds (1129) (color figure online)

Fig. 2 Heat map diagrams of relative expression levels of DEGs in dwdp2 versus dwdp and the category and number of up- and down-regulated transcripts. Red and blue colors indicate relative increases

or decreases in the expression in the two pairwise comparisons. The values are expressed as $\log_{10}(\text{FPKM} + 1)$ (color figure online)

bHLHs, WRKYs, 7 NACs, 5 bZIPs and 4 dehydration responsive element-binding proteins (DREBs) were dramatically up-regulated in the whole plant.

Genes associated with phytohormones were identified such as those related to abscisic acid (ABA), auxins, brassinosteroids, cytokinins (CKs), ethylene, jasmonic acid (JA), and salicylic acid (SA) (Table S3,4). The majority of hormone-related transcripts in the whole plant and stem, e.g., 94.6% (53 out of 56 transcripts) and 78.5% (28 out of 32 transcripts), respectively, showed enhanced expression in drought-stress samples, especially for ABA and SA-related genes. ABA-related genes were observed, including those involved in ABA biosynthesis, such as NCED1 (9-cis-epoxycarotenoid dioxygenase, NCED), ABA2, β -Carotene hydroxylase, and signaling pathways (phospholipase C (PLC) and phosphatase 2C (PP2C)). Most importantly, these candidates accounted for the largest proportion of hormone-related candidates in the both whole plant and stem samples (Table S3,4), and all the unigenes were up-regulated under drought stress.

Transcripts involved in ROS scavenging, including 93 and 51 loci in the whole plant and stem, respectively, were differentially expressed under drought stress, such as catalases (CAT), peroxidases (POD), glutaredoxin (GRX), aldehyde dehydrogenase (ALDH), and thioredoxin (TRX) (Table S3,4). Most up-regulated scavenging enzymes, 76.3% in the whole plant and 74.5% in the stem, indicated their roles in resisting drought stress in *D. wangliangii* (Table S3,4).

A number of genes encoding transporters were differentially expressed under drought stress in the both whole plant and stem of *D. wangliangii*, i.e., 210 and 139 genes, respectively, related with re-establishing both osmotic and ionic homeostasis under stress (Table S3,4). For the whole plant, 28 out of 31 ABC proteins were highly up-regulated in the dry season. Similar to previous reports (Padmalatha et al. 2012), three Na^+/H^+ exchangers in the whole plant were up-regulated. In the whole plant, most transporters for glucose (7), sugar (4), other hexose (2), malate (6), carboxylic acid (2) and CMP-sialic acid (1) showed significant

Fig. 3 Heat map diagrams of relative expression levels of DEGs in dwds2 versus dwds and the category and number of up- and down-regulated transcripts. Red and blue colors indicate relative increases

or decreases in the expression in the two pairwise comparisons. The values are expressed as $\log_{10}(\text{FPKM} + 1)$ (color figure online)

Fig. 4 Number and category of unique DEGs annotated as transcription factors in the *D. wangianguii* transcriptome

up-regulation. With respect to the transporters in the stem, there was a decrease in the components and numbers compared with the whole plant. Nonetheless, many up-

regulated glucose transporters (12) were obtained. In addition, we also observed an up-regulated glycine transporter 1 that was absent in the whole plant. Transcripts

encoding heavy metal transport proteins, such as those associated with cobalt (1), magnesium (1), iron (2), zinc and calcium (1) were up-regulated, indicating an increase in heavy metal ion transport and homeostasis under drought stress, whereas cadmium transporters (2) were down-regulated during the dry season. Eight aquaporin genes [plasma membrane intrinsic proteins (PIPs), basic membrane intrinsic proteins (SIPs) and tonoplast intrinsic proteins (TIPs)] were mostly activated in the whole plant but were lacking in the stem, suggesting their tissue-specific expression (Table S3,4). Particularly, 2 up-regulated NPFs (comp78577_c0; comp53062_c0) and four ABCGs (ABCG. 4, ABCG. 21, ABCG. 35, ABCG.39) in the whole plant associated with ABA transport, and induced NPF (comp72152_c0) and ABCG.11, ABCG.12 in the stem were observed (Table S3,4).

Under drought stress, osmotic adjustment (OA) has been reported to maintain growth, stomatal conductance, and photosynthesis (Chaves and Oliveira 2014). The whole plant genes related to carbohydrate metabolisms, such as trehalose (trehalose-phosphate synthase 1) and sucrose (sucrose synthase 3 and 2UDP-glucose pyrophosphorylases), were highly expressed, whereas transcripts encoding UDP-D-galactose 4-epimerase 1 (UGE1) involved in sucrose metabolism were significantly down-regulated (Table S3). In particular, in the both whole plant and stem, trehalose biosynthesis genes were highly up-regulated. Furthermore, two osmotin-like proteins (OSM) showed down-regulation under drought stress (Table S3,4).

As for the dehydration-responsive genes, 89.7% HSPs (122 out of 136 transcripts) in the whole plant and 31.3% HSPs (26 out of 83) in the stem were down-regulated (Table S3,4). In seedling both whole plant and stem, all six sHSPs were down-regulated. Furthermore, the LEA members in the whole plant accounted for a larger proportion of up-regulated genes (25 out of 26) compared with that of stems (13 out of 24) (Table S3,4). A larger proportion of up-regulated P450 (25 out of 26) in the whole plant was also observed (Table S4).

In addition, 3 lipid transfer proteins and 12 lipases in the whole plant and 3 lipid transfer proteins and 10 lipases in the stem differently expressed in *D. wangliangii* (Table S3,4). While 13 out of 15 transcripts showed significant up-regulation in the whole plant, only 5 out of 13 transcripts were observed to be up-regulated in the stem. Among these DEGs, one lipid transfer protein gene (comp91423_c0) and one extracellular lipase (comp90906_c0) were detected to be down-regulated in both the whole plant and stem (Table S3,4). Intriguingly, lipid transfer proteins and lipases showed a different expression pattern in this study, that is, for the whole plant, 86.7% of transcripts were up-regulated compared with only 38.5% in the stem.

Four out of five xyloglucan endotransglucosylases in the whole plant, and two R2R3-type MYBs in the whole plant and one in the stem showed induced expression in response to water stress (Table S7). Especially, a heterotrimeric GTP-binding protein (G protein) gene significantly up-regulated in the stem but not in the whole plant (Table S3,4).

GO enriched pathways and network analysis

Based upon the 5462 DEGs in the whole plant and 4049 DEGs in the stem, 189 significantly enriched GO pathways were identified (Table S5). These GO pathways were involved in metabolic processes (52), response to stimulus (40), binding (6), transport (7), oxidoreduction (6) and other functions. Several metabolic processes were observed, involving the synthesis of several macromolecules, such as carbohydrates (lipid, hexose, like glucose, monosaccharide, glycosyl), nitrogenous compounds (purine nucleotide, purine ribonucleoside, ribonucleoside), organic acids (monocarboxylic acid, pyruvate, aromatic amino acid), and ATP and cofactors. Water deprivation, osmotic stress, light, cold, radiation, oxidative stress, heat, hormones, jasmonic acid, cadmium, salt and some biotic factors (bacteria, fungi) were the sub-categories under 'response to stimulus'. As for the binding pathways, metal ions (cobalt, copper) and chromatin were the primary factors. Transport enrichment pathways included the following categories: protons, metal ions, hydrogen and transmembrane transport. Oxidoreduction was principally associated with oxidoreduction activity and the oxidoreduction coenzyme metabolic process. In addition, two GO items associated with the lipid metabolic process were detected in the whole plant (Table S5). Compared with the whole plant, 47 GO terms in the stem were evidently enriched, including ATP and nucleoside/ribonucleoside-associated metabolic processes (6), cofactor binding (1), hydrogen and proton transport (2) and oxidoreduction (3) (Table S6). Unexpectedly, no GO terms related to the response to stimulus were perceived in the stem.

Discussion

'Response to stimulus' conferring water deficit tolerance

Based on the analysis of directed acyclic graphs (DAGs), GO networks from the two comparisons were obtained under biological processes, including the 'response to stimulus' and 'metabolic processes' (Fig. 5). Similar to GO enrichment terms in dwdp2 versus dwdp mentioned above, the category 'response to stimulus' was central in the GO network composed of response to biotic and abiotic stresses

Fig. 5 The network of enriched GO terms related to response to stimulus/stress illustrated by a directed acyclic graph. The blue color represents a significant difference for dsdw2 versus dsdw, the green color represents a significant difference for dpdw2 versus dpdw, and the grey color represents a significant difference between the two groups (color figure online)

in dwdp2 versus dwdp (Fig. 5). It is worth noting that the ‘response to stimulus’ is one of the most important adaptive mechanisms for organisms under stress environment. Like the previously reported GO terms associated with water deprivation, response to salt stress, oxidative stress, osmotic stress, temperature stimulus, and hormone stimulus were identified (Xu et al. 2013; Bhardwaj et al. 2015; Li et al. 2015; Tian et al. 2015). Other factors related to the response to various abiotic stresses, namely, substances, endogenous stimulus and defense response, were enriched as well (Xu et al. 2013; Li et al. 2015; Tian et al. 2015). In particular, stimulus to light and radiation was observed for coping with the epiphytic environment. Interestingly, 7 biotic terms were noticed including response to bacteria (GO:0050896; GO:0042742; GO:0009617) and other organisms (GO:0051707, GO:0098542, GO:0009607, GO:0043207) under water deficit, suggesting that response to biotic stresses could contribute to the drought tolerance of *D. wongliangii* (Fig. 5). Enriched responses to various stimuli indicated that crosstalk between different stress responses conferred the prominent drought adaptation for the orchid.

‘Nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes’ related to drought adaptation

Metabolic reactions produce stress-protectant substances including solutes and antioxidants that remove denatured

DNA or proteins (Zhou et al. 2013; Li et al. 2015). Another enriched GO network illustrated by DAGs was associated with ‘nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes’ in dwdp2 (whole plants collected in the rainy season) versus dwdp (whole plants collected in the dry season) (Fig. 6). It consisted of the nucleoside phosphate metabolic process (GO:0006753), nucleotide metabolic process (GO:0009117), nucleoside monophosphate metabolic process (GO:0009123), ribonucleoside metabolic process (GO:0009119), purine ribonucleoside metabolic process (GO:0046128), purine ribonucleoside monophosphate metabolic process (GO:0009167), ribonucleoside monophosphate metabolic process (GO:0009161), purine-containing compound metabolic process (GO:0072521), ribonucleotide metabolic process (GO:0009259), pyridine nucleotide metabolic process (GO:0019362), purine nucleotide metabolic process (GO:0006163), and purine ribonucleotide metabolic process (GO:0009150) (Fig. 6). In addition, the purine ribonucleoside triphosphate metabolic process (GO:0009205), nucleoside triphosphate metabolic process (GO:0009141), ribonucleoside triphosphate metabolic process (GO:0009199), and purine nucleoside triphosphate metabolic process (GO:0009144) were enriched in dwds2 (stems collected in the rainy season) versus dwds (stems collected in the dry season). Nucleotide biosynthesis and metabolism was indicative of close relationship with drought tolerance. For instance, nucleotide biosynthesis and degradation have been suggested in drought responses

Fig. 6 The network of enriched GO terms related to nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes illustrated by a directed acyclic graph. The blue color represents a significant difference for dsdw2 versus dsdw, the green color represents a significant difference for dpdw2 versus dpdw, and the grey color represents a significant difference between the two groups (color figure online)

(Bhardwaj et al. 2013). In addition, nucleotide metabolism was reported to potentially contribute to the drought tolerance of *Hylocereus undatus* (Fan et al. 2014) and *Triticum boeoticum* (Liu et al. 2015). Enriched GO network analysis of dwdp2 (whole plants collected in the rainy season) versus dwdp (whole plants collected in the dry season) (Fig. 6) revealed the represented ‘nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes’ pathway. Based upon the previous reports, this metabolic network should play a key role in water deficit conditions for the epiphytic orchid *D. wongliangii*. However, further experiments remain to be carried out to clarify the relationship of ‘nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes’ with drought response.

Signal transduction pathways

The signaling pathway response to abiotic stress includes key steps such as signal perception, transduction, and responsiveness, resulting in activation of a series of physiological, and metabolic reactions (Joshi et al. 2016). The phosphorylation and dephosphorylation mediated by kinases and phosphatases lead to signal transduction pathways under stress. For instance, the CDPKs and MAPKs are known for their effect on drought stress response (Joshi et al. 2016). The occurrence of MAPK6, a key player in ethylene biosynthesis (Liu and Zhang 2004), suggested induction of the ethylene signaling pathway in *D. wongliangii* under drought (Table S3). Calmodulin (CaMs)/CAM-like (CMLs), CDPKs and CBL-interacting protein kinases (CIPKs) are Ca^{2+} sensors in plant involved in signal transduction pathways induced by stress (Weinl and Kudla 2009). CDPKs act in the signaling pathway by adjusting ABA signaling and reduction of ROS (Asano

et al. 2012). The activation of these signal transduction-associated genes including CDPKs, MAPK, Ca^{2+} signaling, and other kinases in *D. wongliangii* implied their roles by participating in signaling pathways under drought stress. Taken together, the transcripts involved in signaling in the biological processes presented enrichments with respect to the up-regulated categories of dwds2 (stems collected in the rainy season) versus dwds (stems collected in the dry season) (Fig. 3), implying that the signaling class should confer water-deficit tolerance in the stem.

Transcription factors (TFs)

TFs are indispensable for plants in resisting environmental stress (Franco-Zorrilla et al. 2014; Li et al. 2015; Joshi et al. 2016). These genes are regulated by upstream components and activate several functional genes encoding a wide range of proteins involved in abiotic stress (Mizoi et al. 2013; Joshi et al. 2016). ERF family has been extensively studied as a response to abiotic stress, such as drought, and proved to confer tolerance in transgenic plants (Joshi et al. 2016). Overexpression of an ERF in *Trifolium* led to enhanced osmotic stress tolerance (Basu et al. 2016). In the present study, 30 ERFs were up-regulated in the whole plant signifying their function in the drought tolerance for *D. wongliangii*. ZAT10 and ZAT12 in *Arabidopsis* regulate the responses to excess light (EL) and drought stresses mediated by ROS (Mittler et al. 2006), demonstrating the effect on protecting the injury of photosynthesis exposed to drought stress. Most ZEPs contributing to water stress were up-regulated (Osakabe et al. 2014), revealing their roles on the drought tolerance of *D. wongliangii*.

In plants, MYBs are one of the key factors in responses to stress (Zhang et al. 2012). For example, AtMYB60 and

AtMYB61 could modulate stomatal aperture, and then improve drought tolerance in plants (Cominelli et al. 2005). Total 15 MYBs showed up-regulation in the whole plant (Table S3,4). Increasing evidence has disclosed that basic helix-loop-helices (bHLHs) can regulate plant responses to abiotic stress, especially drought (Dong et al. 2014), via JA- and ABA-mediated regulatory networks (Weng et al. 2015). Similar to the bHLHs in rice (Huang et al. 2014), all bHLHs in the whole plant were significantly up-regulated under osmotic stress, implying their positive role in the drought response. In addition, six WRKYs transcripts were dramatically up-regulated in the whole plant. The MAPK cascade has been demonstrated to function via WRKYs to regulate downstream gene expression in response to drought stress (Shen et al. 2012; Gong et al. 2015). The NAC gene family is the plant-specific and the largest TF family (Shao et al. 2015). The overexpression of stress-responsive NAC1 (SNAC1), specifically-expressed in rice guard cells, enhanced stomatal closure, and then drought tolerance in rice (Hu et al. 2006). It was observed that seven NACs TFs were up-regulated in *D. wongliangii*, representing their improvement in drought tolerance of this orchid. Similar to *D. wongliangii*, NAC, MYB-related, and WRKY families were highly represented in *Macrotyloma uniflorum* induced by drought (Bhardwaj et al. 2013). Previous studies have confirmed that the basic leucine zipper (bZIP) can be ABA-inducible, playing an important role in ABA signaling pathway conferring drought stress in plants (Park et al. 2015). Five bZIPs were totally up-regulated in the whole plant under dehydration stress for *D. wongliangii*. Likewise, all four up-regulated dehydration responsive element-binding proteins (DREBs) were found to be responsive to water deficit for *D. wongliangii*, indicating the positive roles in drought tolerance. DREBs have been reported to improve drought tolerance in the plant (Xu et al. 2013; Bhardwaj et al. 2015). The expression of *OsDREB2A* and *OsDREB2B* resulted in enhanced osmotic-tolerance in transgenic plants (Mizoi et al. 2013). Similar to other TFs, 4 DREBs were also induced under osmotic stress in *D. wongliangii*. ABA-responsive cis-element-mediated transcription is controlled by an ABA receptor-related with SNF-related protein kinase 2 (SnRK2) (Umezawa et al. 2010). Unexpectedly, no DEGs encoding *AREB* was observed in the study.

Recently, the protein to protein interactions with different types of TF have been demonstrated as another layer of regulation on plant stress adaptation and secondary metabolite biosynthesis (Nakashima et al. 2014), such as *AREB/ABFs* with NACs, *SNAC* with ABRE region (Joshi et al. 2016). In our case, we identified that ERFs, MYBs, bHLHs, NACs, bZIPs, DREBs, and ZATs in the whole plant, all these members were observed to have increased transcript abundance (Table S3). Further experimental

results are required to verify these interactions between these up-regulated TFs that will benefit us to better understand their functions under drought stress.

Hormonal regulation

Major phytohormones, such as ABA, CKs, auxin, ethylene, and gibberellic acid (GA), enable adaptation to drought stress through synergistic and antagonistic crosstalk for plants (Basu et al. 2016; Le et al. 2012; Padmalatha et al. 2012). Under drought stress, ABA initiates plants adaptation to drought by stomatal closure, induces expression of various stress-related genes, and then reduces plant growth (Basu et al. 2016; Joshi et al. 2016). ABA-related genes accounted for the largest proportion of hormone-related candidates in both comparisons (Table S3,4), and showed up-regulation under drought stress, being in agreement with the results presented for cotton (Padmalatha et al. 2012). This implies the pivotal function of ABA in the drought adaptation of *D. wongliangii*.

Auxin negatively regulates adaptation of drought stress for plants. Auxin homeostasis is maintained by auxin-conjugation and transport and plays a key role in stress adaptation (Tognetti et al. 2012). Previous reports revealed down-regulation of IAA amido synthetase 3 (GH3) under drought stress (Le et al. 2012); conversely, GH3 and auxin receptor proteins were up-regulated in this study, indicating the contribution of auxin to the dehydration tolerance of this orchid. CKs could delay the senescence and death of premature leaf under water deficit. An increase in the endogenous levels of CKs leads to stress adaptation by delaying drought-induced senescence (Basu et al. 2016). Furthermore, our transcriptomic analysis identified 4 CKs-related genes with altered expressions in the drought-stressed whole plant. The majority of these genes were up-regulated under drought stress, which is not inconsistent with results reported previously for *Arabidopsis* (Nishiyama et al. 2011) and soybean (Le et al. 2012), enlightening the various roles of these two hormones in the drought tolerance of *D. wongliangii*. Multiple mutants of the CK receptor histidine kinases (HKs), AHK2, AHK3, and AHK4 enhanced the tolerance to drought for plants (Osakabe et al. 2014), while one HK3 was identified in the whole plant (Table S3,4). JA-related genes formed the third major group that displayed altered expression profiles in the drought-stressed in the whole plant and stem. In the both whole plant and stem, two genes encoding responses to salicylic acid (comp39991_c0; comp92828_c0) were significantly up-regulated (Table S3,4). Gibberellins (GA) usually positively regulate the adaptation of plant to water deficit (Basu et al. 2016), but the genes associated with GA biosynthesis did not show differential expression. Furthermore, brassinosteroids, SA, and strigolactone also have

an equally important role in plant growth and development, despite their less characterization (Basu et al. 2016).

All hormones modulate and interact with each other rather than individually (Ha et al. 2012; Basu et al. 2016). Similar to other enriched GO analyses under stress (Xu et al. 2013; Bhardwaj et al. 2015), one enriched GO term (response to hormone, GO:0009725) was also observed (Fig. 5).

ROS scavenging

Inter-connection between the responses to excess light and drought stresses existed, and 70% genes induced by excess light can be induced by drought as well (Estavillo et al. 2011). EL also stimulates the production of ROS via specific photochemical and biochemical processes (Li et al. 2009). ABA triggers the production of ROS under water deficit, thus dramatically resulting in oxidative damage to photosynthetic apparatus proteins, DNA and lipids (Osakabe et al. 2014). Both EL and drought posed extreme oxidative stress for *D. wangiianii* during water deficit season. To detoxify these radicals, plants adopt multiple defense systems including scavenging enzymes and anti-oxidative metabolites (Fang et al. 2015). Ascorbate-peroxidase encoding genes (APXs) conferred improved water use efficiency and drought tolerance (Estavillo et al. 2011). One up-regulated APX in the whole plant revealed its contribution to the drought tolerance (Osakabe et al. 2014). Furthermore, we noticed that glutathione S-transferases (GST) showed significant differential expression in the both stem and whole plant. Auxins have been reported to induce ROS detoxification enzymes including GST (Tognetti et al. 2012). Based on the transcriptome of *D. wangiianii*, transcripts associated with the auxin signaling pathway were mostly up-regulated (Table S3,4). Therefore, it is suggested that auxin metabolism may interact with the ROS detoxification enzyme system in *D. wangiianii* under drought stress. Consistent with the identification of transcripts mentioned above, oxidoreduction-related GO terms could also be responsible for growth and defense for *D. wangiianii* (Table S5).

Substance transporters

The transport of molecules across the plasma or vacuolar membranes is a primary way in response to drought stress (Le et al. 2012; Padmalatha et al. 2012; Zhang et al. 2014). Under water deficit stress, ion- and water-transport systems could modulate turgor pressure (Osakabe et al. 2014). Aquaporins generally respond to dehydration under drought stress (Padmalatha et al. 2012; Yu et al. 2012). Eight aquaporin genes were mostly activated in the whole

plant, suggesting their roles in drought tolerance of the orchid. Under dehydration stress, ABA transport system is one of the key responses to water deficit. The ABA is transported to guard cells from vascular tissue via passive diffusion and by specific transporters including NRT1/PTR FAMILY 4.6 (NPF4.6), ABC transporter G family member 40 (ABCG40) in vascular tissues (Osakabe et al. 2014). Here, 2 up-regulated NPFs, 4 ABCGs, 1 induced NPF, 1 ABCG.11 and ABCG.12 in the stem were observed (Table S3,4). In fact, ABA-mediated ion transports also play a key role in stomatal responses related to the plant tolerance to water stress (Osakabe et al. 2014).

Osmotic adjustment

Osmolytes also play an essential role in maintaining the stability of membrane and enzyme activity under osmotic stress (Singh and Laxmi 2015; Basu et al. 2016; Joshi et al. 2016). Stomatal closure under drought stress reduces the uptake of CO₂ and nutrient, resulting in altering metabolic pathways of materials (Xiong and Zhu 2002; Basu et al. 2016). Soluble sugars produced in carbohydrate metabolism under drought could regulate osmotic conditions and cell wall integrity (Padmalatha et al. 2012; Xu et al. 2013). The products of the hydrolysis of glucomannans and monosaccharides may be utilized in cellular reactions in pseudobulbs due to water deficit associated with high temperature accelerate catabolic processes (Stancato et al. 2001). Among sugars, trehalose and soluble sugars play a role in water deficit tolerance by maintaining osmotic conditions (Xu et al. 2013). In particular, in the both whole plant and stem, trehalose biosynthesis genes were highly up-regulated (Table S3,4), specifying accumulation of trehalose in the cell. With respect to the DEGs in the stem, one sucrose-phosphate synthase 4 and one OSM34 were significantly down-regulated, while 4 trehalose phosphorylases were all highly activated during the dry season (Table S4). The accumulation of compatible solutes such as proline protects plants from detrimental effects by maintenance of membrane integrity, and stabilization of ROS (Basu et al. 2016). However, no activation of proline biosynthesis genes, an osmolyte and antioxidant, was detected in *D. wangiianii*. Furthermore, kinase including, an *Arabidopsis* HK (AtAHK1) and plasma membrane-localized receptor-like kinases (RLKs) with the ability to mediate osmotic-stress signaling and function as an osmosensor (Osakabe et al. 2014). PP2Cs are essential regulators for improving osmotic tolerance (Komatsu et al. 2013; Joshi et al. 2016), and all PP2Cs (16 genes in the whole plant and 2 in the stem) were induced in response to water deficit (Table S3,4).

Dehydration responsive genes

Transcripts encoding dehydration responsive genes were identified in the whole plant and stem under drought stress (Table S3,4). HSPs, especially sHSPs, have been reported to confer abiotic stress, especially water deficit (Guo et al. 2014). Unexpectedly, 89.7% HSPs in the whole plant and 31.3% HSPs in the stem were down-regulated (Table S3,4). Furthermore, all six sHSPs in the both whole plant and stem of *D. wangliangii* showed down-regulation, indicating that HSPs may not be directly involved in enabling drought tolerance in *D. wangliangii*. LEA proteins function in protecting macromolecules and further increase drought stress tolerance (Yu et al. 2012; Zhang et al. 2014). Previous studies reported that LEA were up-regulated during dehydration (Le et al. 2012; Padmalatha et al. 2012), similar to our observation in *D. wangliangii* under drought stress. Among all DEGs related to dehydration responsive genes, LEA were up-regulated in both comparison groups. Furthermore, the LEA members in the whole plant showed a larger proportion of up-regulated genes compared with stems. Plant cytochrome P450 genes are involved in the biosynthesis of various secondary metabolites and generally exhibited up-regulation under drought stress (Yu et al. 2012). Here, we also observed a larger proportion of up-regulated P450 (25 out of 26) in the whole plant (Table S4).

Lipid metabolism

Generally, drought causes expression of cell wall strengthening and cellular membrane stabilization genes, such as lipid binding proteins and lipid transfer proteins (Zhang et al. 2014). Lipid transfer proteins and lipases contribute to repairing stress-induced damages in membranes under drought stress (Guo et al. 2013). The observation of lipid transfer proteins and lipases confirmed the roles of lipid metabolism on drought adaption. The interaction of lipid metabolic pathways and ABA-dependent signaling function in maintaining the structural and functional integrity of cellular membranes (Golldack et al. 2014).

Root morphology

Under desiccation stress condition, lateral roots were significantly reduced probably through meristem suppression and regulation of the xyloglucan endotransglucosylases gene (Basu et al. 2016). Four out of five xyloglucan endotransglucosylases were induced in the whole plant (Table S7), revealing their role in water stress for *D. wangliangii*. Additionally, the *Arabidopsis* R2R3-type MYB TF (MYB96) was able to activate lateral root

meristem development via ABA signaling cascade (Seo and Park 2009). Two R2R3-type MYBs in the whole plant and one in the stem showed induced expression in response to water stress, indicating their roles on the promotion of lateral root meristem (Table S3,4). Hormonal cross-talk was also implicated as a potential signal in response to water stress to modulate root system architecture (Basu et al. 2016). qDTY12.1 and DEEPER ROOTING 1 (DRO1) gene were proved to increase lateral root and root hair formation and resulted in drought avoidance as well (Uga et al. 2013; Dixit et al. 2015).

Orchid pseudobulbs

Epiphytic orchids are generally exposed to solar radiation and natural air currents and only receive intermittent rainfall. For surviving in drought conditions, epiphytes have to accumulate enough water within their own tissues (Zotz and Tyree 1996). The orchid pseudobulbs, acting as a reservoir for water and carbohydrates (Ng and Hew 2000), may facilitate a reduction in the content of leaf water during drought (Stancato et al. 2001; He et al. 2013). Until now, the molecular mechanism of how the pseudobulbs store water remains a mystery. In *Arabidopsis*, the α subunit of a prototypical heterotrimeric GTP-binding protein (G protein) encoded by a single gene (GPA1) is able to avoid water loss by decreasing the apertures of stomatal pores in the epidermis (Wang et al. 2001). Consistent with this result, we identified a G protein gene which only significantly up-regulated in the stem, but not in the whole plant (Table S3,4). This finding sheds light on the role of GPA1 in high drought tolerance of all orchids with pseudobulbs. Besides, the candidates involved in the ATP metabolic process (GO: 0046034) were observed in the stem as shown by GO networks analysis. Moreover, nucleoside/nucleotide and ribonucleoside/ribonucleotide metabolic processes and response to stimulus were the two most important categories conferring resistance to drought stress in this orchid. In addition, some transcripts were especially up-regulated in the stem of *D. wangliangii* but not in the whole plant, such as triacylglycerol lipase 2-like (comp81679_c1), phospholipase A1-Ibeta2 (comp77687_c0), monoglyceride lipase-like (comp48941_c0), and glycolipid transfer protein (comp86639_c0) (Table S3,4).

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