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Comparative genomics analysis of drought response between obligate CAM and C_3 photosynthesis plants

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ABSTRACT

Crassulacean acid metabolism (CAM) plants exhibit elevated drought and heat tolerance compared to C_3 and C_4 plants through an inverted pattern of day/night stomatal closure and opening for CO_2 assimilation. However, the molecular responses to water-deficit conditions remain unclear in obligate CAM species. In this study, we presented genome-wide transcription sequencing analysis using leaf samples of an obligate CAM species *Kalanchoë fedtschenkoi* under moderate and severe drought treatments at two-time points of dawn (2-h before the start of light period) and dusk (2-h before the dark period). Differentially expressed genes were identified in response to environmental drought stress and a whole genome wide co-expression network was created as well. We found that the expression of CAM-related genes was not regulated by drought stimuli in *K. fedtschenkoi.* Our comparative analysis revealed that CAM species (*K. fedtschenkoi*) and C_3 species (*Arabidopsis thaliana*, *Populus deltoides* 'WV94') share some common transcriptional changes in genes involved in multiple biological processes in response to drought stress, including ABA signaling and biosynthesis of secondary metabolites.

1. Introduction

Abiotic stresses, including drought, heat, chilling, salinity, variant light intensities and qualities, and heavy metals, are the major constraints that affect current global crop biomass production and food security (Lesk et al., 2016). Drought and heat stress are the most critical limiting factors having negative impact on plant development and agricultural productivity, associated with climate change (Fahad et al., 2017). Substantial yield and biomass losses in major crops, including wheat, rice, maize, barley and cotton, have been reported due to water-deficit stress (Kamara et al., 2003; Pettigrew, 2004; Samarah, 2005; Lafitte et al., 2006; Balla et al., 2011; Daryanto et al., 2016; Fahad et al., 2017). For instance, from 1980 to 2015, a decrease of approximate 40% of water on a globe scale resulted in yield reduction of 20.6% in wheat (*Triticum aestivum* L.) and 39.3% in maize (*Zea mays* L.) (Daryanto et al., 2016). Drought disasters caused a reduction in national cereal production by 10.1% on average during 1964–2007 (Lesk et al., 2016). Significantly lower grain yield of rice under drought stress was observed in comparison with well-watered condition (Lafitte et al., 2006). The reduction in grain yield of barley (*Hordeum vulgare* L.) and cotton (*Gossypium hirsutum*) ball production was also observed under drought conditions (Pettigrew, 2004; Samarah, 2005).

Plants that perform the specialized photosynthesis of crassulacean acid metabolism (CAM), which show the highest water-use efficiency

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and enhanced drought and heat tolerance in comparison with C_3 and C_4 photosynthesis plants, provide a biological solution to challenges caused by the warmer and drier environments (Borland et al., 2014; Yang et al., 2015, 2017). CAM species open stomata at night when temperature is lower, for minimizing water loss via evaporation, and assimilate CO_2 nocturnally. CO_2 is then converted to malate by phospho*enol*pyruvate carboxylase (PEPC) and accumulated in the vacuole as malic acid before the light period (Borland et al., 2014, 2015; Yang et al., 2015, 2017). During daytime, the stomata are closed to prevent the water loss through transpiration when temperature is higher. CO_2 is released from malate through decarboxylation process and re-fixed via ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBISCO) mediated Calvin–Benson cycle (Borland et al., 2014, 2015; Yang et al., 2017).

The CAM plasticity includes two typical operation modes based on evolutionary history and environmental context: The first mode is obligate or constitutive CAM (Herrera, 2008; Silvera et al., 2010; Borland et al., 2014), which always exhibits CAM pathway in mature photosynthetic tissues irrespective of environmental conditions. It can be divided into "strong (or typical) CAM" and "weak CAM" based on the relative proportion of net atmospheric CO_2 fixation in the dark to the light over a 24-h period, with dark CO_2 fixation contributing >70% and <1/3 considered as strong and weak CAM, respectively (Winter and Holtum, 2002; Nelson and Sage, 2008). The second mode is facultative or inducible CAM or C_3-CAM intermediate (Herrera, 2008; Silvera et al., 2010; Borland et al., 2014). The species that perform facultative CAM can switch between C_3 and CAM photosynthesis depending on environmental factors and stimuli, such as drought, salt, photoperiod, photosynthetic photon flux (PPF), and nutrient deficiency. For instance, the CAM photosynthesis was induced in *Guzmania monostachia* after treated with 30% polyethylene glycol solution or withholding of irrigation, and the nocturnal acid accumulation and phosphoenolpyruvate carboxylase (PEPC) activity were up-regulated significantly under drought condition as well (Mioto and Mercier, 2013; Carvalho et al., 2017). Further, Brilhaus et al. demonstrated the transcript of genes encoding CAM-cycle enzymes was elevated upon water deficiency through transcriptome-sequencing (RNA-Seq) in a facultative CAM species of *Talinum triangulare*, and also identified key transcription components involved in the regulation of the C_3-CAM transition (Brilhaus et al., 2016).

Recently, diel transcriptional profiles from multiple CAM species, such as *Phalaenopsis equestris*, *Ananas comosus*, *Agave* spp., *Kalanchoë fedtschenkoi*, *Yucca* spp. and *Sedum album,* have been created, providing rich genomic resources for functional investigation of core CAM gene expression and regulatory modules (Gross et al., 2013; Cai et al., 2015; Ming et al., 2015; Abraham et al., 2016; Yang et al., 2017; Heyduk et al., 2019; Wai et al., 2019; Zhang et al., 2020). In this study, to reveal gene expression responsive to drought stress in a typical obligate CAM species, we performed an RNA-Seq analysis using mature leaves of *K. fedtschenkoi* under water-deficit treatments at two time points of dawn (2-h before the start of light period) and dusk (2-h before the start of dark period). On the basis of our analysis of RNA-seq data, we identified differentially expressed genes responding to drought stimuli in *K. fedtschenkoi*. We generated a gene co-expression network for identifying molecular regulatory modules underlying drought response in *K. fedtschenkoi*. Furthermore, we performed a comparative analysis of transcript profiles of drought-responsive genes between *K. fedtschenkoi* and two C_3 photosynthesis species (*Arabidopsis thaliana* and *Populus deltoides*).

2. Results

2.1. Differentially expressed genes (DEGs)

The Pearson correlation analysis and principal component analysis were firstly performed for assessment of the quality of our RNA-seq data. The results showed that three biological replicates for each treatment were closely grouped together, and the samples collected at dawn and dusk were separated well (Fig. 1a), indicating the high reproducibility and reliability of our data.

Next, we conducted the temporal comparison (dusk-vs-dawn) under individual treatments (i.e., well-watered control, moderate drought and severe drought) (Fig. 1b; Supplementary Table S1). In total, we identified 6270 DEGs at dusk versus dawn under control condition, of which 3159 and 3111 genes were down- and up-regulated, respectively (Fig. 1c). Under the moderate drought condition, 5487 DEGs were identified at dusk versus dawn, of which 2769 and 2718 genes were down- and up-regulated, respectively. For the severe drought condition, 5814 DEGs were identified from the comparison at dusk versus dawn, of which 2967 and 2847 genes were down- and up-regulated, respectively. As shown in the Venn diagram (Fig. 1c), a total of 4115 DEGs between dusk and dawn were shared by three treatments (control, moderate drought, and severe drought), of which 2089 and 2026 genes were down- and up-regulated, respectively, at dusk in comparison to dawn. We found that differential expression of a wide range of transcription factor (TF) genes between dawn and dusk in the shared DEG list under the three conditions (control, moderate drought, and severe drought), including 199 TF genes that were down-regulated and 157 TFs that were up-regulated at dusk relative to dawn (Supplementary Table S2). A total of 755 DEGs were specifically responsive to severe drought but not moderate drought, among which 356 genes (47%) including 20 TFs were down-regulated and 399 genes (53%) including 39 TFs were up-regulated at dusk relative to dawn (Supplementary Figs. 1–2). Specifically, an ABA responsive gene of *ABF2* (Kaladp0011s1227) was down-regulated at dusk-vs-dawn under the severe drought condition. Two circadian rhythm regulatory genes of PIF3 (Kaladp0034s0177 and Kaladp0057s0097) were up-regulated significantly ($p < 0.01$) by the severe drought stress in the comparison of dusk to dawn (Supplementary Figs. 1–2).

We then performed the drought condition comparison for the analysis of DEGs responsive to drought treatments at time points of dusk and dawn (Fig. 1b; Supplementary Table S1). Compared to the control condition, only 41 and 26 DEGs were identified at dawn under the moderate drought and the severe drought conditions, respectively (Supplementary Table S3). Particularly, *PHR2* (Kaladp0044s0064), encoding a blue-light receptor 2, was up-regulated significantly ($p < 0.05$) at dawn by both moderate and severe drought stresses (Supplementary Table S3). Another gene of *SOUL-1* (Kaladp0068s0260), encoding a SOUL heme-binding family protein, which is involved in red or far-red light signaling pathway, was up-regulated under the severe drought treatment (Supplementary Table S3). A drought responsive gene of *KCS11* (Kaladp0062s0077) was up-regulated as well by the severe drought stress. Furthermore, 12 DEGs were observed that were up-regulated under both moderate and severe drought conditions at dawn, including several essential genes in the biosynthesis of flavonoids, such as *PAL2*, *C4L*, *4CL3*, *CHS* and *MYB21* (Supplementary Table S5). At dusk, two genes, *CKX6* (Kaladp0039s0276) and *AITR1* (Kaladp0674s0024*)* were significantly ($p < 0.01$) down- and up-regulated, respectively, under the severe drought condition compared to the control condition, whereas no DEGs were identified in the comparisons of MD-vs-control and SD-vs-MD (Supplementary Tables S1 and S3). These results indicate that circadian rhythm plays an important role in regulation of gene expression, whereas drought stress has less impact on gene expression in *K. fedtschenkoi*.

Also, our DEG analysis of the interaction between time and drought stress revealed that eight and 16 DEGs (dusk-vs-dawn) were significantly ($p < 0.05$) affected by the moderate drought and severe drought conditions at dusk-vs-dawn (Supplementary Figs. 3a–b and Supplementary Table S4), respectively, and two DEGs (dusk-vs-dawn; Kaladp0091s0140 and Kaladp0095s0583) were affected by both MD and SD treatments (Supplementary Fig. 3c).

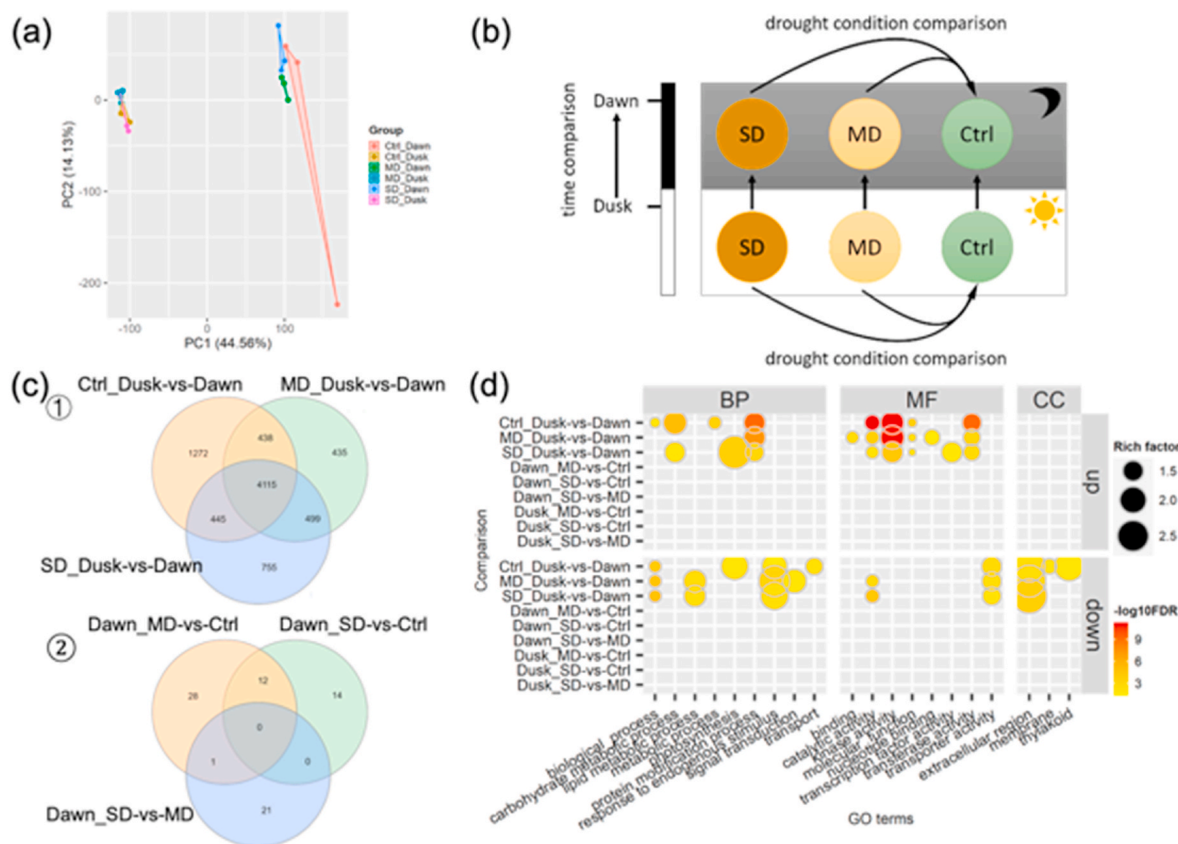

Fig. 1. Transcriptomic comparison of *Kalanchoë fedtschenkoi* under drought stresses.
(a) Principal component analysis (PCA) of the six groups of transcriptome data. **(b)** Schematic of sample collection and comparisons. The leaf samples were collected at dawn (2-h before the light period) and dusk (2-h before the dark period) under control (Ctrl), moderate drought (MD), and severe drought (SD) conditions. For differentially expressed gene (DEG) identification, the comparisons were classified into time comparisons (dusk-vs-dawn) and drought condition comparisons (MD-vs-Ctrl, SD-vs-Ctrl, and SD-vs-MD). **(c)** Venn diagrams represent DEGs overlapped in different comparisons. **(d)** Gene ontology (GO) enrichment analysis of up- and down-regulated DEGs from the comparisons of time and drought conditions. Detailed DEGs enrichment of biological process (BP), molecular function (MF), and cellular component (CC) are shown in Supplementary Figs. 4–6.

2.2. Functional predication of DEGs

According to three major gene ontology (GO) categories of biological process (BP), molecular function (MF), and cellular component (CC), we performed the GO enrichment analysis for the DEGs identified from the time comparison and drought condition comparison (Fig. 1d; Supplementary Figs. 4–6). For the temporal comparison of dusk-vs-dawn, DEGs under the moderate drought condition were significantly ($p < 0.05$) enriched in “glutamine biosynthetic process (GO:0006542)”, “inositol biosynthetic process (GO:0006021)” and “polyol biosynthetic process (GO:0046173)” on the BP category. DEGs under the severe drought condition were significantly ($p < 0.05$) enriched in “cysteine biosynthetic process (GO:0019344)”, “cysteine biosynthetic process from serine (GO:0006535)”, “cysteine metabolic process (GO:0006534)”, “L-serine metabolic process (GO:0006563)” and “serine family amino acid metabolic process (GO:0009070)” on the BP category (Supplementary Fig. 4). To get a better functional overview of genes responsive to drought stress in *K. fedtschenkoi*, the GO enrichment analysis was conducted on up- and down-regulated DEGs separately (Fig. 1d). On the comparison of dusk-vs-dawn, we found that genes up-regulated at dusk relative to dawn under the severe drought condition were significantly ($p < 0.05$) enriched in “photosynthesis (GO:0015979)”, whereas genes down-regulated under control (well-watered) condition were enriched in “photosynthesis” at dusk-vs-dawn. The GO term of “lipid metabolic process (GO:0006629)” was enriched for down-regulated genes at dusk-vs-dawn under the moderate and severe drought conditions (Fig. 1d). However, no enrichment was identified on the comparisons of drought conditions (Fig. 1d). These results indicate that drought stress had an impact on the temporal expression pattern of genes relevant to photosynthesis and lipid metabolism in *K. fedtschenkoi*.

On the category of MF, DEGs from comparison of dusk-vs-dawn under control condition and moderate drought conditions were significantly ($p < 0.05$) enriched in “inositol-3-phosphate synthase activity (GO:0004512)” and “intramolecular lyase activity (GO:0016872)”, whereas DEGs from comparison of dusk-vs-dawn under the severe drought condition were enriched in “inositol-3-phosphate synthase activity (GO:0004512)”, “serine *O*-acetyltransferase activity (GO:0009001)”, and “serine *O*-acyltransferase activity (GO:0016412)” (Supplementary Fig. 5).

DEGs from the comparison of drought-vs-control conditions at dawn were highly enriched in “lipid biosynthetic process (GO:0008610)” and “lipid metabolic process (GO:0006629)” (Supplementary Fig. 5). The GO terms of “transferase activity, transferring acyl groups (GO:0016740)” and “transferase activity, transferring acyl groups other than amino-acyl groups (GO:0016746)” were enriched in the DEGs sets of drought-vs-control treatments (moderate- and severe-drought) at dawn. The up-regulated DEGs at dusk relative to dawn under the severe drought treatment were significantly ($p < 0.05$) enriched in “transcription factor activity (GO:0003700)” while the down-regulated DEGs were enriched in “catalytic activity (GO:0003824)” at dusk-vs-dawn under the moderate and severe drought treatments (Fig. 1d). However, no enrichment was observed for the DEGs from the comparisons of drought conditions (Fig. 1d).

2.3. Co-expression network

To explore the high-expression correlation of genes in response to drought stress in *K. fedtschenkoi*, a WGCNA co-expression network was constructed by using the identified DEGs sets. Based on the transcript profiles, we characterized 6 co-expression modules with different color labels, which have varied module sizes number of DEG (Fig. 2a–b). The largest module of 'turquoise' contains 3959 genes and the smallest module of 'black' only has 64 genes. The expression under drought conditions at dawn and dusk in module 'black' was higher than that of normal condition, especially under severe drought, and this module was enriched in 'hydrolase activity' based on the GO enrichment analysis (Fig. 2c). Module 'red' also indicated induced expression at dawn under drought conditions relative to that of normal condition and was functionally enriched in 'lipid metabolic process'. The expression of DEGs in module 'green' showed down-regulation at dawn under drought conditions in comparison to that of control, which was functionally enriched in 'terpene synthase activity' and 'carbon-oxygen lyase activity'. The modules of 'blue' and 'brown' showed opposite expression change under drought treatment with low expression and up-regulated expression at dawn, respectively. Module 'turquoise' did not show expression change of DEGs between drought treatment and control, which was functionally enriched in 'photosynthesis' and 'protein kinase activity'. We also performed an enrichment analysis for transcription factors (TFs) from these modules, and identified that AP2-EREBP TFs were enriched in module 'black' and MYB TFs were enriched in module 'red' (Fig. 2d).

2.4. Core CAM genes under drought conditions

Although obligate CAM plants constitutively perform CAM photosynthesis irrespective of environmental irrigation conditions, we still analyzed the expression pattern of CAM-related genes to observe their response under water-deficit stress. The genes of *beta-carbonic anhydrase (β-CA), phosphoenolpyruvate carboxylase (PEPC), phosphoenolpyruvate carboxylase kinase (PPCK), NAD(P)-malate dehydrogenase (MDH) aluminum-activated malate transporter (ALMT)* are core components that regulate carboxylation process and malic acid accumulation in vacuoles during night. No statistical differences were observed between drought treatments and control in terms of transcript profiles of these CAM-carboxylation genes based on our comparison analysis. The genes, including *tonoplast dicarboxylate transporter* (*TDT*), *NAD(P)-malic enzyme*

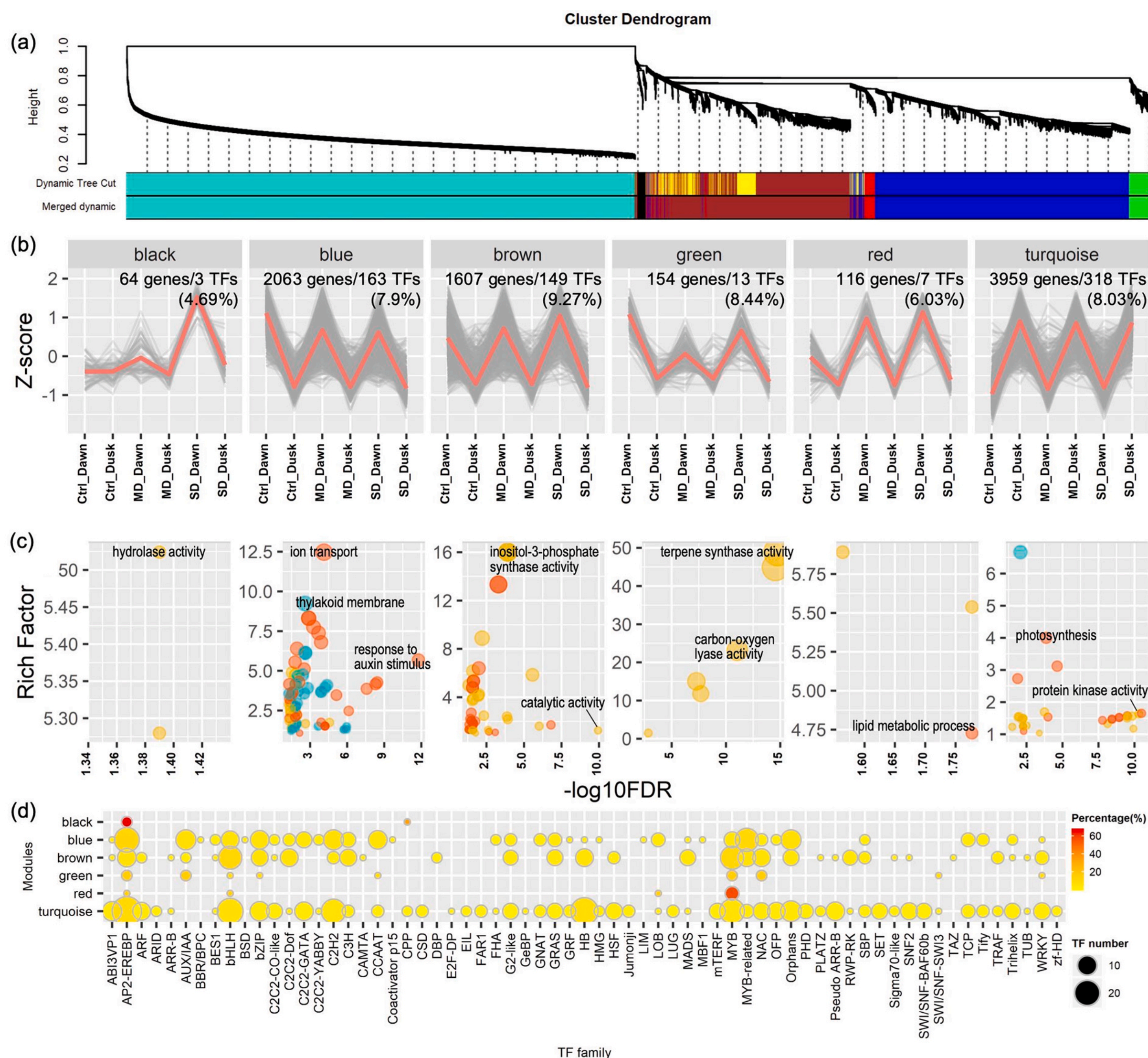

Fig. 2. Weighted gene co-expression network analysis (WGCNA) of DEGs in *Kalanchoë fedtschenkoi* under drought stresses. (a) Cluster dendrogram of DEGs in *Kalanchoë fedtschenkoi*. Different colors in merged modules column represent six different modules (MEs). **(b)** Z-score normalized expression patterns of DEGs in different modules. **(c)** GO enrichment analysis of DEGs in different MEs. **(d)** Statistic of TF number in different TF families of DEGs in different co-expression MEs.

[*NAD(P)-ME*], and *pyruvate phosphate dikinase* (*PPDK*), that are involved in CAM decarboxylation process did not show obvious expression pattern change under drought conditions relative to the control (Fig. 3; Supplementary Table S6).

2.5. Circadian rhythm and stomatal movement genes under drought conditions

Because the circadian system plays critical roles in regulating the special photosynthesis in CAM plants, we investigated the transcript profiles of core genes involved in the central circadian oscillators in response to drought stimuli (Fig. 4a; Supplementary Table S7). We found that the gene *CHE* (Kaladp0032s0054, CCA1 HIKING EXPEDITION) was down-regulated under drought treatments at dawn. However, we did not observe statistical differences for the expression of other genes that involve in circadian clock signaling under the drought stress conditions at both dawn and dusk in *K. fedtschenkoi* (Fig. 4a).

After comparison of the transcript profiles of stomatal movement genes between normal condition and drought treatment (Fig. 4b; Supplementary Table S7), a homolog of a 2C type protein phosphatase *ABI1* (*HAB1*) (Kaladp0048s0509) was identified that was highly induced by water-deficit stress at dawn (Fig. 4b). We then extracted a subnetwork of HAB1 (Fig. 5a), and identified several known abscisic acid (ABA) responsive genes that were co-expressed with *HAB1* (Fig. 5b), including

min — max
Zscore

	Dusk_Ctrl	Dusk_MD	Dusk_SD	Dawn_Ctrl	Dawn_MD	Dawn_SD	
Kaladp0018s0287	-0.83	-0.88	-0.88	0.63	0.79	1.17	*β-CA*
Kaladp0018s0289	-0.84	-0.88	-0.88	0.52	0.90	1.18	
Kaladp0034s0051	-0.88	-0.99	-0.92	1.14	0.93	0.72	
Kaladp0081s0143	0.51	1.31	0.66	-0.89	-0.87	-0.72	
Kaladp0095s0055	0.81	0.95	1.06	-1.05	-0.90	-0.87	*PEPC*
Kaladp0048s0578	0.90	1.06	0.71	-1.02	-0.96	-0.69	
Kaladp0011s1355	-0.60	-0.33	-0.50	-0.72	0.49	1.65	
Kaladp0062s0055	0.62	0.96	0.99	-1.10	-0.73	-0.74	
Kaladp0037s0517	-0.89	-0.88	-0.89	0.72	0.46	1.47	*PPCK*
Kaladp0604s0001	1.20	0.99	0.66	-0.96	-1.01	-0.87	
Kaladp0082s0192	0.94	0.66	1.20	-0.96	-1.00	-0.84	
Kaladp0058s0569	0.89	0.91	1.06	-1.15	-0.81	-0.90	*MDH*
Kaladp0082s0194	0.84	0.92	1.12	-1.01	-0.91	-0.96	
Kaladp1038s0012	0.84	0.93	1.09	-1.12	-0.87	-0.88	
Kaladp0062s0038	-0.78	-0.88	-0.95	0.48	0.91	1.22	*ALMT*
Kaladp0011s0027	-0.82	-0.42	-0.74	0.74	1.69	-0.45	
Kaladp0091s0013	-0.92	-0.97	-0.56	0.26	0.86	1.34	
Kaladp0042s0251	-0.98	-0.96	-0.91	0.69	1.15	1.00	*TDT*
Kaladp0015s0134	-0.50	-0.67	-0.95	-0.00	0.53	1.59	*NAD(P)-ME*
Kaladp0472s0027	0.89	0.93	0.99	-1.27	-0.84	-0.70	
Kaladp0092s0166	-0.93	-0.91	-0.92	0.85	1.21	0.70	
Kaladp0039s0092	-0.86	-0.92	-0.89	0.80	0.97	0.89	*PPDK*
Kaladp0076s0229	-0.85	-0.80	-0.87	0.76	0.89	0.88	
Kaladp0060s0363	0.93	0.96	1.02	-0.98	-0.97	-0.97	*PPDK-RP*

Fig. 3. Expression pattern of core CAM genes in response to drought stress at different time points. Z-score used for normalization of gene transcript profiles and for generation of Heatmap figures. The Z-scores were shown in the figure accordingly. There is no significant ($P < 0.05$) differential expression between the drought treatments (MD, SD) and control in each time point (dawn and dusk). *β*-CA, β type carbonic anhydrase; MDH, malate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; PPCK, PEPC kinase; ALMT tonoplast aluminum-activated malate transporter; NAD(P)-ME, NAD(P)$^+$-dependent malic enzyme; TDT, tonoplast dicarboxylate transporter; PPDK, pyruvate phosphate dikinase; PPDK-RP, PPDK regulatory protein. Dawn, 2-h before the start of light period and Dusk, 2-h before the dark period; Ctrl, normal irrigation used as control; MD, moderate drought; SD, severe drought.

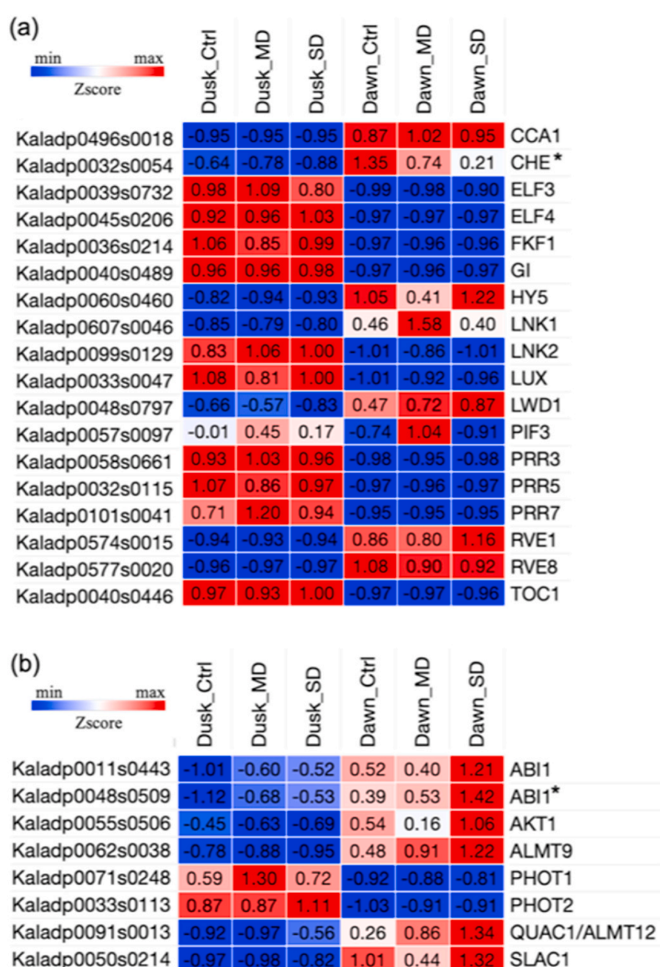

Fig. 4. Expression pattern of circadian rhythm and stomatal movement genes in response to drought stress at different time points. (a) Expression patterns of circadian rhythm genes under drought stress. **(b)** Expression patterns of stomatal movement genes under drought stress. Z-score used for normalization of gene transcript profiles and for generation of Heatmap figures. The Z-scores were shown in the figure accordingly. Dawn, 2-h before the start of light period and Dusk, 2-h before the dark period; Ctrl, normal irrigation used as control; MD, moderate drought; SD, severe drought. "*" indicates significant ($p < 0.05$) differential gene expression between the drought treatment and the control.

genes encoding three *CBL*-interacting protein kinases (CIPKs) (Kaladp0351s0028, Kaladp0090s0086, and Kaladp0964s0018), NAC (no apical meristem) domain transcriptional regulator superfamily protein (Kaladp0082s0047), a heat shock transcription factor C1 (HSFC1) (Kaladp0024s0811), and basic-leucine zipper (bZIP) transcription factor family protein 5 (ABI5) (Kaladp0011s0290 and Kaladp0911s0004). These genes co-expressed with *HAB1* were also up-regulated by water-deficit stress at dawn, although they did not show significantly differential expression relative to the control (Fig. 5b).

2.6. Comparison of drought responsive genes between K. fedtschenkoi and C_3 species

To examine molecular differences underlying responsive adaptation to drought stress between CAM and C_3 species, we performed comparative analysis of transcriptomes among *K. fedtschenkoi*, *Arabidopsis thaliana,* and *Populus deltoides* 'WV94'. First, we constructed the WGCNA co-expression networks based on previous published datasets and characterized 8 expression models for both *Arabidopsis* and *Populus*, which were divided into two clusters of drought-inhibited and drought-

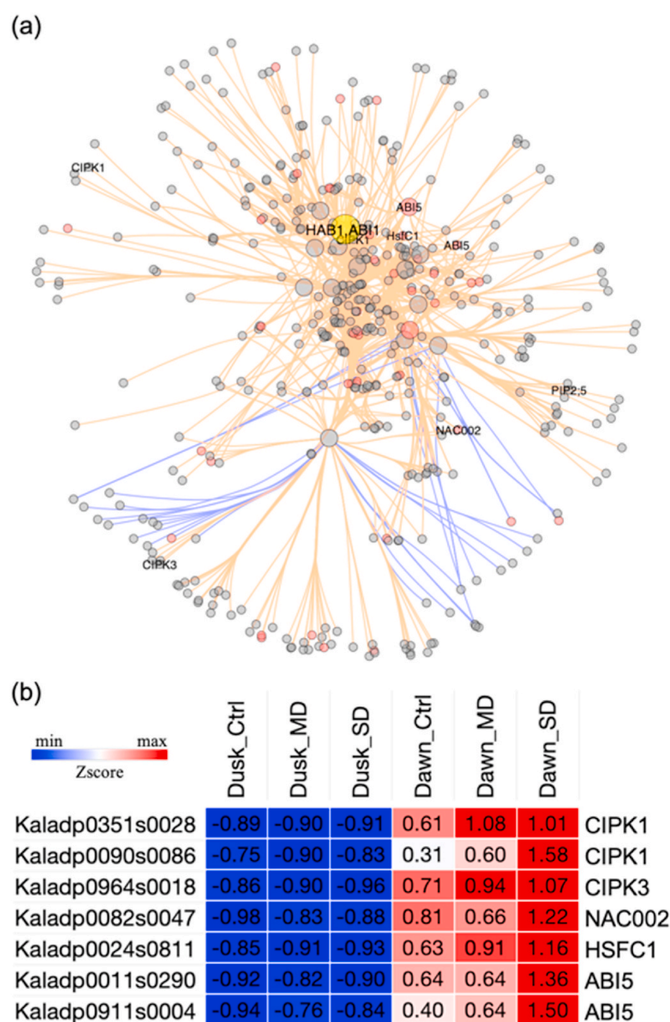

Fig. 5. Co-expression network of an ABA-signaling regulatory gene ABI1 (*Kaladp0048s0509*). (a) Co-Expression network of ABI1. Large and small nodes represent the primary and secondary co-expressed genes, respectively; red nodes represent transcription factors. Orange and blue edges represent positive and negative correlations, respectively. (b) Expression pattern of ABI1 co-expression genes. Z-score used for normalization of gene transcript profiles and for generation of Heatmap figures. The Z-scores were shown in the figure accordingly. Dawn, 2-h before the start of light period and Dusk, 2-h before the dark period; Ctrl, normal irrigation used as control; MD, moderate drought; SD, severe drought.

induced genes (Fig. 6). Comparative analysis was executed between *K. fedtschenkoi* and *Arabidopsis thaliana,* and between *K. fedtschenkoi* and *Populus deltoides* 'WV94' (Supplementary Table S8). We identified 30 up-regulated genes and 113 down-regulated genes that were shared by *K. fedtschenkoi* and *Arabidopsis*, and 96 up-regulated genes and 772 down-regulated genes that were shared by *K. fedtschenkoi* and *Populus* (Supplementary Table S8). Particularly, the transcripts of genes encoding phenylalanine ammonia lyase (PAL) (Kaladp0058s0598), chalcone synthase (CHS) (Kaladp0081s0076 and Kaladp0081s0078), cinnamate 4-hyroxylase (C4H) (Kaladp0098s0220), 4-coumarate-CoA ligase (4CL) (Kaladp0058s0423 and Kaladp0538s0001), and MYB domain protein 12 (MYB12) (Kaladp0008s0500) were up-regulated by water-deficit stress in *K. fedtschenkoi*, which displayed similar expression profile changes as their orthologs in Arabidopsis under drought conditions (Supplementary Table S5).

3. Discussion

Due to drier and warmer environments associated with climate change, genetic improvement of crop plants for sustainable bioenergy and food production in semi-arid and arid regions is becoming urgently needed (Borland et al., 2014; Stewart, 2015; Yang et al., 2015). This need can be partially met by exploitation of CAM photosynthesis, which enables high water-use efficiency as compared to C_3 and C_4 plants (Cushman, 2001; Borland et al., 2009; Matiz et al., 2013; Yang et al., 2015, 2019). For example, the drought-responsive genes identified in this study can be used to engineer "CAM-on-demand" systems in C_3 crops, in which the engineered crops can switch from C_3 to CAM under drought conditions and back to C_3 when drought stress disappears, using biosystems design or synthetic biology approaches (Yang et al., 2020; Yuan et al., 2020).

In facultative CAM species, the transcripts encoding core CAM-cycle enzymes, including β-CA, PEPC, PPCK, MDH, ALMT, NAD(P)-ME and PPDK, were up-regulated by water-deficit stress, resulting in the shift of the photosynthetic pathway from C_3 to CAM (Cushman et al., 2008; Dever et al., 2015; Brilhaus et al., 2016). According to our transcriptomic data analysis in *K. fedtschenkoi*, we did not observe significant expression differences for all core CAM genes between drought treatment and control at both dawn and dusk time points (Fig. 3), providing molecular evidence to explain how constitutive CAM regulatory pathway is not responsive to water-deficit stress. It was recently reported that the nocturnal CO_2 fixation in the wild-type plants of another *Kalanchoë* species (*K. laxiflora*) was significantly reduced at 18 days after withholding of water (Boxall et al., 2020), indicating that no-watering treatment of 18 days can cause severe drought stress in *Kalanchoë*. Therefore, it can be assumed that the *K. fedtschenkoi* plants in this study were under drought stress when the leaf samples were collected at 19 days after the beginning of severe drought treatment (i.e., no-watering treatment). This assumption is further supported by the molecular evidence that multiple genes, such as *PHR2* (Kaladp0044s0064), *SOUL-1* (Kaladp0068s0260), *CKX6* (Kaladp0039s0276) and *KCS11* (Kaladp0062s0077), *PAL2*, *C4L*, *4CL3*, *CHS* and *MYB12*, were significantly ($p < 0.05$) up-regulated in *K. fedtschenkoi* after 19-day water-deficit treatment (Supplementary Tables S3 and S5) consistent with the drought-responsive expression of their homologous genes in other species such as chickpea, *Agrostis stolonifera*, tobacco and Arabidopsis (Ahmad et al., 1998; Khanna et al., 2006; Xu and Huang, 2018; Khandal et al., 2020; Ahmed et al., 2021; Lohani et al., 2022). In addition, we analyzed the transcript profiles of circadian related-genes under drought stress condition and found that the expression of *CHE*, a circadian rhythm regulatory gene, was down-regulated by drought stress at dawn but not at dusk. In *Arabidopsis*, CHE is a critical functional component of the circadian clock system, which represses the expression of CCA1 through binding to its promoter region (Pruneda-Paz et al., 2009). This study focuses on the drought response in an obligate CAM species. In the future, it would be interesting to compare drought responsive gene expression between obligate and facultative CAM species, such as *Yucca* spp. (Heyduk et al., 2019), *Talinum triangulare* (Brilhaus et al., 2016), *Sedum album* (Wai et al., 2019), *Portulaca* (Ferrari et al., 2020; Gilman et al., 2022) and *Isoetes* (Wickell et al., 2021).

Flavonoids are a class of naturally produced polyphenolic secondary metabolites in plants, which play important roles in response to external stimuli of fungal parasites, herbivores, pathogens and ultraviolet (UV) radiation (Dixon and Pasinetti, 2010; Santos et al., 2017). Application of flavonoids on plants leads to enhanced abiotic stresses tolerance, including salt and drought, through reducing of the oxidative damage (Ahmed et al., 2021; Lohani et al., 2022). A group of key enzymes are involved in the central biosynthesis pathway of flavonoids, including phenylalanine amonialyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumaroyl-coenzyme A ligase (4CL), chalcone synthase (CHS), chalcone flavanone synthase (CHI), flavanone 3β-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), flavonol synthase (FLS), isoflavonoid

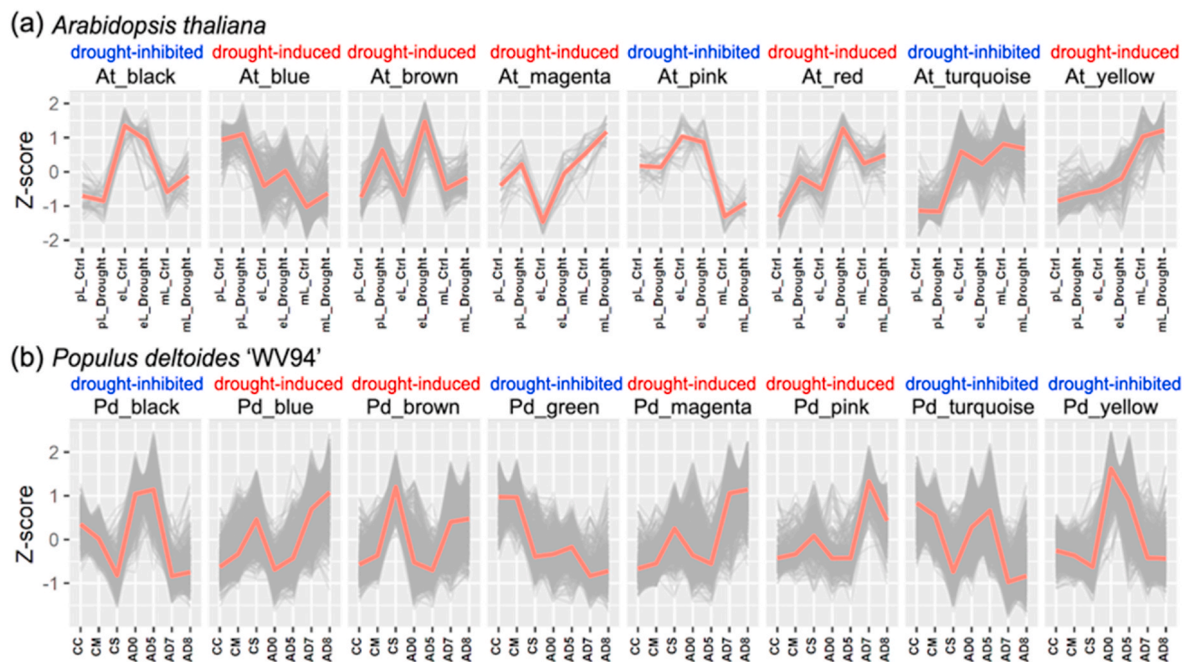

Fig. 6. Drought responsive genes in C_3 species *Arabidopsis thaliana* and *Populus deltoides* 'WV94'. (a) Different developmental stages of *Arabidopsis* leaves, including proliferating leaf (pL), expanding leaf (eL), and mature leaf (mL), were treated under control (Ctrl) and drought conditions. The expression data was obtained from GEO accession GSE16474 (Skirycz et al., 2010; Garcia et al., 2018). **(b)** Poplar plants were treated under cyclic drought experiment: cyclic control (CC), cyclic mild (CM), and cyclic severe (CS) and acute drought experiment: acute day 0, 5, 7, and 8 (AD0, AD5, AD7, and AD8, respectively). Different WGCNA co-expression modules in each species labeled "drought-induced" or "drought-inhibited" represent the genes in this module were induced or repressed during drought treatment, respectively. The color names, i.e., black, blue etc. indicate individual expression modules in these two species.

synthase (IFS), and anthocianin synthase (ANS) (Koes et al., 1994; Santos et al., 2017). It has been demonstrated that overexpression of C4H and 4CL improves drought stress tolerance in *Fraxinus mandschurica* and up-regulation of CHS, F3H and DFR increases drought tolerance in tobacco, rice, alflafla and Arabidopsis as well (Cui et al., 2014; Nakabayashi et al., 2014; Chen et al., 2019a; Chen et al., 2019b; Feyissa et al., 2019; Lohani et al., 2022). In this study, through our RNA-seq analysis, we identified several genes encoding the enzymes of PAL2, C4L, 4CL3, and CHS and a transcription factor of MYB12 for activation of the promoters of CHS, F3H, and FLS, which were up-regulated in both CAM and C_3 photosynthesis species under drought condition (Fig. 7) (Wang et al., 2016a; Wang et al., 2017), indicating the molecular response of flavonoid regulatory signaling to water-deficit stress is shared by CAM and C_3 species.

Plants have developed various adaptive and defense mechanisms to regulate stomatal morphology and movement for reducing water evaporation and increasing water-use efficiency in response to extreme drought environments (McAdam and Brodribb, 2012; Saradadevi et al., 2017). It is thought that water-deficit stress could strongly impact stomatal conductance and stomatal movement which is associated with increased amount of abscisic acid (ABA), a crucial stress responsive phytohormone (Brodribb and McAdam, 2013). The ABA signaling cascade contains four core components, i.e., ABA receptors (PYR/-PYL/RCAR family), type 2C protein phosphatase (PP2C), SNF1-related protein kinase 2 (SnRK2) and/or open stomata 1(OST1), and bZIP transcription factors like ABI5, and/or slow anion channel-associated 1 (SLAC1) (Umezawa et al., 2010; Dong et al., 2015; Hauser et al., 2017). The PP2C family plays a global role in the negative regulation of ABA signaling, which can be classified into 10 or more subfamilies (Raghavendra et al., 2010; Fuchs et al., 2013; Singh et al., 2016). HAB1/ABI1 and ABI2 belong to the subfamily of clade A PP2C, which has been demonstrated to be involved in ABA responsive signaling via interacting with ABA receptors and protein kinase SnRK2s (Fujii et al., 2009; Santiago et al., 2012; Soon et al., 2012). The elevated transcript abundance of ABI1 under drought condition in *K. fedtschenkoi* at dawn and dusk (Fig. 4b) implies that ABA signaling is affected by drought stimuli. According to subnetwork analysis of ABI1, we found that CBL-interacting protein kinases (CIPKs), NAC (no apical meristem) domain transcriptional regulator superfamily protein, and basic-leucine zipper (bZIP) transcription factor family protein 5 (ABI5) were co-expressed with ABI1 (Fig. 5). Notably, the CIPKs are involved in signaling pathways of osmotic stress and ABA in Arabidopsis, tobacco, and wheat (Chen et al., 2013; Wang et al., 2016b; Cui et al., 2018). The NAC domain transcriptional regulator superfamily proteins play important roles in response to ABA and water deprivation in Arabidopsis (Fujita et al., 2004; Tran et al., 2004). It has been documented that ABI5 is a regulator of seed germination, early seedling development and seed maturation through ABA signaling in Arabidopsis and legumes (Skubacz et al., 2016; Zinsmeister et al., 2016). Taken together, the results indicate that ABA signaling transduction is regulated by drought stress in *K. fedtschenkoi*.

4. Conclusion

In this study, we identified DEGs and co-expressed gene modules responsive to drought stress in an obligate CAM species *K. fedtschenkoi*. We provided molecular evidence that core CAM pathway is not regulated by drought stimuli in constitutive CAM species. Furthermore, we found that *K. fedtschenkoi* shares some commonality with C_3 species (*Arabidopsis thaliana* and *Populus deltoides* 'WV94') in gene expression relevant to ABA signaling and secondary metabolism in response to drought stress, as summarized in Fig. 7.

5. Material and methods

5.1. Plant material and sample collection

The freshly generated *Kalanchoë fedtschenkoi* (ORNL diploid

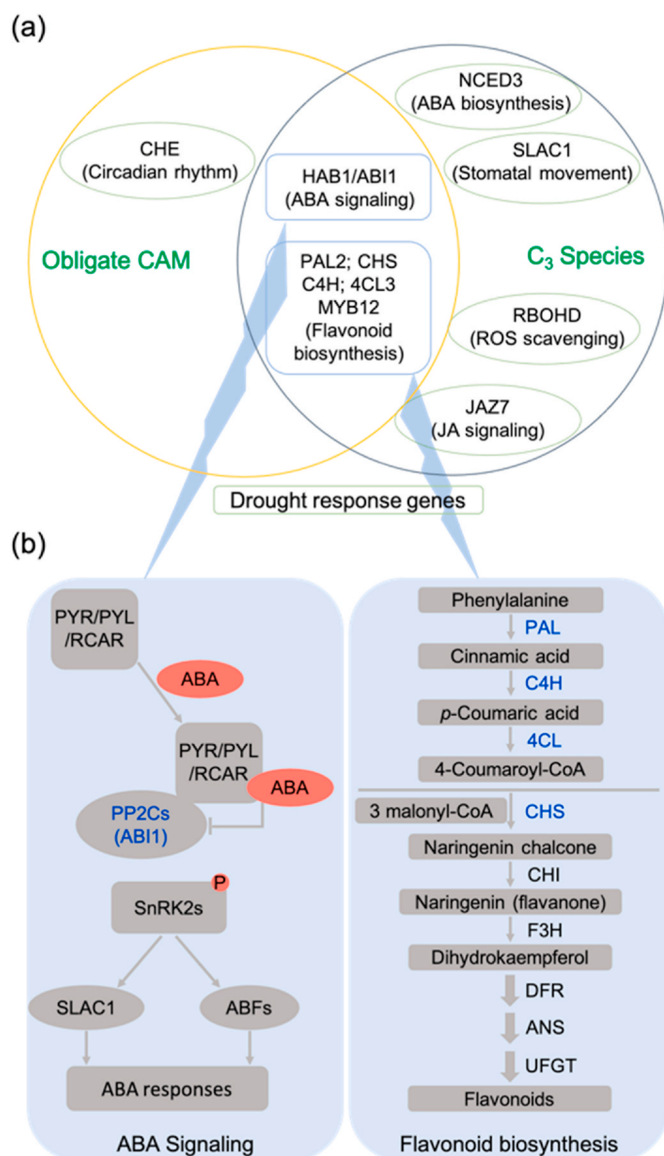

Fig. 7. Comparison of gene regulation responding to drought stress between obligate CAM species (*K. fedtschenkoi*) and C_3 species (*Arabidopsis thaliana* and *Populus deltoides* 'WV94'). (a) Summary of genes responding to drought stress in obligate CAM species and C_3 species. NCED3: nine-*cis*-epoxycarotenoid dioxygenase 3; SLAC1: slow anion channel-associated 1; RBOHD: respiratory burst oxidase homolog D; JAZ7: jasmonate ZIM-domain 7; CHE: CCA1 hiking expedition. (Geiger et al., 2009; Mittler and Blumwald, 2015; Kalladan et al., 2019; Meng et al., 2019). **(b)** Schematic diagrams showing ABA signaling and flavonoid biosynthesis pathways in plants. The genes shared by *K. fedtschenkoi* and C_3 species in response to drought stress were highlighted in blue.

accession M2) plants were grown in a growth chamber on a 12 h/12 h photoperiod with a photon flux density of 250 μmol m^{-2} s^{-1} and day/night temperatures 25 °C/18 °C, respectively. After growing in soil for 4 weeks, watering of 1/3 of the plants was stopped to induce severe drought stress, and 1/3 of the plants were watered with 200 mL water per pot every other day to induce a moderate drought treatment, and 1/3 of the plants were watered with full irrigation every other day as controls. Two weeks (14 days) after the beginning of drought treatments, the soil moisture (SM) was 40% ± 3%, 20% ± 3% and 2% ± 3% for the control, moderate drought and severe drought treatments, respectively, as measured by using a *Delta-T* SM150 device (*Delta-T Devices*, Cambridge, UK). A total of 19 days after the beginning of drought treatments, the mature leaves (leaf pairs of 4th, 5th and 6th below the shoot apex) from all treated plants were collected and immediately frozen in liquid nitrogen, and then stored at −80 °C for further processing. Leaf samples were collected from three individual plants to represent three biological replicates for each treatment at each time point.

5.2. Total RNA extraction

For total RNA isolation, approximately 100 mg of tissue powdered by grinding leaves in liquid nitrogen was pre-treated with 850 μl of CTAB buffer (1.0% β-mercaptoethanol freshly added) and incubated at 56 °C for 5 min. The sample mixture was then extracted with 600 μl chloroform:isoamylalcohol (24:1) and centrifuged at full-speed for 10 min at room temperature. The clear supernatant was carefully transferred into a filter column provided in the Spectrum™ Plant Total RNA Kit (Sigma, Cat. No. STRN250-1 KT) and then centrifuged for 1 min at full-speed to remove the plant tissue debris. The remaining steps were performed with the Spectrum™ Plant Total RNA Kit according to manufacturer's instructions. An on-column DNase treatment was applied for removing of residual genomic DNA contaminations. The final RNAs were eluted with 50 μl of RNase free water from the binding columns and RNAs quantity and quality were examined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of 3 μg RNA for each sample was prepared and shipped to HudsonAlpha Institute for Biotechnology (Huntsville, Al, USA) for library preparation and transcriptomic sequencing.

5.3. Library construction and RNA-seq

The RNA samples were used for sequencing library construction using the Illumina TruSeq Stranded mRNA library prep kit (Illumina Inc., San Diego, CA). Illumina RNASeq w/PolyA Selection, Plates: Plate-based RNA sample prep was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Illuminas TruSeq Stranded mRNA HT sample prep kit utilizing poly-A selection of mRNA following the protocol outlined by Illumina in their user guide: https://support.illumina.com/sequencing/sequencing_kits/truseq-stranded-mrna.html, and with the following conditions: total RNA starting material was 1 μg per sample and 8 cycles of PCR was used for library amplification. The prepared libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed with other libraries, and the pool of libraries was then prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v4, and Illumina's cBot instrument to generate a clustered flow cell for sequencing. Sequencing of the flow cell was performed on the Illumina HiSeq 2500 sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 2 × 150 indexed run recipe. The filtered RNA-seq reads of each library were aligned to the *Kalanchoë fedtschenkoi* reference genome (Yang et al., 2017) using the TopHat2 software program (Kim et al., 2013). Reads that mapped uniquely to one locus were counted and raw gene counts were generated using FeatureCounts (Liao et al., 2013). The value of transcripts per million (TPM) was used for gene expression level in this study (Li and Dewey, 2011).

5.4. Data analysis

Identification of differentially expressed genes (DEGs) between treatments and control, or between time points was conducted with the DESeq2 (v1.2.10) software program (Love et al., 2014), with the model of "design(dds) <- ~ stress + time + stress:time" to analyze the impact of time, stress and their interaction. The cut-off for significant DEGs was a false discovery rate (FDR) corrected p-value <0.05, along with |log2 (fold change)| >1 in gene expression. Two strategies were used for

identifying DEGs, including temporal comparison and drought condition comparison (Fig. 1b). The comparison conducted between samples collected at two time points (i.e., dusk and dawn) under individual treatments (control, moderate drought and severe drought), was defined as the temporal comparison (Supplementary Table S1). The drought condition comparison was defined as the comparison of severe/-moderate drought versus control or severe drought versus moderate drought treatments at the same time point (i.e., dusk or dawn). (Supplementary Table S1).

The R package WGCNA was applied for construction of a weighted gene co-expression network with the log2 normalized TPM values and WGCNA used softPower 12, minModuleSize 20, and MEDissThres 0.10 as the threshold (Langfelder and Horvath, 2008). Gene Ontology (GO) enrichment analyses were performed using BiNGO (Maere et al., 2005) with all genes in the genome as background. The drought treatment datasets of *Arabidopsis* and *Populus* used for comparison analysis in this manuscript were obtained from published data (Skirycz et al., 2010; Garcia et al., 2018). Reciprocal BLAST best hits (Lohani et al., 2022) were used to identify the orthologous genes among *K. fedtschenkoi*, Arabidopsis, and poplar. All analysis was run with default parameters.

Data availability

All raw short reads are available in the NCBI SRA database (SRA accessions: SRP156058 – SRP156075).

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Disclosure

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Credit author statement

X.Y., R.H. and J.Z. conceived and designed the research. R.H., S.J., and D.L. performed the experiments. J.Z., A.S., A.L., M.W., V.N., C.D., K. K., J.S., H.L., and P.R. analyzed the data. R.H. and J.Z. drafted the manuscript. X.Y., J.G.C., W.M., T.J.T., and G.A.T. revised the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2022.153791.

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