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Identification of *CoDREB* Genes for Drought and Cold Tolerance in *Camellia oleifera*

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Abstract: *DREB* is a plant-specific transcription factor family that plays a pleiotropic regulatory role in response to abiotic stresses such as drought and cold. In this study, we identified 51 *DREB* genes of *Camellia oleifera*. These CoDREBs ranged from 88 to 518 amino acids (average/median 259/237 aa). The predicted molecular weights (MW) of the CoDREB proteins ranged from 9.7 kDa to 59.6 kDa, and the isoelectric points (*p*I) ranged from 4.62 to 10.44. A gene structure analysis showed that 43/51 (84.3%) *CoDREBs* were intronless, and the number of exons varied from one to three. Then, we focused on the response of *CoDREB* genes in terms of plant drought and cold acclimation. Under short-/long-term drought stress, *CoDREB1.2/4.1/4.4/4.8/4.12/4.15/5.1/5.3/5.5/6.2* have different regulations in response to long-term drought response, and *CoDREB1.4/2.5/4.6/4.1/6.3/6.5* specifically in the short term. Additionally, in response to mild/severe drought and followed by recovery, we found that *CoDREBs* may be involved in a complex drought-responsive regulatory network. Under cold stress, *CoDREB5.2* and *CoDREB6.5* are significantly up-regulated, and *CoDREB* may participate in the regulation of the low-temperature response of *C. oleifera*.

Keywords: *Camellia oleifera* Abel; *CoDREB*; drought stress; cold stress

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1. Introduction

Agricultural security and sustainability are jeopardized by a variety of potential risks, such as global environment/climate changes, poverty, the Russo-Ukrainian War (interlinked global crises and conflicts), and even the COVID-19 pandemic. To maintain high levels of production in the agricultural sector, plant breeders/researchers are focusing on predictable potential hazards, such as environmental changes that cause abiotic stresses (unfavorable growth conditions) during plant growth, which inevitably affect crop yield every year. During long-term natural selection pressures, sessile plants have evolved a complex set of molecular and physiological mechanisms to cope with abiotic/biotic stresses and breeders expanding numerous tools and mining molecular resources to develop new improved crop varieties [1,2]. Therefore, it is critical to understand underlying mechanisms regarding desired plant breeding goals.

Camellia oleifera Abel. (commonly known as tea oil, Theaceae) is one of four major woody oil plants that are widely distributed in subtropical mountain areas of the Yangtze River basin and South China, with elevations ranging from about 200 to 2000 m [3,4]. The plantation area of *C. oleifera* spans approximately 4.4 million hectares, with an annual output of over 2.6 million tons of seeds and, as a result, oil yields of more than 0.65 million tons [5]. Precipitation in the main tea-oil-producing areas is mostly concentrated from March to June, accounting for 60% to 70% of the total precipitation during the year; precipitation from July to September (the annual dry season in South China) is relatively low, accounting for

about 20% of the annual precipitation, and is susceptible to high temperatures and drought, while the critical period for tea oil synthesis/accumulation is from July to August [6]. Accordingly, despite being a drought-resistant tree, (seasonal) drought stress is regarded as the major threat to *C. oleifera* growth [7], causing wilting; decreases in photosynthesis, oil contents, flower buds, and fruits setting; even the death of drought-sensitive cultivars that is mainly induced by cell death caused by reactive oxygen species (ROS) accumulation [8,9]. Plants have developed drought-resistance strategies that involve a series of morphological, biochemical, physiological, and molecular factors [10,11]. Similar to tea and other crops, tea oil also modifies its leaf structure based on the degree of drought in order to meet the needs for survival and growth [12], increasing peroxidase (POD) activity and malonaldehyde (MDA) content [7]. Moreover, due to recent advances in high-throughput sequencing, a transcriptomic analysis of *C. oleifera* can be used to identify core differentially expressed genes (DEGs) in response to various degrees of drought stress [5,7,13]. However, research on *C. oleifera*-specific drought-resistant regulatory networks/mechanisms remains far from being used in molecular assistance breeding or simply providing genetic resources to speed up the breeding process.

Dehydration-responsive element-binding (DREB) proteins bind to specific DNA motifs (DRE/CRT element, A/GCCGAC), and are found to be important in activating the expression of target stress-inducible genes [14–16]. As reported, the overexpression of *DREB2* gene can improve the drought tolerance of the *Arabidopsis* [14]. Additionally, *DREB1* is rapidly and transiently induced by cold stress, and the overexpression of *DREB1* leads to increased stress tolerance in rice and *Arabidopsis* [14,17]. To date, *DREB* has been widely identified in the plant kingdom and the role of *DREB* has frequently been reviewed. Gene discovery leads to improved drought stress tolerance in plants via gene transfer. The present study comprehensively integrates the latest *C. oleifera* transcriptome studies on various levels of drought stress (both short-/long-term and severe). Simultaneously, as *C. oleifera* is perennial non-wood economic species and a cold-area-adaptive subspecies, cold-responsive *DREB* is an important resource for future molecule assistance breeding. A publicly available RNA-seq dataset related to *C. oleifera* was also analyzed to identify potential *DREBs*, and preliminarily characterized DREBs may be involved in combined drought and cold stress responses. We identified 18 *DREBs* involved in short-/long-term drought responses, both together and each individually, and cross-validated the function of genes involved in mild/severe drought stresses. *CoDREB5.2* and *CoDREB6.5* were identified as key genes for cold adaptation. Finally, the combined results suggest that *CoDREB5.2* and *CoDREB6.5* may be involved in both drought and cold stress adaptation.

2. Materials and Methods

2.1. Homologs Sequence Identification of DREB Genes in C. oleifera

To aid in the comprehensive identification and analysis of *DREB* gene family in *C. oleifera*, sequences of putative *DREB* genes were obtained from National Center for Biotechnological Information (NCBI, https://www.ncbi.nlm.nih.gov/), accessed on 14 March 2022. GenBank and The *Arabidopsis* International Resource (TAIR, https://www.arabidopsis.org/), accessed on 14 March 2022. The chromosome-scale reference genome of diploid progenitor *C. oleifera* "Nanyongensis" (CON, 2n = 2x = 30) was downloaded from Zenodo.org released on 9 December 2021 (https://zenodo.org/record/5768785#.Y7b3H3ZBwQ8, accessed on 14 March 2022) [18]. *CoDREB* genes were identified with a released procedure: *CoDREB* genes were first searched with TBtools "BLAST GUI Wrapper" [19] with *AtDREBs* to obtain candidate *C. oleifera* DREB-family members; the HMM (Hidden Markov Model) profile PF00847 (AP2 domain) was retrieved from the Pfam database (https://pfam.sanger.ac.uk/), accessed on 14 March 2022. to identify putative DREB genes with highly conserved amino acid residues 14th Valine (V) and 19th glutamic acid/valine/leucine/alanine (E/V/L/A) [20]. The molecular weight (MW) and isoelectric point (*p*I) were predicted using the ProtParam tool (https://web.expasy.org/protparam/), accessed on 14 March 2022.

2.2. Phylogenetic Analysis of DREB Proteins and Nomenclature

CoDREB and its homologs were imported into MEGAX (version 10.2.6, https://www.megasoftware.net), accessed on 25 March 2022 and the sequence alignment was performed using the default "ClustalW" settings [21]. Phylogenetic trees were then constructed using the "Neighbor-joining" algorithm with default parameters, and the '.nwk' phylogenetic tree was made using iTol online (https://itol.embl.de), accessed on 25 March 2022 [22]. According to *Arabidopsis* A1–A6 nomenclature, *CoDREB* genes have been designated based on their location on the phylogenetic tree.

2.3. Gene Structure and MEME Conserved Motif Analysis

Conserved motifs of CoDREB proteins were predicted using the MEME suite version 5.4.1 (https://meme-suite.org/meme/index.html), accessed on 25 March 2022 [23] with default parameters except the "motifs should find" was set to 10. The coding sequences (CDS) and the structure of all genes were graphically displayed with TBtools function "Gene Structure View" [19]. The chromosome-scale reference genome annotated structure information "gff" file of *C. oleifera* was downloaded from Zenodo.org released on 9 December 2021 (https://zenodo.org/record/5768785#.Y7b3H3ZBwQ8, accessed on 14 March 2022) [18].

2.4. RNA-Seq Data

Raw data of RNA-seq were downloaded from The European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/browser/home), accessed on 29 October 2022 [24] through using "IBM aspera" (https://www.ibm.com/products/aspera), accessed on 29 October 2022 that was installed through "Bioconda" [25]. Short-/long-term drought stress RNA-seq data were "PRJNA355046" and "PRJNA875963" [5,7]; "PRJNA309526" for mild and severe drought stress [13] and "PRJNA292037" for cold acclimation of wild *C. oleifera* cultivars at series location and elevation [4]. Raw data (raw reads) in fastq format were first qualified with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), accessed on 29 October 2022 for Q20, Q30, GC-content and sequence duplication levels; the data were then processed in Hisat2 version 2.2.1 [26] for read alignment to the *C. oleifera* reference genome. The reads were subjected to fragments per kilobase of transcript per million fragments mapped (FPKM) conversion to obtain the expression value of genes and transcripts. In-house R scripts were used to analyze gene expression and generate heatmaps. A heatmap was generated using ggplot2, reshape2, gplots, and dplyr packages in R version 4.1.2 [27].

3. Results

3.1. Genome-Wide Identification and Phylogenetic Analysis of DREB Genes in C. oleifera

A total of 198 proteins containing AP2/ERF domain(s) (PF00847) were originally obtained in the diploid *C. oleifera* var. The "Nanyongensis" (CON) (2n = 2x = 30) genome was identified via stepwise procedures, including local BLAST and HMM searches, which were processed at an E-value cutoff of 1E-5 [18] (Figure 1A). These genes contained at least one ap2 domain. The 14th valine (V) and 19th glutamic acid/valine/leucine/alanine (E/V/L/A), used to confirm conserved amino acid residues, were processed to identify 51 candidate *DREB* genes in *C. oleifera*. These CoDREBs ranged from 88 to 518 amino acids (average/median 259/237 aa). The predicted molecular weights (MW) of the CoDREB proteins ranged from 9.7 kDa to 59.6 kDa, and the isoelectric points (*p*I) ranged from 4.62 to 10.44 (Table S1).

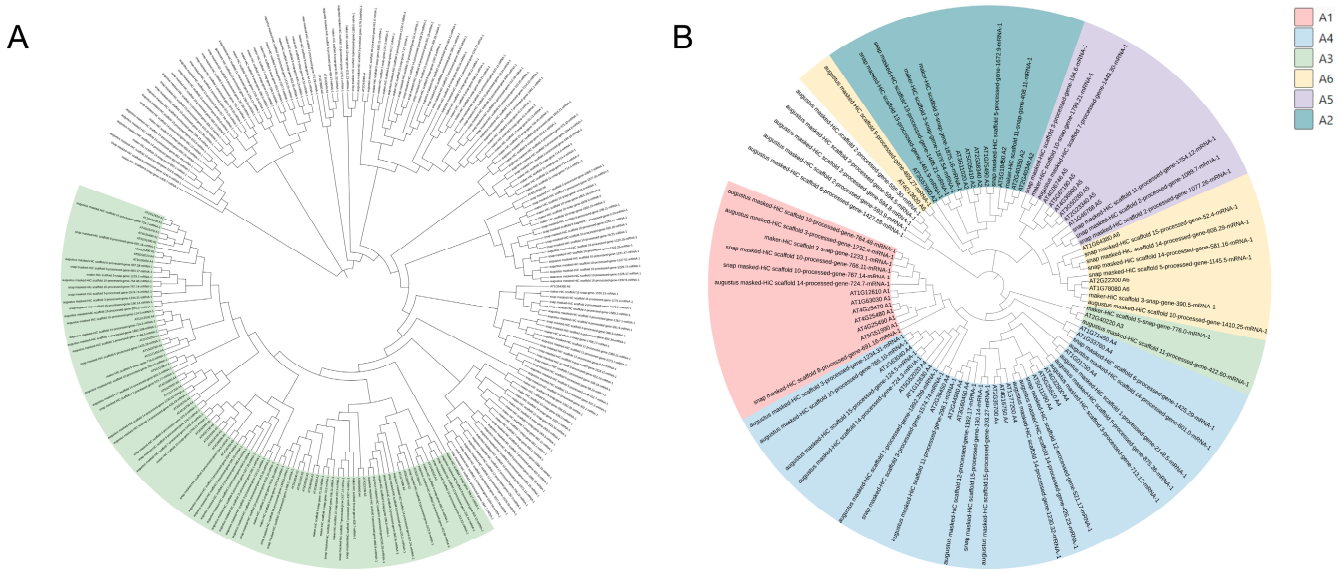

Figure 1. Phylogenetic analysis of *CoDREBs* using BLAST and other programs; a total of 198 proteins containing AP2/ERF domain(s) were obtained in the diploid *C. oleifera* var. "Nanyong" (CON) (2n = 2x = 30) genome. (**A**) Multiple sequence alignments were performed for 51 *CoDREBs* and 40 *AtDREBs*, and phylogenetic analysis was performed in MEGAX. A total of 51 *CoDREB* genes could be classified into six groups, namely A1 (with 7 members), A2 (with 6 members), A3 (with 2 members), A4 (with 18 members), A5 (with 6 members) and A6 (with 7 members). (**B**).

3.2. Phylogenetic Relationship, Conserved Motifs and Genes Structure Analysis and Nomenclature of CoDREBs

To investigate the evolutionary relationships of *CoDREB* genes, multiple sequence alignments of 51 putative *CoDREBs* and 40 *AtDREBs* were processed, followed by a phylogenetic analysis in MEGAX. The results show that 51 *CoDREB* genes can be classified into six groups, namely A-1 (with seven members), A-2 (6), A3 (2), A-4 (18), A-5 (6) and A-6 (7) (Figure 1B). Five genes that are evolutionarily distant from the main groups were not classified and are almost never expressed in RNA-seq treatments (Figure S1). Furthermore, in silico conserved motif prediction (MEME suite) and the exon–intro structure were analyzed to overview the structure characteristics of *CoDREBs*. A total of 10 motifs were predicted and named as Motifs 1 to 10 (Figure 2). All proteins included Motif 1 and 2, which consist of the AP2 structural domain. None of genes contained all 10 motifs. However, the motif diversity is relatively conserved in all groups, with slight differences among subfamilies. The previous five non-classified genes have highly conserved motifs, especially regarding Motif 5. A gene structure analysis showed that most *CoDREBs* were intronless, 43/51 (84.3%), and the number of exons varied from one to three (Figure 2). Additionally, we suggested a uniform nomenclature for *CoDREB*, excluding those unclassified genes based on phylogenetic and structural studies to facilitate the future investigation of expression. (Table 1). The "Seq id" values from the GFF file, gene loci, and 14th and 19th amino acid residues are all listed. Interestingly, the 19th leucine was only found in the A-5 and A-6 subfamilies (Table 1).

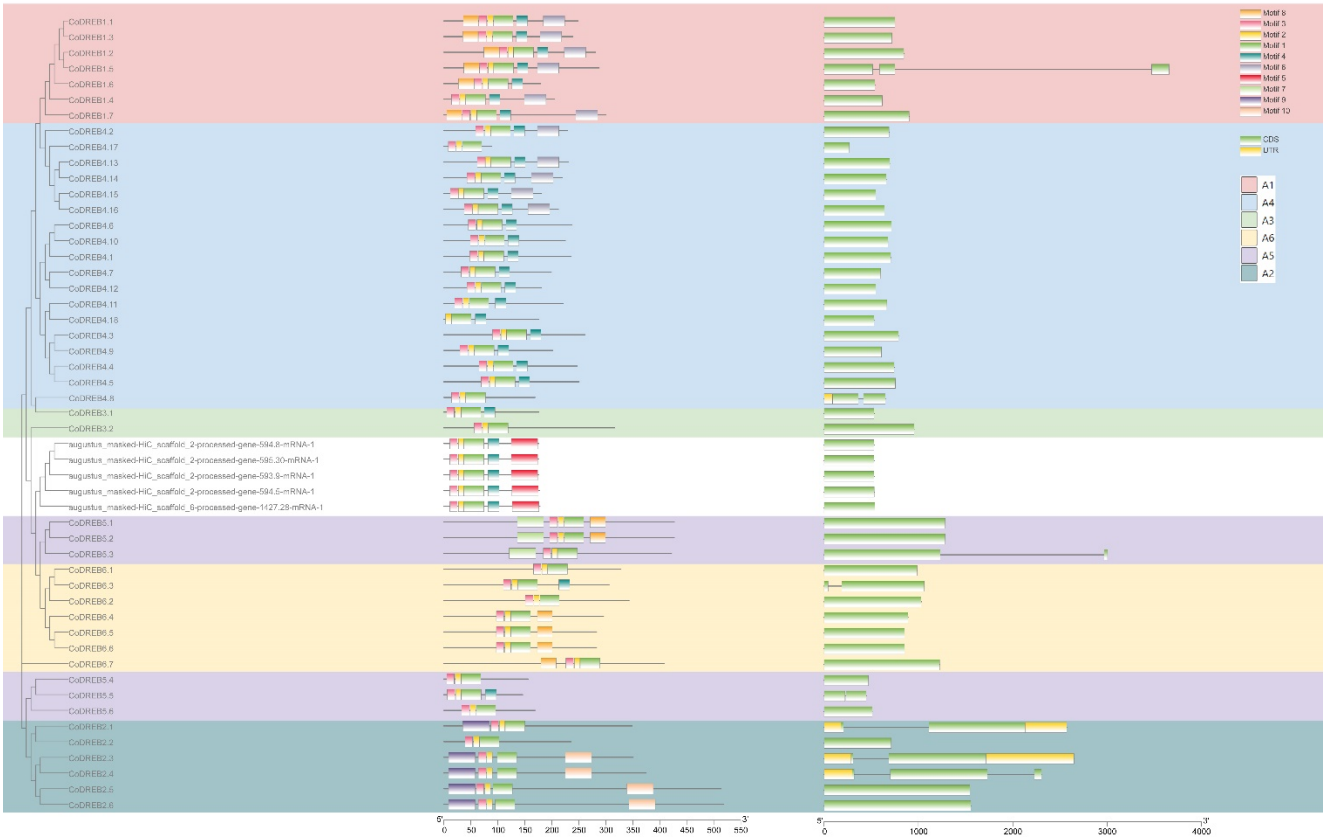

Figure 2. We used MEME suite version 5.4.1 to predict the silico conserved motif prediction and exon–intro structure of *CoDREBs* protein; a total of 10 motifs were predicted and named as Motifs 1 to 10.

Table 1. The seq-id, gene loci, and 14th valine (V) and 19th glutamic acid/valine/leucine/alanine (E/V/L/A) amino acid residues of 51 *CoDREBs*.

Symbol	Seq Id	14th	19th	Source	Strand	Start	End
CoDREB1.1	augustus_masked-HiC_scaffold_10-processed-gene-764.48-mRNA-1	V	E	HiC_scaffold_10	–	76409702	76410451
CoDREB1.2	augustus_masked-HiC_scaffold_3-processed-gene-1232.4-mRNA-1	V	E	HiC_scaffold_3	–	123283087	123283932
CoDREB1.3	snap_masked-HiC_scaffold_10-processed-gene-766.11-mRNA-1	V	E	HiC_scaffold_10	–	76623619	76624338
CoDREB1.4	augustus_masked-HiC_scaffold_14-processed-gene-724.7-mRNA-1	V	E	HiC_scaffold_14	+	72478180	72478797
CoDREB1.5	maker-HiC_scaffold_3-snap-gene-1233.1-mRNA-1	V	E	HiC_scaffold_3	–	123362118	123365776
CoDREB1.6	snap_masked-HiC_scaffold_10-processed-gene-767.14-mRNA-1	V	E	HiC_scaffold_10	–	76705366	76705905
CoDREB1.7	snap_masked-HiC_scaffold_8-processed-gene-691.16-mRNA-1	V	E	HiC_scaffold_8	–	69154923	69155825

Table 1. *Cont.*

Symbol	Seq Id	14th	19th	Source	Strand	Start	End
CoDREB2.1	maker-HiC_scaffold_11-snap-gene-408.11-mRNA-1	V	E	HiC_scaffold_11	+	40854256	40856829
CoDREB2.2	snap_masked-HiC_scaffold_5-processed-gene-1672.9-mRNA-1	V	E	HiC_scaffold_5	+	167280494	167281204
CoDREB2.3	maker-HiC_scaffold_3-snap-gene-1875.16-mRNA-1	V	E	HiC_scaffold_3	−	187547455	187550105
CoDREB2.4	maker-HiC_scaffold_3-snap-gene-1876.54-mRNA-1	V	E	HiC_scaffold_3	−	187645199	187647503
CoDREB2.5	snap_masked-HiC_scaffold_13-processed-gene-1466.21-mRNA-1	V	E	HiC_scaffold_13	−	146604874	146606418
CoDREB2.6	snap_masked-HiC_scaffold_13-processed-gene-1461.9-mRNA-1	V	E	HiC_scaffold_13	+	146118529	146120085
CoDREB3.1	maker-HiC_scaffold_5-snap-gene-776.0-mRNA-1	V	E	HiC_scaffold_5	+	77602969	77603618
CoDREB3.2	augustus_masked-HiC_scaffold_11-processed-gene-422.60-mRNA-1	V	E	HiC_scaffold_11	+	42215560	42216513
CoDREB4.1	augustus_masked-HiC_scaffold_14-processed-gene-1230.32-mRNA-1	V	E	HiC_scaffold_14	−	123064670	123065377
CoDREB4.10	snap_masked-HiC_scaffold_12-processed-gene-521.17-mRNA-1	V	E	HiC_scaffold_12	-	52181645	52182322
CoDREB4.11	augustus_masked-HiC_scaffold_3-processed-gene-713.12-mRNA-1	V	A	HiC_scaffold_3	+	71361910	71362575
CoDREB4.12	augustus_masked-HiC_scaffold_14-processed-gene-601.0-mRNA-1	V	E	HiC_scaffold_14	+	60115087	60115632
CoDREB4.13	augustus_masked-HiC_scaffold_3-processed-gene-1234.31-mRNA-1	V	E	HiC_scaffold_3	−	123398472	123399167
CoDREB4.14	augustus_masked-HiC_scaffold_10-processed-gene-765.10-mRNA-1	V	E	HiC_scaffold_10	−	76529118	76529777
CoDREB4.15	augustus_masked-HiC_scaffold_15-processed-gene-124.5-mRNA-1	V	E	HiC_scaffold_15	+	12454548	12455093
CoDREB4.16	augustus_masked-HiC_scaffold_14-processed-gene-724.3-mRNA-1	V	E	HiC_scaffold_14	+	72440566	72441204
CoDREB4.17	augustus_masked-HiC_scaffold_1-processed-gene-1892.209-mRNA-1	V	E	HiC_scaffold_1	+	189269071	189269337
CoDREB4.18	augustus_masked-HiC_scaffold_5-processed-gene-875.36-mRNA-1	V	A	HiC_scaffold_5	−	87523685	87524215
CoDREB4.2	snap_masked-HiC_scaffold_3-processed-gene-1574.74-mRNA-1	V	E	HiC_scaffold_3	−	157422246	157422935
CoDREB4.3	augustus_masked-HiC_scaffold_12-processed-gene-1192.17-mRNA-1	V	E	HiC_scaffold_12	−	119269647	119270435

Table 1. *Cont.*

Symbol	Seq Id	14th	19th	Source	Strand	Start	End
CoDREB4.4	snap_masked-HiC_scaffold_15-processed-gene-190.14-mRNA-1	V	E	HiC_scaffold_15	+	19021440	19022183
CoDREB4.5	augustus_masked-HiC_scaffold_15-processed-gene-203.27-mRNA-1	V	E	HiC_scaffold_15	−	20340240	20340995
CoDREB4.6	augustus_masked-HiC_scaffold_14-processed-gene-426.23-mRNA-1	V	E	HiC_scaffold_14	+	42621443	42622156
CoDREB4.7	augustus_masked-HiC_scaffold_1-processed-gene-2148.5-mRNA-1	V	E	HiC_scaffold_1	+	214865752	214866351
CoDREB4.8	snap_masked-HiC_scaffold_6-processed-gene-1425.29-mRNA-1	V	E	HiC_scaffold_6	−	142541920	142542450
CoDREB4.9	augustus_masked-HiC_scaffold_11-processed-gene-298.1-mRNA-1	V	E	HiC_scaffold_11	−	29817495	29818103
CoDREB5.1	snap_masked-HiC_scaffold_2-processed-gene-1077.26-mRNA-1	V	L	HiC_scaffold_2	−	107715224	107716507
CoDREB5.2	snap_masked-HiC_scaffold_2-processed-gene-1089.7-mRNA-1	V	L	HiC_scaffold_2	+	108932798	108934081
CoDREB5.3	snap_masked-HiC_scaffold_11-processed-gene-1254.12-mRNA-1	V	L	HiC_scaffold_11	+	125398486	125401490
CoDREB5.4	augustus_masked-HiC_scaffold_7-processed-gene-1443.30-mRNA-1	V	E	HiC_scaffold_7	−	144370313	144370783
CoDREB5.5	maker-HiC_scaffold_10-snap-gene-1799.21-mRNA-1	V	E	HiC_scaffold_10	+	179923482	179923929
CoDREB5.6	snap_masked-HiC_scaffold_3-processed-gene-194.6-mRNA-1	V	E	HiC_scaffold_3	−	19425828	19426337
CoDREB6.1	snap_masked-HiC_scaffold_5-processed-gene-1145.5-mRNA-1	V	L	HiC_scaffold_5	+	114517410	114518396
CoDREB6.2	augustus_masked-HiC_scaffold_10-processed-gene-1410.25-mRNA-1	V	L	HiC_scaffold_10	−	141067137	141068168
CoDREB6.3	maker-HiC_scaffold_3-snap-gene-390.5-mRNA-1	V	L	HiC_scaffold_3	−	39027029	39028091
CoDREB6.4	snap_masked-HiC_scaffold_15-processed-gene-52.4-mRNA-1	V	L	HiC_scaffold_15	+	5199287	5200177
CoDREB6.5	snap_masked-HiC_scaffold_14-processed-gene-581.16-mRNA-1	V	L	HiC_scaffold_14	−	58133946	58134797
CoDREB6.6	snap_masked-HiC_scaffold_14-processed-gene-608.29-mRNA-1	V	L	HiC_scaffold_14	+	60806950	60807801
CoDREB6.7	augustus_masked-HiC_scaffold_5-processed-gene-469.27-mRNA-1	V	L	HiC_scaffold_5	−	46960303	46961529

3.3. Expression Profiles of 46 CoDREB Genes in Response to Short-/Long-Term Drought Stress

DREB is a plant-specific transcription factor family that has been widely studied for its pleiotropic regulatory roles in response to abiotic stresses, such as drought and cold. However, similar research on tea oil is currently scarce. Thus, to gain insights into the transcriptional regulation of *CoDREB* genes involved in drought response, a publicly available RNA-seq dataset of *C. oleifera* was studied during long-term drought (0 d to 12 d, PRJNA875963) and comparatively short-term drought (0 to 36 h, PRJNA355046). Details on data processing could be found in the Section 2. The overall alignment ratio of reads was quantified using a quality control package "fastQC" that revealed ratios between 78.58% and 85.02%. Both experiments compared drought-tolerant (T) and drought-sensitive (S) cultivars of *C. oleifera*. The expression pattern of *CoDREBs* was group-plotted using a heatmap (Figure 3). In general, almost two-thirds of the *CoDREB* genes were up-/down-regulated during drought stresses. However, half of these genes similarly induce/reduce the response to drought stress, demonstrating a general drought adaptation pathway. Instead, we focused on genes that are differentially regulated in expression level between cultivars under long-/short-term drought stress, or even both types of stress. *CoDREB1.2/4.1/4.4/4.8/4.12/4.15/5.1/5.3/5.5/6.2* were only differentially regulated in response to long-term drought stress, *CoDREB1.4/2.5/4.6/4.16/6.3/6.5* were specifically regulated in response to short-term stress, and only *CoDREB3.1/4.8/5.2* were identified for both types of stress (Figure 3). All 18 *CoDREBs* were identified and labeled, and the changes in *CoDREB* expression patterns between drought-tolerant and -sensitive cultivars may play an important role in regulating drought adaptation in *C. oleifera*.

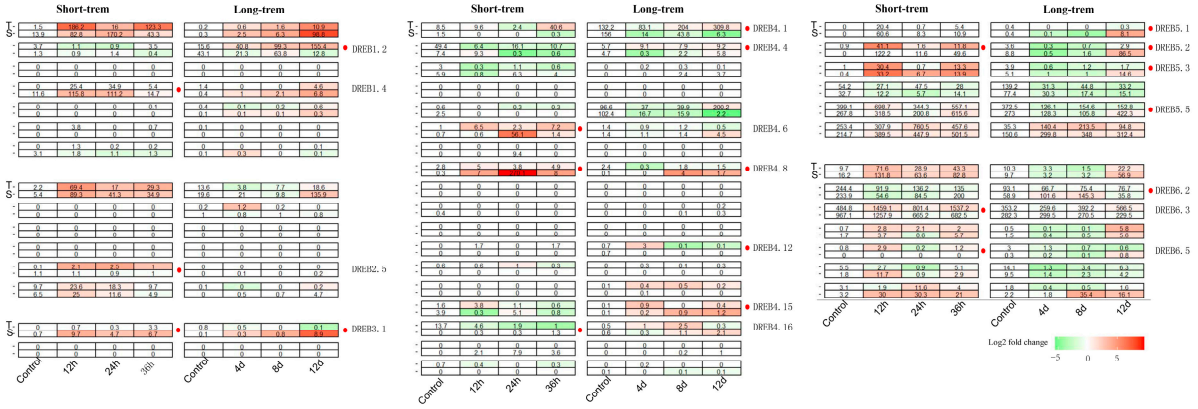

Figure 3. Expression analysis of short-/long-term drought stress with *CoDREBs*, T: Drought-tolerant cultivars of *C. oleifera*, S: Drought-sensitive cultivars of *C. oleifera*, Long-term: 0 d to 12 d, Short-term: 0 to 36 h.

3.4. CoDREBs in Response to Mild, Severe Drought and Followed by Recovery

Despite the negative consequences of drought stress, severe environmental circumstances can lead to interesting adaptations in plants that allow them to survive and reproduce [28]. In this scenario, to extend our understanding of possible biological functions of *DREBs*, the transcriptome data of *C. oleifera* subjected to mild, severe drought stress and followed by recovery were analyzed. As illustrated in Figure 4A, the expression pattern is relatively varied in each *CoDREB* subfamily, indicating that the *CoDREBs* may be involved in a complex drought-responsive regulatory network. We further classified the 46 *CoDREBs* into six clades based on expression profiling; Clades 1–5 were significantly differentially expressed during drought stress, and Clade 6 was not (Figure 4B,C).

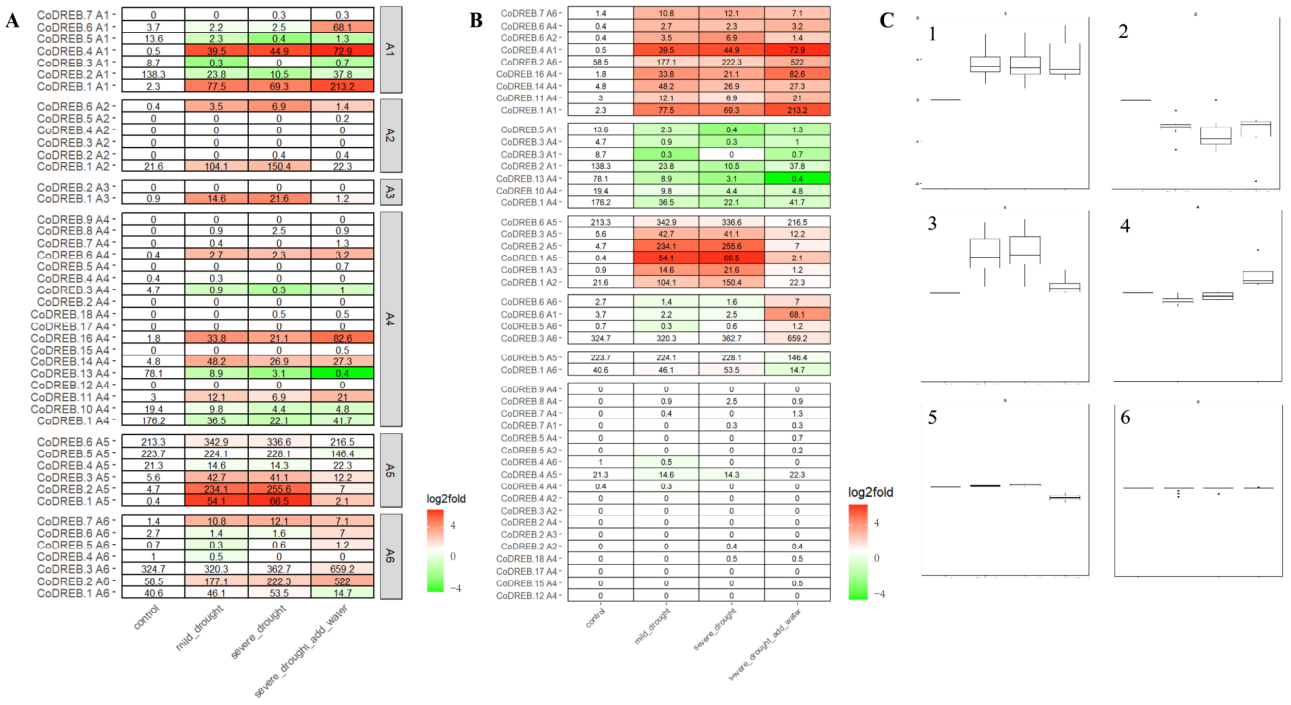

Figure 4. Expression analysis of mild, severe drought stress and severe drought with *CoDREB* expression. (**A**) According to the expression profile, 46 *CoDREBs* are divided into six Clades. (**B**,**C**). Color means the up/down of gene expression, red means the up and green means the down. The darker the color, the higher the expression.

3.5. Transcriptome Analysis of Wild C. oleifera Grown from Different Latitudes and Elevations to Discover CoDREBs Involved in Cold Acclimation

To investigate the response of *CoDREB* genes regarding cold stress acclimation and preliminarily explore the potential candidate of *DREB* involved in cold- and drought-stress-related "crosstalk", we analyzed the transcriptomes of wild tea oil cultivars grown in different locations and elevations [4]. Evidently, cold stress increases with latitude and elevation (Figure 5). *C. oleifera* wild cultivars were harvested in November or December when the perennial plant's cold adaptation occurs. A schematic complex transcriptional regulatory network is involved in this adaptation process, in which *DREB1* functions as a master regulator [29]. In this study, *CoDREB5.2* and *CoDREB 6.5* were significantly up-regulated with a temperature decrease from 9.8 to 2, and this might be involved in the cold acclimation of tea oil. (Figure 5).

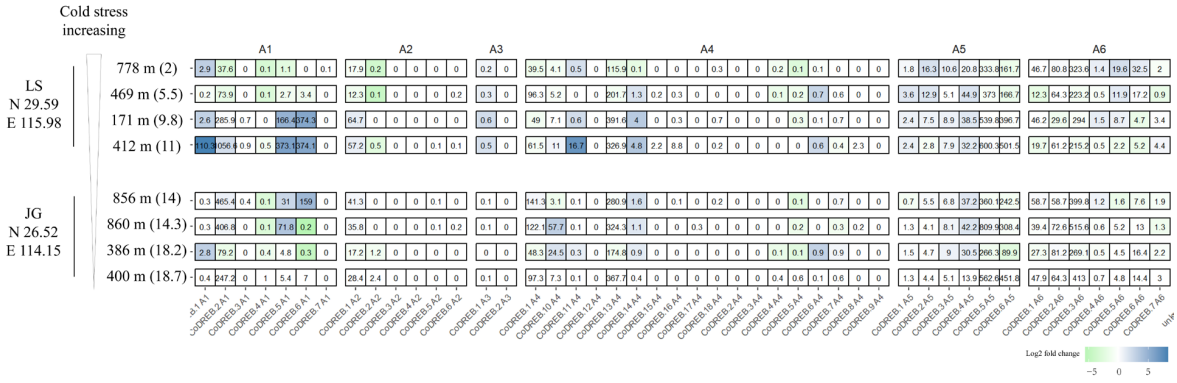

Figure 5. Expression analysis of wild *C. oleifera* cultivars grown in different locations and elevations with *CoDREBs*; (2)–(18.7) indicates the temperature, LS: Lu Mountain, JG: Jinggang Mountain, N: Latitude, E: Longitude. Color means the up/down of gene expression, blue means the up and green means the down. The darker the color, the higher the expression.

4. Discussion

The *AP2/EREBP* superfamily was originally thought to contain plant-specific transcription factors until several AP2 domain-containing HNH endonucleases were found in cyanobacteria (*Trichodesmiumerythraeum*), ciliate (*Tetrahymena thermophile*), and viruses (*Enterobacteria phage* Rb49 and *Bacteriophage Felix* 01) [30]. The *AP2/ERF* transcription factors are known to regulate diverse processes of plant development and stress responses (reviewed by [31]). With the same AP2 domain, the valine (14th V) was present in all *DREB* members but relatively inconsistent at the 19th E/V/L/A, with four candidates identified, whereas alanine (14th A) and aspartic acid (19th D) were conserved in the ERF proteins [32–35]. With the availability of numerous plant whole-genome sequences, *DREB* has been widely identified and investigated in many plant species. However, limited studies have been undertaken on non-timber wood species with *DREB* genes, and even fewer studies have integrated different perspectives to analyze the changes in expression during abiotic stress. Herein, with the release of the CON genome [18], we first identified 51 *DREB* genes in *C. oleifera*. In comparison to *Arabidopsis* (57, genome size 125 Mb), rice (56, 480 Mb), *Vitis vinifera* (38, 400 Mb), alfalfa (172, 800 Mb), jujube (25, 440 Mb) and *Saccharum spontaneum* (83, 3.36 Gb) [36–40], only 51 *CoDREBs* with a genome size of 2.97 Gb were identified. This might be because the aligned diploid CON genome was previously identified as a wild progenitor of cultivated tetraploid/polyploidy *C. oleifera* [18], demanding an in-depth genome sequencing of cultivated polyploid *C. oleifera*. Additionally, this finding might be supported by the discovery of *DREB* genes in tea (*Camellia sinensis*), in which only 45 *CsDREB* genes were identified prior to the recent publication of high-resolution genome sequencing data [41–43]. Furthermore, the de novo assembly of *C. oleifera* RNA-seq raw reads resulted in 66,570 unigenes, which is a value 1.43-fold higher than the CON-released 46,617 genes [13,18]. This result cross-validates the limitation of the CON for a cultivated *C. oleifera* transcriptome experiment. We simultaneously analyzed four different RNA-seq datasets used to standardize gene assembly and alignment within the same framework.

With the 51 identified putative *CoDREB* genes, the structure analysis of *CoDREBs* revealed that motif diversity is relatively conserved in all sub-families with slight differences among these subfamilies (Figure 2). Additionally, we obtained a few group-specific motifs (Figure 2), such as Motif 4/5/9/10. Even with a conserved 14th V, *CoDREBs* containing Motif 5 were phylogenetically separated from the other *CoDREBs* (Figure 1). Moreover, four genes in this subgroup were nearly rarely expressed in response to drought or cold stress (Figure S1). Taken together, these genes may be involved in different abiotic stress responses, as the *DREB* family was first identified in response to drought and cold stress, as well as salt stress [33]. For genes induced during short-/long-term drought stress, groups with diverse expression patterns ranging between tolerant (T) and sensitive (S) were separated from the rest (Figure 3). We identified 18 *CoDREBs* as the candidate drought-responsive genes that potentially participate in a regulatory network. *CoDREB1.4/2.5/4.6/4.16/6.3/6.5* were specifically and differently regulated between T/S cultivars in response to relatively short-term drought stress. During this period, KEGG enrichment analysis revealed that both biochemical pathways and the signal transduction pathway were activated, including "biosynthesis of secondary metabolites", "vitamin B6 metabolism", "anthocyanin biosynthesis", and "phosphatidylinositol signaling system" [7]. Chlorophyll content (Chl), peroxidase (POD) activity, malonaldehyde (MDA) concentration, and soluble sugar content were all found to be different between the two cultivars; therefore, the antioxidant capacities of T and S cultivars differed. For long-term drought stress, fewer biochemical and physiological parameters were measured [5]. PIF7, a *DREB1* negative regulator under circadian control in *Arabidopsis*, significantly decreased in sensitive (S) cultivars but was highly expressed in tolerant (T) cultivars; this may indicate the involvement of the circadian clock system in drought stress response. The perception of drought stress response signaling is rapid, and most dehydration-responsive genes are induced by the plant hormones abscisic acid (ABA) and ethylene (ET) [44]. Therefore, because the most enriched pathways and metabolites are involved in antioxidants but not signal transduction, we hypothesized that the genes

encoding these 18 *CoDREBs* are critical for drought acclimation but not for drought signal perception. Furthermore, additional specialized and in-depth experiments with *CoDREBs* during drought stress are still required.

To understand the possible biological functions of CoDREBs involved in drought tolerance, transcriptome data of C. oleifera subjected to mild and severe drought stresses and followed by recovery were analyzed. Evidently, the expression patterns of CoDREBs are inconsistent within subfamilies (Figure 4A). In another transcription factor gene family in alfalfa [45], cold-responsive bHLH genes with similar expression patterns are clustered with promoter region transcription factor-binding sites (TFBS), rather than structural and conserved motifs (TFBS). Thus, we further classified the 46 CoDREBs into six clades based on expression profiling; Clades 1–5 were significantly differentially expressed during drought stress and Clade 6 was not (Figure 4B,C). We deduced that the genes in Clades 1–5 may be involved in multiple biological processes that have been activated by changing the expression patterns of anti-oxidants in dehydrated plants [46–49]. For instance, genes in Clade 1 that are constitutively expressed both during mild/severe drought stresses and after recovery are expected to be involved in ROS-scavenging activities that require a specific duration of time to eliminate the accumulated dose after drought water recovery. Moreover, Clade 3 genes are sensitive to external drought stresses, suggesting that they may be engaged in the signal transduction regulatory network. All of these findings show that *DREB* genes are involved in multiple processes of drought stress adaptation and are worthy of future research.

Regarding cold stress, stomatal opening is inhibited and demonstrates marked changes in lipid composition, mainly in membranes, as well as for stomatal closure during drought stress [50]. Both cold and drought stresses are accompanied by phytohormone accumulation and other signaling components, such as ROS, nitric oxidase (NO), and Ca^{2+}, while the mechanisms themselves require extensive research. In reality, plants grown in dry and semi-arid regions, such as tea and tea oil, are simultaneously exposed to combined stresses, such as cold and drought stresses [51]. The *DREB* gene family was reported to be involved in cold and drought adaptation. Thus, combined with previous drought RNA-seq data, we adaptively analyzed *CoDREB* expression patterns in cultivars under long-term cold/freezing conditions (Figure 5). Collectively, *CoDREB5.2* and *CoDREB6.5* that are significantly up-regulated with a temperature decrease from 9.8 to 2 might be involved in tea oil cold acclimation. Interestingly, *CoDREB5.2* and *6.5* were both characterized as drought-responsive elements in a previous analysis. We suggest that these two *CoDREB* genes could be interesting molecular targets available for the improvement of plant resistance to drought and cold stresses. On the other hand, we predicted that *CoDREB5.2* and *6.5* might be involved in cold–drought stress signal crosstalk.

Drought and cold stresses are extreme conditions faced by *Camellia oleifera* during its growth and affect the growth and development of *Camellia oleifera*. Improving the drought tolerance and cold tolerance of *Camellia oleifera* has always been a major focus of research. Through the analysis of the structure and biological function of the *CoDREB* gene, we found that this gene can be used to improve the ability of *Camellia oleifera* to adapt to drought and cold stresses.

5. Conclusions

Overall, our study found that the changes in *CoDREB* expression patterns between drought-tolerant and -sensitive cultivars play an important role in regulating drought adaptation in *C. oleifera*. A transcriptome analysis of wild *C. oleifera* grown from different latitudes and *elevations* also found that *CoDREBs* were involved in regulating *C. oleifera* to adapt to cold stress. Drought and cold stresses refer to extreme weather conditions that hurt plants and other living organisms. Both of these can cause physical and physiological damage to the organisms they affect. Gene discovery leads to improved drought/cold stress tolerance in plants via gene transfer. With the improvement of the transgenic system of *C. oleifera*, it will increase our understanding of the function of *CoDREB* in resisting the drought and cold

stresses of *C. oleifera*. *CoDREB* is a new potential target of engineering trees used to resist extreme weather conditions and promote the development of the *C. oleifera* industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb14010019/s1, Figure S1: Expression analysis of short-/long-term drought stress with five unknown genes. Table S1: The Molecular weight (MW) and isoelectric point (pI) of 51 CoDREBs.

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