Transcriptome Profiling of Abiotic Responses to Cold and Drought Stress of *Cinnamomum kanehirae*

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Stout camphor tree (*Cinnamomum kanehirae*) is an economically and socially valuable timber species that is conserved in Taiwan Province, China. In this study, Illumina sequencing technology was used to identify genes in *C. kanehirae* and analyze their levels under drought and low temperature stresses. The obtained reads generated 27,885 single genes, including 8,136 that were differential. Based on the results of Kyoto Encyclopedia of Genes and Genomes enrichment, the primary metabolic pathways in response to drought and low temperature stress included sucrose and starch metabolism, photosynthesis, glycolysis and sugar metabolism synthesis, phenylpropanoid biosynthesis, plant-pathogen interaction, and flavonoid biosynthesis, as well as the identification of transcription factors (TFs) with different patterns of expression. Finally, the RNA-Seq data were validated using real-time fluorescence quantitative analysis to identify TFs with different patterns of expression. These data provide a valuable resource for further research on the molecular mechanisms of drought and low temperature stress in *C. kanehirae* and contribute to the exploration of drought and cold resistance genes.

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Keywords: Cinnamomum kanehirae; Drought stress; Cold stress; Transcriptome; qRT-PCR

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INTRODUCTION

Drought and low temperatures are the major abiotic stresses in the plant growth cycle that severely affect plant growth and development (Zhou *et al*. 2015). Plants respond to drought and low temperature stress by regulating various physiological and biochemical processes, such as stomatal closure, the activation of respiration, and the inhibition of cell growth and photosynthesis (Mishra *et al*. 2014). Wang (2014) and Galeano *et al*. (2019) showed that teak (*Tectona grandis* L.) and rubber trees (*Hevea brasiliensis* Muell. Arg.) respond to drought stress by regulating bioenergy synthesis, antioxidant enzymes, and osmotic regulation. Indian sandalwood (*Santalum album*) and *Populus* respond to low temperature stress by inducing the accumulation of malondialdehyde, proline, antioxidants, and soluble carbohydrates (Jiang *et al*. 2011; Zhang *et al*. 2017). Several studies have shown that drought and low temperature stress can lead to changes in gene expression, which has important implications for the transcriptional regulation of plants as they respond to drought and low temperature stress (Deng *et al*. 2019). Riahi *et al*. (2013) found that 9-*cis*-epoxy carotenoid dioxygenase (NCED) is a key enzyme in the biosynthetic

pathway of abscisic acid (ABA), which cleavages carotenoids to form the plant hormone ABA. In turn, this leads to the closure of grape (*Vitis vinifera*) stomata, reduces water loss by transpiration, and enhances the promotion of plant drought resistance. By activating Ca^{2+} channels, cryogenic stress triggers a series of signaling pathways, such as Ca^{2+}-dependent protein kinases (CDPKs), mitogen-activated protein kinases (MAPKs), and calcineurin B-like (CBL) proteins, among others, which interact with other Ca^{2+} signals, participate in mediating the plant perception of environmental signals, and modulate downstream environmental stress responses, thereby responding to low-temperature stress (Yuan *et al*. 2018). Wang *et al*. (2013) found that CDPKs play an important role under low temperature stress in tea (*Camellia sinensis*). Potential genes related to cold tolerance in sugarcane (*Saccharum officinarum*) have also been investigated, and the genes related to the regulation of membrane system stability were found to be the determinants of cold tolerance differences, while the key genes that encode protein kinase activity, starch metabolism, and calcium signaling were associated with cold tolerance (Huang *et al*. 2021). In recent years, the mechanisms of stress response of woody plant have attracted increasing amounts of attention, and the molecular mechanisms of drought and low temperature stress reactions of camphor have been studied less intensively.

High-throughput sequencing is an important technique for studying plant molecules, since it enables the identification and characterization of new large-scale transcriptional data (Martin and Wang 2011; Lawson *et al*. 2020). In recent years, increasing numbers of researchers have used transcriptome sequencing to study changes in the levels of expression of drought-tolerant and cold-tolerant genes in plants (Dong *et al*. 2014; Yang *et al*. 2015). For example, a study of *Salix cupularis* found that the NAC, WRKY, MYB and disease-resistant genes, such as chalcone synthase, serine/threonine protein kinase PBS1, LRR receptor protein kinase, and lectin-like protein kinase, were significantly expressed under drought stress (Xu *et al*. 2021). In *Ammopiptanthus mongolicus*, 1,636 unigenes involved in the transcripts for cold stress were identified. The largest gene families were the ethylene-responsive element binding factor family (ERF), followed by the basic helix-ring-helix family (bHLH), the acetylene family, the homologous domain-leucine zip family (HD-ZIP), and the WRKY family (Pang *et al*. 2013).

Stout camphor tree (*Cinnamomum kanehirae*) is an evergreen broad-leaved tree in the Lauraceae family, which is an endemic conservation tree species in Taiwan Province of China; the tree is distributed in mountainous areas at 200 to 2,000 m above sea level and is one of the most valuable timber species in Taiwan (Hung *et al*. 2014). *C. kanehirae* can live for a long time and has a large diameter. Its wood is widely used in furniture, carvings, and building materials, making it highly valuable economically (Hung *et al*. 2017). With the increasing demand for *C. kanehirae*, the wild camphor resources in Taiwan have become rare and precious because the trees have been plundered or cut down in large quantities, thus seriously damaging their natural ecology. Most of the existing natural forests of camphor are old trees; the mother trees are scattered; they are not easily pollinated; collecting the seeds is difficult, and the rate of seed germination is low, which renders the camphor trees endangered (Kuo *et al*. 2010). Our group introduced *C. kanehirae* to be planted in Yunnan Province in 2015 and found that the climate in central Yunnan differs greatly from the origin of *C. kanehirae*. The low temperature and drought in this region limit the planting of *C. kanehirae* (Zhang *et al*. 2021). Therefore, the introduction and domestication of *C. kanehirae* is the key to solving the problem of whether

this species can be successfully cultivated in central Yunnan. However, no data on the mechanism of response of *C. kanehirae* to drought and low temperature stress have been generated.

This study included transcriptome analyses on the leaves of *C. kanehirae* under drought and low temperature stresses with the following objectives: 1) to obtain key differential genes under different types of stresses, 2) to investigate the molecular regulatory mechanisms of *C. kanehirae* under drought and low temperature conditions, and 3) to verify the detection results by qRT-PCR analysis.

EXPERIMENTAL

Plant Materials

Annual *C. kanehirae* seedlings with good growth conditions were planted in the greenhouse at the Bailong Campus of Southwest Forestry University (Kunming, China), and the growth conditions of the control group (CMKZ) were as follows: 80% water holding capacity in the field, normal watering (five times/d) at room temperature (25 °C). The low temperature treatment involved the placement of the *C. kanehirae* seedlings in incubator for 2 h at 4 °C (CMK4) and 15 °C (CMK15), respectively, and the rest of the conditions were the same. The drought treatment (CMKG) included a lack of watering for 12 consecutive days. The relative soil moisture content in the pots was monitored by weighing in combination with a ProCheck soil moisture meter (Decagon Devices, Inc., Pullman, WA, USA). The levels of moisture were monitored until they went down to 40%, and two-thirds of the leaves of *C. kanehirae* had curled. Three replicates of each treatment were used during which leaf changes were observed and recorded, and the leaves of different treatments were collected, stored in liquid nitrogen and sent to Personalbio (Shanghai, China) for transcriptome sequencing.

RNA Isolation and Construction of RNA-seq Libraries

As described by Yang *et al*. (2020), the total RNA of camphor leaf tissue under different treatments was extracted using the Trizol reagent. The integrity and concentration of RNA were determined using 1% agar gel electrophoresis and quantified with a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). The polyA structure of the magnetic bead and mRNA were used to isolate the mRNA, so that the fragmentation of mRNA was reversed into a double-stranded structure. When the second strand of cDNA was synthesized, the 5' end was repaired; the 3' end was added to A, and a 300 bp long cDNA fragment was recovered for PCR amplification. Finally, the cDNA library was obtained and sequenced using an Illumina NovaSeq platform (San Diego, CA, USA).

Data Processing and Annotation

The RNA-Seq raw sequencing data was filtered using Cutadapt, and the filtered clean reads were further targeted to the reference genome (ASBRC_Ckan_1.0) using HISAT2 (http://ccb.jhu.edu/software/hisat2/index.shtml), with up to two mismatches allowed. To obtain comprehensive information on gene function, the genes were searched using *BLAST*, and the following databases were annotated: NCBI Non-redundant Protein Sequences (Nr), eggNOG, SwissProt, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO).

Differential Expression Analysis

GO enrichment and KEGG pathway analyses were performed based on the established GO database (gene Ontology, http://geneontology.org/) and the *C. kanehirae* gene annotation database. The P-value of differential genes was calculated using a hypergeometric distribution method. The criterion for significant enrichment was $P < 0.05$, and the GO terms and KEGG pathway of the differential genes were significantly enriched compared with the whole genomic background, which helped to determine the primary biological functions exercised by the differential genes. The levels of gene expression were differentially analyzed using DESeq, and the conditions for the differentially expressed genes (DEGs) were as follows: expression difference multiple|log2FoldChange|>1, and significance $P < 0.05$.

Quantitative Real-time PCR (qRT-PCR) Analysis

The accuracy of RNA-Seq data was determined using qRT-PCR, and three technical replicates were performed for each sample. The RNA from the sample was stored at -80 °C, and the primers were designed as required for qRT-PCR using Primer Premier 5.0 software. The sequences are shown in Table 1. Amplification was performed using the real-time RT-PCR system, and expression was calculated using the 2~ΔΔ Ct method (Yang *et al*. 2017).

Table 1. Primer Sequences

Primer Name	Sequence (5'-3')
CKAN_02528500F	TGCAGTATAACATCTTGTAC
CKAN_02528500R	AATAGCACAGCTTATGTCAT
CKAN_02421400F	GAGAATTTGGCGACCGAGTC
CKAN_02421400R	TTCTGGTATTTTGATCGGTA
CKAN_00179400F	GAGAGCCATATATTGTTGCT
CKAN_00179400R	TAACCGGGTCCAAATACAGG
CKAN_01330000F	GAACCACTACGACATACCAC
CKAN_01330000R	AGCGACCAGGAGGGTATGTT
CKAN_00809200F	AGACCGAGCTGATCAGGTTC
CKAN_00809200R	ACAAGGTCACCGGAGGCGGT
CKAN_00351400F	TTATGTTGGCAGGATTGGCA
CKAN_00351400R	GTGTTCTCATCCACCATTTC
CKAN_01502300F	CAGTTGGACGTGACATCTTC
CKAN_01502300R	TAAGGCTGCTAAACTGTCAT
CKAN_02148700F	GACGTCCTCAAGAACCGGAT
CKAN_02148700R	CCTTGCAGAGGCAGCTCATG
CKAN_02523600F	AGAGCCAAGTTGCAGCATCT
CKAN_02523600R	ACTATTCCAAGAGGATTCCA

RESULTS AND DISCUSSION

Transcriptome Data Analysis

The *C. kanehirae* leaf transcriptome was sequenced using a NovaSeq high-throughput sequencing platform, and the data obtained were processed as shown in Table 2. The results showed that the average number of Raw Reads in each sample was 42,485,470, and the average number of Clean Reads obtained after filtering was 38,466,679. The alignment rate with the total number of sequences in the reference genome (ASBRC_Ckan_1.0) was 87.06%, respectively, and these data show that the high-throughput sequencing platform produced high-quality data for the transcriptome sequencing of stout camphor, which can be analysed in the next step.

Table 2. Statistical Results

Sample	Raw Reads	Clean Reads	Total Mapped	Multiple Mapped	Uniquely Mapped
CMK15	41336864	37481532	32739505 (87.35%)	1634936(4.99%)	31104569 (95.01%)
CMK4	39892944	36289978	31326941(86.32%)	1498405 (4.78%)	29828536 (95.22%)
CMKG	45193744	40915854	35873411 (87.68%)	1778766 (4.96%)	34094645 (95.04%)
CMKZ	43518326	39179350	34009116 (86.80%)	1817853 (5.35%)	32191263 (94.65%)

Annotation of Gene Function

All the Unigenes obtained were functionally annotated in the GO, KEGG, eggNOG, SwissProt, and Nr databases, and the resulting annotation information is shown in Fig. 1 (see Supplementary Table. S1). A total of 27,885 genes were found to be validly functionally annotated. Among them, the GO database annotated 13,121, accounting for 47.1%; the KEGG database annotated 11,165, accounting for 40.0%; the eggNOG database annotated 26,812, accounting for 96.2%; SwissProt annotated 23,012, accounting for 82.6%; and Nr annotated 26,865, accounting for 96.3%. Unigenes were commented on the most frequently in the Nr database.

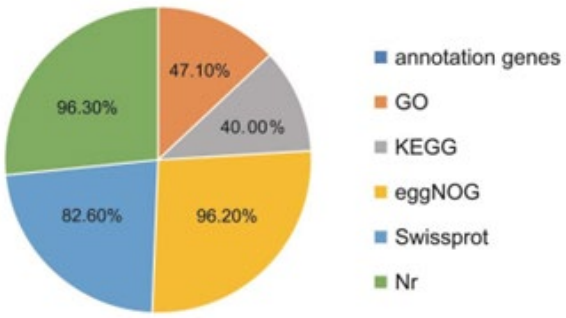


Fig. 1. Gene function annotation

Differently Expressed Genes

The fragments per kilobase of transcript per million mapped reads (FPKM) method was used to calculate the abundance of the screened differential genes, and 8,136 differential genes were identified (Fig. 2). A total of 1,301, 1,370, 1,429, 1,430, 1,398, and 1,207 DEGs were identified in CMKZ_vs_CMK4 (834 upregulated and 468 downregulated), CMK15_vs_CMK4 (1,068 upregulated and 302 downregulated), CMKG_vs_CMKZ (839 upregulated and 590 downregulated), CMKG_vs_CMK4 (902 upregulated and 528 downregulated), CMKZ_vs_CMK15 (484 upregulated and 914 downregulated), and CMKG_vs_CMK15 (538 upregulated and 669 downregulated). The three treatment groups CMKG_vs_CMKZ, CMKG_vs_CMK4, and CMKZ_vs_CMK4 were selected to produce Venn diagrams, which showed that 24 genes were co-expressed in the three treatment groups, including 19 that were upregulated and five that were down regulated (Fig. 3 and Table. S2). This result suggests that co-expressed DEGs may play an important role in the response of *C. kanehirae* to cold and drought stresses.

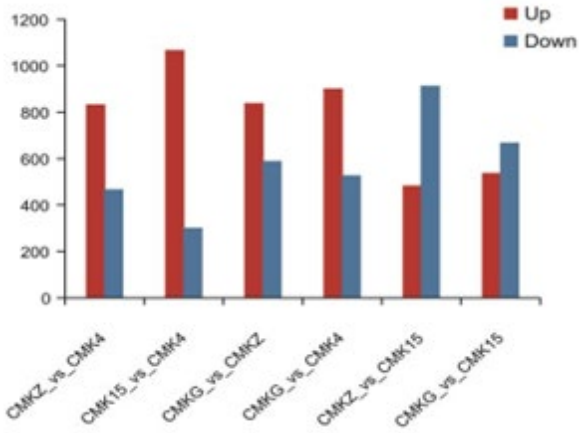


Fig. 2. The number of differentially expressed genes (DEGs).The x-coordinate is the treatment group and the ordinate is the number of genes

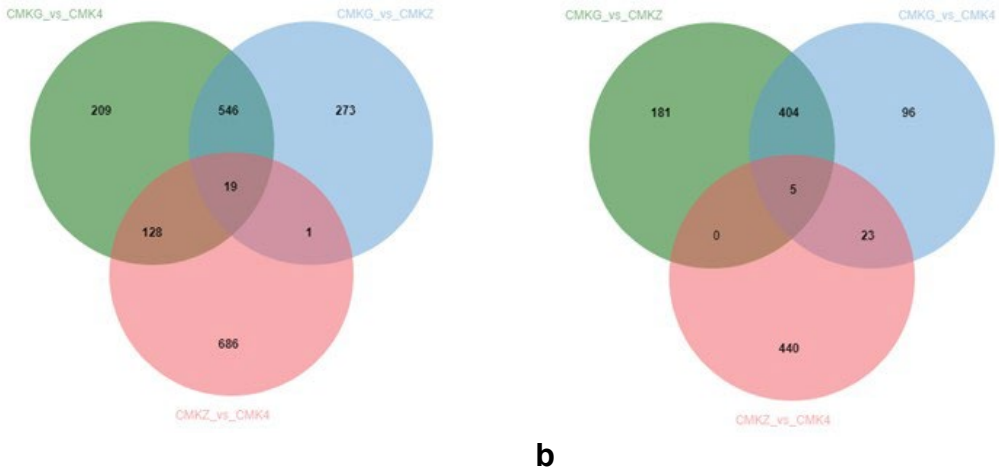


Fig. 3. Venn diagrams showing the number of differentially expressed genes (DEGs). (a)Venn diagrams showing the up-regulated DEGs; (b)Venn diagrams showing the down-regulated DEGs

GO Functional Enrichment Analysis of DEGs

In this study, GO annotation was used to classify the possible functions of single feature genes. A total of 13,121 single genes were successfully assigned to at least one GO term. The GO terms primarily included biological process (BP), cellular component (CC), and molecular function (MF). The functional enrichment of the differential GO genes in the *C. kanehirae* leaves varied under different treatments. The four treatment groups CMKZ *vs*. CMK4, CMKG *vs*. CMKZ, CMKG *vs*. CMK4, and CMKG *vs*. CMK15 DGEs were primarily enriched in cellular components, while the two treatment groups CMKZ *vs*. CMK15 and CMK15 *vs*. CMK4 DEGs were primarily enriched in biological processes (Fig 4). Under drought stress, the top three DEGs were primarily enriched in membrane (228 upregulated and 107 downregulated), followed by photosystem I (14 upregulated) and carbohydrate metabolic process (46 upregulated and 32 downregulated). Under low temperature stress, the top three DEGs were primarily enriched in cell periphery (55 upregulated and 21 downregulated), followed by cell wall (25 upregulated and six downregulated) and external encapsulating structure (25 upregulated and six downregulated) shown in Table. S3. The results suggest that the biological responses of *C. kanehirae* may change differently under drought and low temperature stresses.

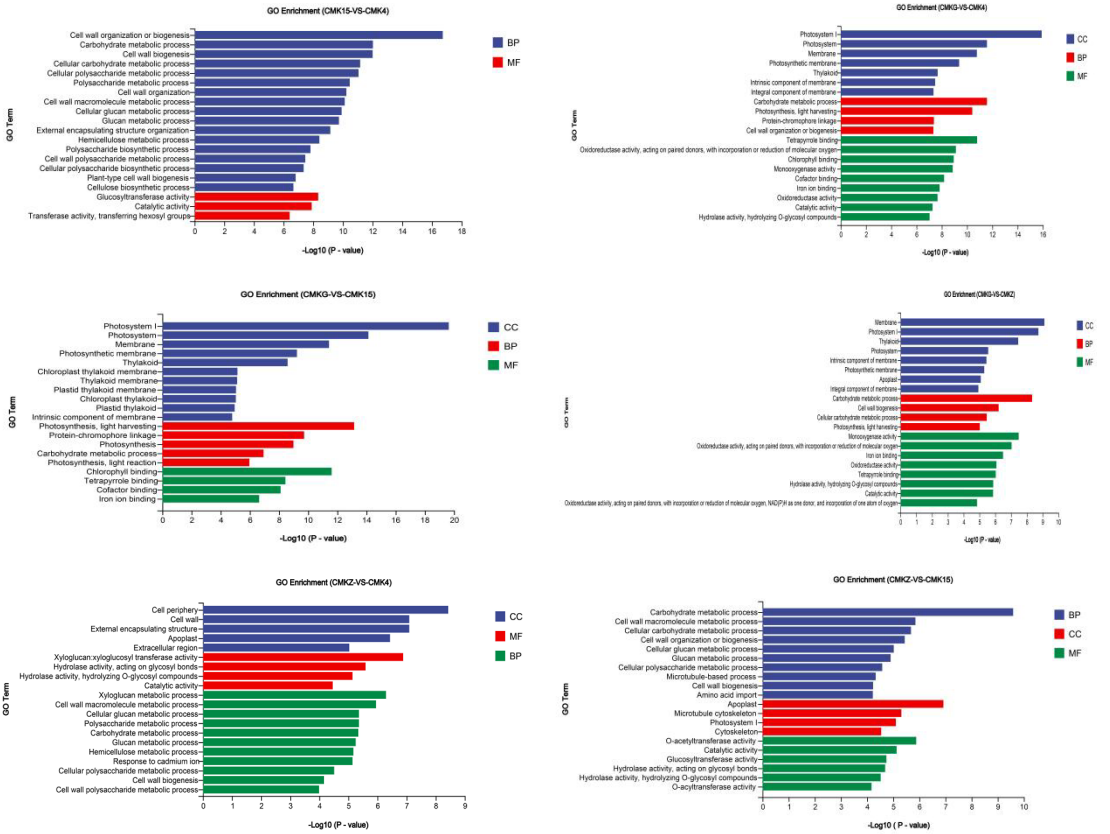


Fig. 4. GO enrichment of *Cinnamomum kanehirae* unigenes. The x-coordinate is GO Term, and the ordinate is -log10 (p-value) enriched by GO Term

Enrichment Analysis of the DEGs by KEGG

To identify several major pathways related to drought and low-temperature stress, an enrichment analysis of the KEGG pathway for all the up- and downregulated DEGs was performed. Drought and low temperature treatment resulted in 1,058 single genes that were

upregulated, and 1,673 single genes that were downregulated. All the DEGs were associated with 94 metabolic pathways. The 20 most significantly enriched pathways are shown in Fig. 5 and Table. S4. Under drought stress, the three most significantly enriched metabolic pathways were starch and sucrose metabolism, photosynthesis, and glycolysis/ gluconeogenesis. Under low temperature stress, the three most significantly enriched metabolic pathways were phenylpropanoid biosynthesis, plant-pathogen interaction, and flavonoid biosynthesis.

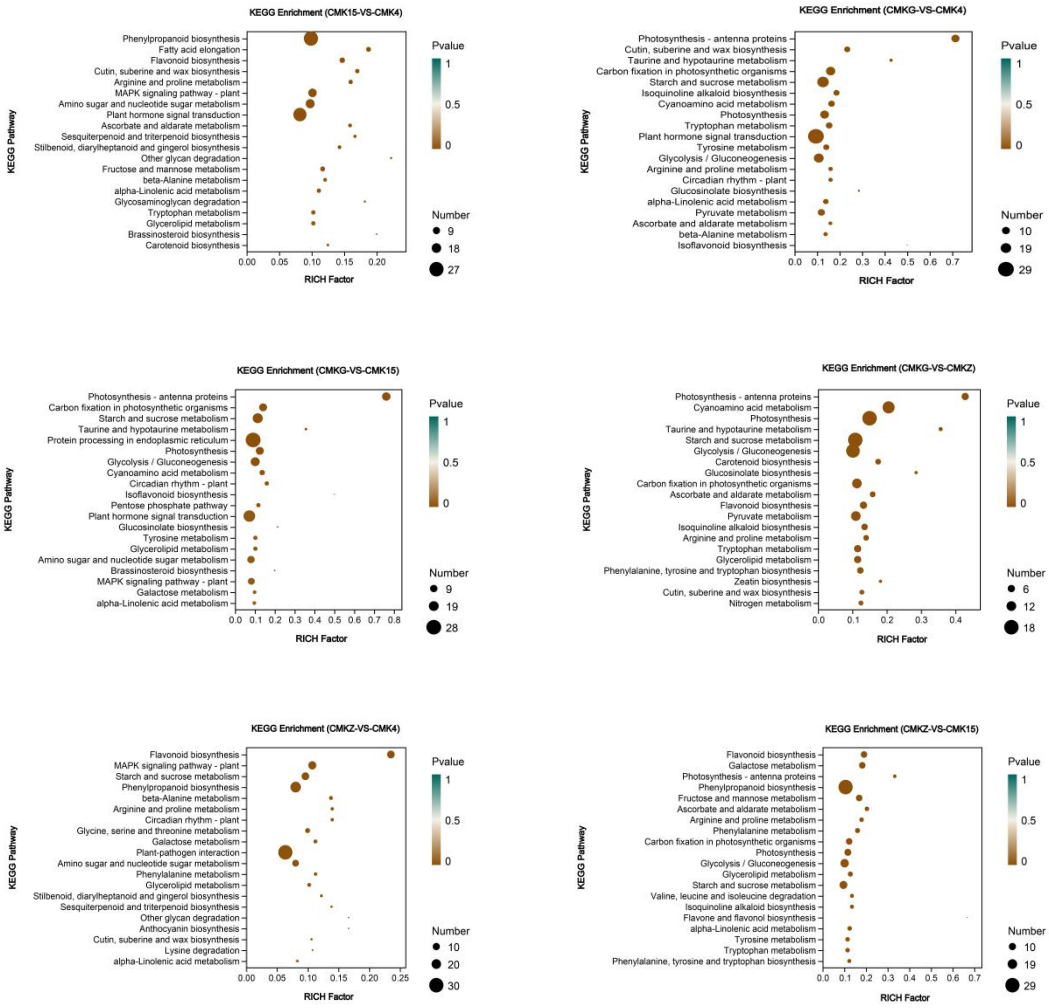


Fig. 5. KEGG enrichment of *Cinnamomum kanehirae* unigenes. The x-coordinate is rich Factor; the ordinate is Pathway; the size of the midpoint in the figure represents the number of differentially annotated genes (up-regulated or down-regulated, which is related to the gene set selected during analysis) in the corresponding Pathway; the depth of the color represents the significance level

Identification of Differentially Expressed Genes

To quantify the amount of gene expression, each single gene was analyzed using FPKM values, and the range of FPKM values for each sample is shown in Fig. 6 and Table. S5. A total of 42 genes were significantly differentially expressed under drought and low temperature stresses. Under drought stress, Chloroplast photosystem II PsbW protein

(*CKAN_00961700*), which encodes chlorophyll a/b binding proteins (*CKAN_02133400*, *CKAN_02608400*, *CKAN_00460200*, and *CKAN_01326200*) and the photosystem II reaction center PSB28 protein (*CKAN_ 02671000*) were all down regulated. The genes that encoded 9-*cis*-epoxycarotenoid dioxygenase (*CKAN_01050200*), cytochrome P450 (*CKAN_02528500*) and four genes that encode β-glucosidase (*CKAN_02421400*, *CKAN_00177300*, and *CKAN_ 00179400*, *CKAN_02600400*) were upregulated. Five genes that encoded the aldehyde dehydrogenase family *CKAN_00414400*, *CKAN_01048900* and *CKAN_00822700* were downregulated under drought stress, and *CKAN_00822700* and *CKAN_02523600* were upregulated under low temperature stress, indicating that the aldehyde dehydrogenase family responds to both drought and low temperature stress. Under low temperature stress, the genes that encoded Ca^{2+} signaling-related genes, such as calcium-dependent protein (*CKAN_01155700*), calcium-binding protein (*CKAN_00501100*, *CKAN_02399000*, *CKAN_00302700*) and arginine metabolism-related genes, including Δ-1-pyrroline-5- carboxylate synthase (*CKAN_01502300*) and arginine decarboxylase (*CKAN_02148700*), were up regulated, indicating that Plant-pathogen interaction and Arginine and proline metabolism play an important role in low temperature stress.

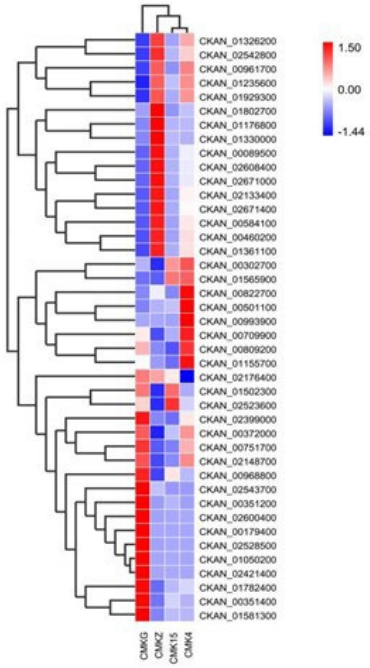
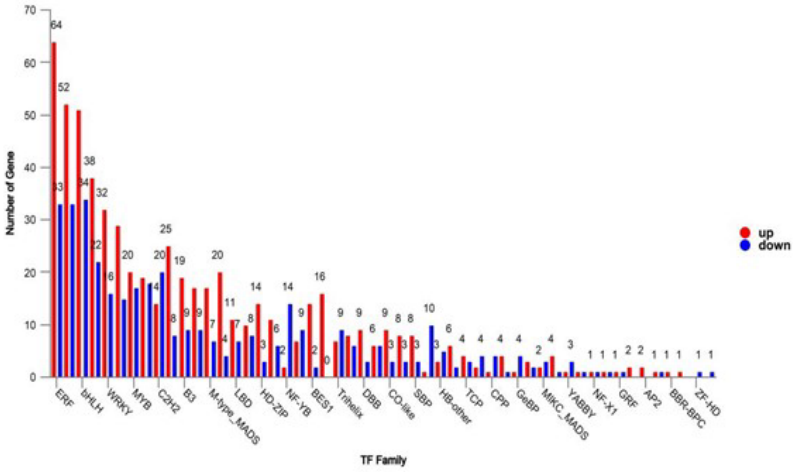


Fig. 6. Heat map of 42 differentially expressed genes. Horizontally represent genes, and each column represents a sample. Red represents high expression genes, blue represents low expression genes

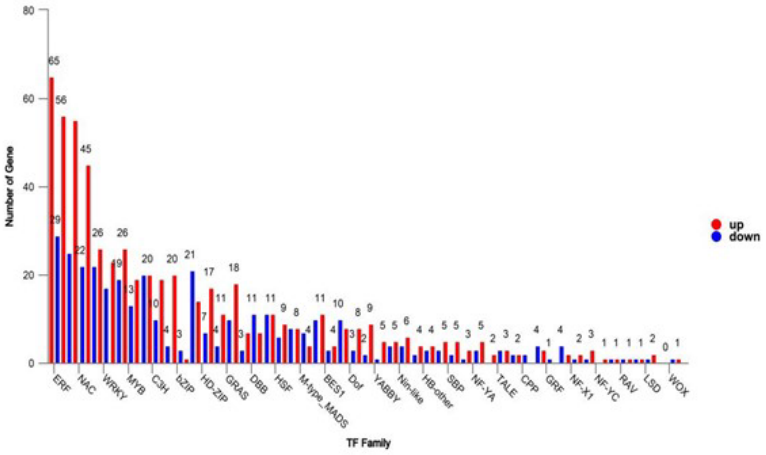
Transcription Factors With High Transcript Levels

A total of 17,082 DEGs were found to be members of 58 TF families. A total of 52 transcription factors (TFs) with high levels of expression were screened under drought and low temperature stress, including NAC, MYB, ERF, bHLH, WRKY and other family TFs (Fig. 7 and Table. S6). Among them, the levels of expression of two genes of the NAC family (*CKAN_01625800* and *CKAN_02660300*), one gene of the bHLH family

(*CKAN_00822700*), and one gene of the ERF family (*CKAN_00641900*) changed similarly under drought and low temperature stresses. Other TFs responded differently under different stress conditions. For example, the level of expression of WRKY (*CKAN_00395900*) was upregulated under drought stress and downregulated under low temperature stress. The level of expression of MYB (*CKAN_02004700*) was downregulated under drought stress and upregulated at low temperature. In this study, the levels of expression of two WRKY33 TFs (*CKAN_00993900* and *CKAN_00751700*) and one uncommon TF MYC (*CKAN_00968800*) that were encoded in the MAPK cascade signaling pathway were upregulated under low temperature stress. These TFs are known to play important roles in plant development and responses to abiotic stress (Yao *et al*. 2019).



a



b

Fig. 7. Analysis of differentially expressed transcription factor genes in Cinnamomum kanehirae under drought and cold stress. (a)CMKG_vs_CMKZ; (b) CMKZvsCMK4.The x-coordinate is different transcription factor family, and the ordinate is the number of different genes falling into this transcription factor family

Validation of Transcriptome Data by qRT-PCR Analyses

Nine candidate genes were selected randomly for qRT-PCR analysis to validate the results of RNA-Seq. They represented various functional classes or pathways, including photosynthesis, sucrose and starch metabolism, hormone signaling, and TFs. Enrichment analysis suggested that these genes were involved in the responses to drought and low temperature stress. The results showed that the patterns of expression of both methods were consistent, confirming the reliability of the transcriptome results (Fig. 8 and Table. S7).

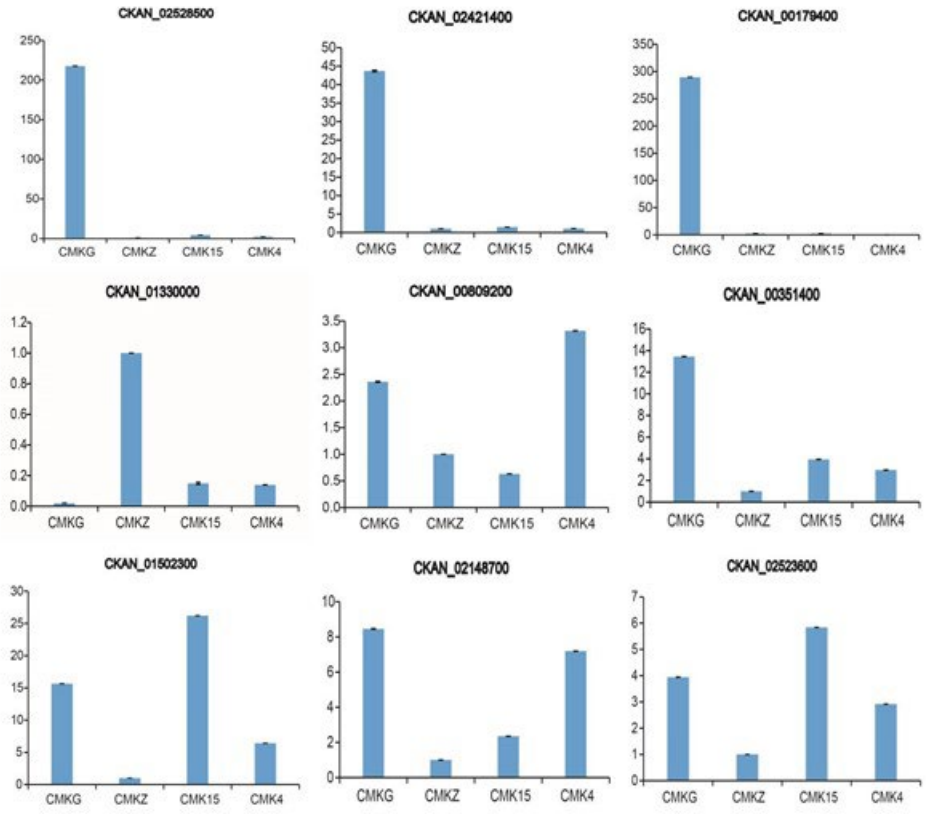


Fig. 8. The expression profiles of nine transcripts in *Cinnamomum kanehirae* by qRT-PCR. The x-coordinate is different treatment group, and the ordinate is DEGs expression changes

Drought is one of the most serious abiotic factors that affects plant growth and development. It inhibits plant growth and yields by interfering with various physiological and biochemical processes, including photosynthesis, chlorophyll synthesis, plant signaling, and carbohydrate metabolism. Plant tissues respond to drought and increase their resistance by altering their external morphological configuration, osmoregulation, metabolic regulation, and production of stress-resistant substances to reduce the damage caused by drought (Anjum *et al*. 2017). In this study, the KEGG pathway enrichment analysis under drought stress showed that the DEGs were significantly enriched in starch and sucrose metabolism, photosynthesis (Dong *et al*. 2014), and glycolysis/ gluconeogenesis (Wang *et al*. 2020), and other pathways were significantly enriched. Photosynthesis, a key process of primary metabolism, has a central role in plant productivity under drought conditions (Pinheiro and Chaves 2011). Its production of carbohydrates, some amino acids, lipids, proteins, and organic acids is directly involved in a variety of biochemical reactions, such as glycolysis, amino acid metabolism and lipid

metabolism, thus, making photosynthesis the most fundamental material and energy metabolic pathway in biology (Feng *et al*. 2022). Previous studies found that the relative level of expression of the chlorophyll a/b binding proteins, PetE, PetF, PsbP, PsbQ, PsbW, and Psb28 were progressively downregulated in tomato (*Solanum lycopersicum*) under water stress, and this downregulation was suppressed by the addition of exogenous silicon (Zhang *et al*. 2018). In this study, 20 DEGs related to the photosynthetic pathway were identified under drought stress. The chlorophyll a/b binding protein, psbQ, psbB, photosystem I reaction center subunit and photosystem II related PsbW and PSB28 were significantly differentially expressed, and the chlorophyll a/b binding protein was downregulated under drought stress. The results of both studies were basically consistent.

Plant hormones play an important role in the plant response and adaptation to abiotic stresses, and ABA is an important stress hormone that is key to plant stress responses (Zhang *et al*. 2021). The key step in its synthesis is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED3) (Khan *et al*. 2019). The homologous genes of NCED1 were found to be upregulated under drought stress in maize (*Zea mays*) (Lu *et al*. 2017). In this study, the expression of a gene that encodes a 9-*cis*-epoxycarotenoid dioxygenase was upregulated in expression under drought stress, which is consistent with the findings of Lu *et al*. (2017). This suggests that there is a conserved regulation of the drought-induced response to *C. kanehirae*. Nambara *et al*. (2005) showed that there are at least two pathways for the catabolism of ABA. One is the oxidative pathway, which is mediated by a class of cytochrome P450 monooxygenases. The other is the glycol-coupled pathway, in which ABA is inactivated in *Arabidopsis* mutants in the form of ABA glycosyl esters that are stored in the vesicles or in an ectoplasmic pool. When the plants are exposed to drought conditions, the β-glucosidase AtBG1 in *Arabidopsis* releases ABA from the glucosyl ester form, which also plays an important regulatory role in the drought response of plants (Seki *et al*. 2007). β-glucosidases have key roles in defense against drought stress and in scavenging reactive oxygen species (ROS), and they are upregulated at higher levels in drought-tolerant plants than in those that are sensitive to drought (Kadam *et al*. 2012). Several studies have shown that the induction of β-amylase can help the plants to adapt to stress conditions by hydrolyzing more starch into maltose, which acts as a precursor for soluble sugar metabolism (Prasch *et al*. 2015). For example, the levels of expression of 12 genes annotated as β-glucosidases were upregulated in barley (*Hordeum vulgare*) under drought stress (Hong *et al*. 2020). In this study, the levels of expression of all four DEGs that encoded β-glucosidases were upregulated under drought stress, and the findings of both studies were largely consistent.

Temperature is a key factor in the regulation of plant growth and development, and under natural conditions, plants are more sensitive to temperature changes and can regulate more DEGs in response to adversity stresses when there is a significant change in temperature. Low temperature is one of the major environmental stresses that afflicts plants during growth (Tripathy *et al*. 2014). In this study, the KEGG pathway enrichment analysis of *C. kanehirae* under low temperature stress showed that the DEGs were significantly enriched in phenylpropanoid biosynthesis, plant-pathogen interaction (Gao *et al*. 2021), and flavonoid biosynthesis (Romero *et al*. 2020) pathways. Signal transduction pathways play a key role in the response to low temperature stress (Janska *et al*. 2010), and Ca^{2+} plays an important role in the response of plant to cold stress as the most important second messenger of signal transduction. Plants sense changes in the cytoplasmic membrane through low temperature signaling sensors. For example, rice (*Oryza sativa*) senses cold stimuli through cold tolerance difference 1 (COLD1) (Ma *et al*. 2015). Many Ca^{2+} signaling

pathways are triggered under cold stress, such as Ca^{2+}-dependent protein kinases (CDPKs), mitogen-activated protein kinases (MAPKs), calcium regulated calcineurin b-like protein (CBL), and calmodulin (Wang *et al*. 2013). Yang *et al*. (2010) identified a novel calcium/calmodulin receptor-like kinase (CRLK1) that is essential for plant cold tolerance. The CDPK family is a large gene family whose members have different functions and are involved in a variety of different signaling pathways (Romeis *et al*. 2001). Under low temperature stress, Song *et al*. (2013) found that the level of expression of CDPK4 was significantly upregulated in *Populus simonii*. In this study, the level of expression of CDPK8 was significantly upregulated under low-temperature stress, and both studies suggest that CDPK could be an important signaling component in the responses of plants to the stress of low temperatures.

The mitogen-activated protein kinase cascade reaction is an evolutionarily conserved signal transduction module that regulates extracellular signal transduction to the nucleus. The cascade consists of three main components, MAPK kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK interconnected by phosphorylation events. These kinases transmit messaging to the sensor and play a role in intracellular and extracellular signaling in plants (Sinha *et al*. 2011). In *Arabidopsis*, the MAPKKK protein AtANP1 initiates a phosphorylation cascade with AtMPK3 in response to cold stress, thereby regulating freezing tolerance in Arabidopsis (Wei *et al*. 2021). Under cold stress, the levels of expression of the genes that encode MAPK have been found to be upregulated in multiflora rose (*Rosa multiflora*) (Zhang *et al*. 2016). In this study, the level of expression of a gene that encodes MAPK was upregulated under low temperature stress, suggesting that the MAPK cascade response could be involved in the response of *C. kanehirae* to cold. Proline is an imidic acid that has an important osmoprotective effect during low temperature stress in plants by significantly increasing its content in tissues when plants are subjected to low temperature stress (Liang *et al*. 2013). In this regard, Kong *et al*. (2019) conducted a study of the differential expression of genes related to proline metabolism under low temperature stress in pepper (*Capsicum annuum*) and found that the levels of expression of the genes that encoded aldehyde dehydrogenase (ALDH), polyamine oxidase (PAO), ornithine decarboxylase, proline 4-hydroxylase (P4HA), and arginine decarboxylase-related genes were upregulated. In contrast, the levels of expression of the genes that encoded 1-pyrroline-5-carboxylate dehydrogenase (P5CDH) were downregulated (Kong *et al*. 2019). In this study, the level of expression of related genes that encode polyamine oxidase, the aldehyde dehydrogenase family, glutamic decarboxylase, arginine decarboxylase and Δ-1-pyrroline-5-carboxylate synthase were upregulated. In a study by Wang *et al*. (2014) on *Lilium lancifolium*, two genes that encoded Δ-1-pyrroline-5-carboxylate synthase were significantly upregulated by 0.88- and 1.29-fold, respectively, after cold stress, indicating that different plants vary in their mechanisms of response to the genes related to proline metabolism under cold stress. This also indicates that proline plays an important role in osmoprotection under low temperature stress.

TFs are important components of abiotic stress signal transduction pathways and play important regulatory roles in environmental stress-induced gene regulatory networks (Gao *et al*. 2015). This study identified important TFs known to be involved in plant stress resistance, including NAC, ERF, bHLH, WRKY, and MYB. Two genes encoded by NAC and bHLH and one gene encoded by ERF showed similar changes in their levels of expression under drought and low temperature stresses. Other TFs responded differently under different stress conditions. For example, one gene encoded by WRKY was

upregulated under drought stress and downregulated under low temperature stress, while one gene encoded by MYB was downregulated under drought stress and upregulated at low temperature. WRKY is one of the largest families of TFs in plants that not only control plant growth and development but also play important roles in various stress responses. Studies in grape (*Vitis vinifera*) have shown that low temperature stress altered the levels of expression levels of WRKY members, and some of them are involved in ABA-dependent signaling pathways in response to cold stress (Wang *et al*. 2014). In this study, two WRKY33 TFs and an uncommon MYC TF that were encoded in the MAPK cascade signaling pathway were upregulated under low temperature stress. Previous studies have found that the expression of WRKY33 in the MYC2 and MAPK signaling pathways of Chinese Yew (*Taxus chinensis*) is upregulated under low temperature stress (Meng *et al*. 2017). The results of both are largely consistent.

CONCLUSIONS

1. In this study, the transcriptome of *C. kanehirae* samples under drought and low temperature stress was sequenced using an Illumina NovaSeq platform. A comprehensive reference transcriptome database was obtained by deep sequencing and assembly.
2. A sequencing and bioinformatics analysis of the DEGs identified transcripts that were produced in response to drought and low temperature stress, including common up- and downregulated genes and TFs.
3. This study revealed several features of the metabolic regulatory strategy of *C. kanehirae* in response to drought and low temperature stress. These findings will help to identify key genes that play a central role in the abiotic stress response of *C. kanehirae* and provide a theoretical basis for molecular breeding research to improve the resistance of *C. kanehirae* to these stresses.

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APPENDIX

Supplementary Files

Table S1. Statistics of Gene Function Annotation

annotation genes	GO	KEGG	eggNOG	Swissprot	Nr
27885	13121	11165	26812	23027	26865

Table S2. The Number of Differentially Expressed Genes (DEGs)

Control_vs_Treat	Up	Down	Total
CMKZ_vs_CMK4	834	468	1302
CMK15_vs_CMK4	1068	302	1370
CMKG_vs_CMKZ	839	590	1429
CMKG_vs_CMK4	902	528	1430
CMKZ_vs_CMK15	484	914	1398
CMKG_vs_CMK15	538	669	1207

Table S3. GO Enrichment of Unigenes

PG	GO.ID	Category	Term	Up	Down	DEG
CMKG-vs-CMKZ	GO:0016020	CC	membrane	228	107	335
	GO:0009522	CC	photosystem I	14	0	14
	GO:0005975	BP	carbohydrate metabolic process	46	32	78
	GO:0004497	MF	monooxygenase activity	22	18	40
	GO:0009579	CC	thylakoid	36	2	38
	GO:0016705	MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	23	22	45
	GO:0005506	MF	iron ion binding	16	22	38
	GO:0042546	BP	cell wall biogenesis	14	2	16
	GO:0016491	MF	oxidoreductase activity	76	60	136
	GO:0046906	MF	tetrapyrrole binding	27	17	44
	GO:0004553	MF	hydrolase activity, hydrolyzing O-glycosyl compounds	20	20	40
	GO:0003824	MF	catalytic activity	280	186	466
	GO:0009521	CC	photosystem	17	0	17
	GO:0044262	BP	cellular carbohydrate metabolic process	23	9	32
	GO:0031224	CC	intrinsic component of membrane	142	72	214
	GO:0034357	CC	photosynthetic membrane	26	2	28
	GO:0048046	CC	apoplast	12	3	15
	GO:0009765	BP	photosynthesis, light harvesting	9	0	9
	GO:0016021	CC	integral component of membrane	140	69	209
	GO:0016709	MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen	14	6	20
CMKZ-	GO:0071944	CC	cell periphery	55	21	76
	GO:0005618	CC	cell wall	25	6	31

vs-CMK 4	GO:0030312	CC	external encapsulating structure	25	6	31
	GO:0016762	MF	xyloglucan:xyloglucosyl transferase activity	8	2	10
	GO:0048046	CC	apoplast	13	2	15
	GO:0010411	BP	xyloglucan metabolic process	8	2	10
	GO:0044036	BP	cell wall macromolecule metabolic process	13	3	16
	GO:0016798	MF	hydrolase activity, acting on glycosyl bonds	26	15	41
	GO:0006073	BP	cellular glucan metabolic process	15	5	20
	GO:0005976	BP	polysaccharide metabolic process	21	7	28
	GO:0005975	BP	carbohydrate metabolic process	43	18	61
	GO:0044042	BP	glucan metabolic process	15	5	20
	GO:0010410	BP	hemicellulose metabolic process	9	2	11
	GO:0004553	MF	hydrolase activity, hydrolyzing O-glycosyl compounds	24	10	34
	GO:0046686	BP	response to cadmium ion	4	0	4
	GO:0005576	CC	extracellular region	27	6	33
	GO:0044264	BP	cellular polysaccharide metabolic process	16	5	21
	GO:0003824	MF	catalytic activity	236	153	389
	GO:0042546	BP	cell wall biogenesis	10	2	12
	GO:0010383	BP	cell wall polysaccharide metabolic process	9	2	11

Table S4. KEGG Enrichment of Unigenes

PG	PathwayID	Pathway	level1	level2	Up	Down	DEG
CMK G-vs-CMK Z	ko00196	Photosynthesis - antenna proteins	Metabolism	Energy metabolism	9	0	9
	ko00460	Cyanoamino acid metabolism	Metabolism	Metabolism of other amino acids	6	9	15
	ko00195	Photosynthesis	Metabolism	Energy metabolism	18	0	18
	ko00430	Taurine and hypotaurine metabolism	Metabolism	Metabolism of other amino acids	1	4	5
	ko00500	Starch and sucrose metabolism	Metabolism	Carbohydrate metabolism	8	10	18
	ko00010	Glycolysis / Gluconeogenesis	Metabolism	Carbohydrate metabolism	7	10	17
	ko00906	Carotenoid biosynthesis	Metabolism	Metabolism of terpenoids and polyketides	4	3	7
	ko00966	Glucosinolate biosynthesis	Metabolism	Biosynthesis of other secondary metabolites	0	4	4
	ko00710	Carbon fixation in photosynthetic organisms	Metabolism	Energy metabolism	9	3	12
	ko00053	Ascorbate and aldarate metabolism	Metabolism	Carbohydrate metabolism	5	2	7
	ko00941	Flavonoid biosynthesis	Metabolism	Biosynthesis of other secondary metabolites	7	2	9
	ko00620	Pyruvate metabolism	Metabolism	Carbohydrate metabolism	7	5	12
	ko00950	Isoquinoline alkaloid biosynthesis	Metabolism	Biosynthesis of other secondary metabolites	2	6	8
	ko00330	Arginine and proline metabolism	Metabolism	Amino acid metabolism	3	4	7
	ko00380	Tryptophan metabolism	Metabolism	Amino acid metabolism	5	4	9
	ko00561	Glycerolipid metabolism	Metabolism	Lipid metabolism	6	3	9
	ko00400	Phenylalanine, tyrosine and tryptophan biosynthesis	Metabolism	Amino acid metabolism	2	6	8
	ko00908	Zeatin biosynthesis	Metabolism	Metabolism of terpenoids and polyketides	2	2	4
	ko00073	Cutin, suberine and wax biosynthesis	Metabolism	Lipid metabolism	4	2	6
	ko00910	Nitrogen metabolism	Metabolism	Energy metabolism	4	2	6
CMK Z-vs-CMK 4	ko00941	Flavonoid biosynthesis	Metabolism	Biosynthesis of other secondary metabolites	3	13	16
	ko04016	MAPK signaling pathway - plant	Environmental	Signal transduction	12	5	17

			Information Processing				
	ko00500	Starch and sucrose metabolism	Metabolism	Carbohydrate metabolism	7	9	16
	ko00940	Phenylpropanoid biosynthesis	Metabolism	Biosynthesis of other secondary metabolites	10	12	22
	ko00410	beta-Alanine metabolism	Metabolism	Metabolism of other amino acids	6	2	8
	ko00330	Arginine and proline metabolism	Metabolism	Amino acid metabolism	6	1	7
	ko04712	Circadian rhythm - plant	Organismal Systems	Environmental adaptation	3	4	7
	ko00260	Glycine, serine and threonine metabolism	Metabolism	Amino acid metabolism	7	3	10
	ko00052	Galactose metabolism	Metabolism	Carbohydrate metabolism	6	2	8
	ko04626	Plant-pathogen interaction	Organismal Systems	Environmental adaptation	21	9	30
	ko00520	Amino sugar and nucleotide sugar metabolism	Metabolism	Carbohydrate metabolism	12	2	14
	ko00360	Phenylalanine metabolism	Metabolism	Amino acid metabolism	2	5	7
	ko00561	Glycerolipid metabolism	Metabolism	Lipid metabolism	7	1	8
	ko00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	Metabolism	Biosynthesis of other secondary metabolites	2	4	6
	ko00909	Sesquiterpenoid and triterpenoid biosynthesis	Metabolism	Metabolism of terpenoids and polyketides	5	0	5
	ko00511	Other glycan degradation	Metabolism	Glycan biosynthesis and metabolism	3	0	3
	ko00942	Anthocyanin biosynthesis	Metabolism	Biosynthesis of other secondary metabolites	0	3	3
	ko00073	Cutin, suberine and wax biosynthesis	Metabolism	Lipid metabolism	5	0	5
	ko00310	Lysine degradation	Metabolism	Amino acid metabolism	3	1	4
	ko00592	alpha-Linolenic acid metabolism	Metabolism	Lipid metabolism	3	3	6

Table S5. Cross-expression of 42 genes

Gene_ID	CMKG	CMKZ	CMK15	CMK4
CKAN_02133400	2.93	226.00	43.67	100.19
CKAN_02608400	4.87	937.61	183.71	310.61
CKAN_00460200	3.19	1362.75	279.53	706.23
CKAN_01326200	7.52	812.83	220.78	583.68
CKAN_01330000	1.01	53.40	8.23	7.31
CKAN_00961700	3.25	855.41	193.57	758.22
CKAN_02542800	6.93	350.70	108.80	211.40
CKAN_01361100	4.39	1713.64	416.98	879.75
CKAN_02671000	2.66	112.15	22.66	38.76
CKAN_00584100	15.62	976.52	198.83	470.75
CKAN_01929300	10.62	1329.57	435.08	1100.83
CKAN_00089500	12.12	980.52	201.44	348.17
CKAN_02671400	12.59	763.61	161.93	324.46
CKAN_01235600	21.43	1838.58	760.45	1609.48
CKAN_00179400	322.43	0.11	0.12	0.00
CKAN_02600400	19.03	0.11	0.42	0.44
CKAN_02421400	57.06	1.30	1.88	1.37
CKAN_00351200	235.57	10.99	25.83	12.39
CKAN_01050200	3.66	0.00	0.05	0.00
CKAN_02528500	207.98	0.45	4.19	2.10
CKAN_00993900	44.09	44.02	39.43	470.46
CKAN_01581300	178.84	7.10	32.83	30.42
CKAN_00751700	593.42	77.39	127.83	413.78
CKAN_00968800	125.52	16.68	72.64	47.85
CKAN_00372000	90.86	31.22	55.69	81.85
CKAN_01565900	312.01	230.40	1548.86	1649.39
CKAN_00809200	107.85	45.65	28.57	151.34
CKAN_00709900	54.45	13.64	33.67	98.17
CKAN_01782400	313.23	29.98	85.07	104.73
CKAN_00822700	0.00	6.22	0.00	22.89
CKAN_00351400	506.95	37.67	148.95	111.56
CKAN_02523600	296.80	75.46	440.67	220.06
CKAN_01502300	288.57	18.46	299.22	118.28
CKAN_02148700	702.21	83.27	195.99	599.21
CKAN_00501100	1.81	16.65	21.12	117.89
CKAN_01155700	63.92	30.63	15.13	151.41
CKAN_02399000	125.89	17.36	9.72	60.03
CKAN_00302700	85.91	50.65	133.30	151.21
CKAN_01176800	5.96	153.10	23.69	25.82
CKAN_01802700	31.04	378.74	21.47	63.49
CKAN_02543700	578.46	83.21	22.69	24.19
CKAN_02176400	570.08	527.82	452.96	174.05

Table S6. Analysis of Differentially Expressed Transcription Factor Genes in *Cinnamomum kanehirae* under Drought and Cold Stress

PG	family	Up	Down	Total
CMKG-vs-CMKZ	ERF	64	33	97
	NAC	52	33	85
	bHLH	51	34	85
	MYB_related	38	22	60
	WRKY	32	16	48
	FAR1	29	15	44
	MYB	20	17	37
	G2-like	19	18	37
	C2H2	14	20	34
	C3H	25	8	33
	B3	19	9	28
	GRAS	17	9	26
	M-type_MADS	17	7	24
	ARF	20	4	24
	LBD	11	7	18
	HSF	10	8	18
	HD-ZIP	14	3	17
	bZIP	11	6	17
	NF-YB	2	14	16
	Nin-like	7	9	16
	BES1	14	2	16
	ARR-B	16	0	16
	Trihelix	7	9	16
	STAT	8	6	14
	DBB	9	3	12
	GATA	6	6	12
	CO-like	9	3	12
	E2F/DP	8	3	11
	SBP	8	3	11
	NF-YC	1	10	11
	HB-other	3	5	8
	NF-YA	6	2	8
	TCP	4	3	7
	Dof	2	4	6
	CPP	1	4	5
	CAMTA	4	1	5
	GeBP	1	4	5
	S1Fa-like	3	2	5
	MIKC_MADS	2	3	5
	TALE	4	1	5

	YABBY	1	3	4
	EIL	1	1	2
	NF-X1	1	1	2
	SRS	1	1	2
	GRF	1	1	2
	LSD	2	0	2
	AP2	2	0	2
	HB-PHD	1	1	2
	BBR-BPC	1	0	1
	Whirly	1	0	1
	ZF-HD	0	1	1
	HRT-like	0	1	1
CMKZ-vs-CMK4	ERF	65	29	94
	bHLH	56	25	81
	NAC	55	22	77
	MYB_related	45	22	67
	WRKY	26	17	43
	C2H2	23	19	42
	MYB	26	13	39
	FAR1	19	20	39
	C3H	20	10	30
	Trihelix	19	4	23
	bZIP	20	3	23
	ARR-B	1	21	22
	HD-ZIP	14	7	21
	ARF	17	4	21
	GRAS	11	10	21
	G2-like	18	3	21
	DBB	7	11	18
	B3	7	11	18
	HSF	11	6	17
	GATA	9	8	17
	M-type_MADS	8	7	15
	CO-like	4	10	14
	BES1	11	3	14
	STAT	4	10	14
	Dof	8	3	11
	LBD	8	2	10
	YABBY	9	1	10
	GeBP	5	4	9
	Nin-like	5	4	9
	NF-YB	6	2	8

	HB-other	4	3	7
	S1Fa-like	4	3	7
	SBP	5	2	7
	MIKC_MADS	5	1	6
	NF-YA	3	3	6
	HB-PHD	5	0	5
	TALE	2	3	5
	BBR-BPC	3	2	5
	CPP	2	2	4
	E2F/DP	0	4	4
	GRF	3	1	4
	CAMTA	0	4	4
	NF-X1	2	1	3
	ZF-HD	2	1	3
	NF-YC	3	0	3
	HRT-like	1	1	2
	RAV	1	1	2
	TCP	1	1	2
	LSD	1	1	2
	AP2	2	0	2
	WOX	0	1	1
	VOZ	1	0	1

Table S7. The Expression Profiles of Nine Transcripts by qRT-PCR

Gene-ID	CMKG	CMKZ	CMK15	CMK4
CKAN_02528500	217.7247	1.0000	4.3793	2.1893
CKAN_02421400	43.6847	1.0000	1.4493	1.0527
CKAN_00179400	289.4653	1.0000	1.0110	0.0000
CKAN_01330000	0.0193	1.0000	0.1493	0.1390
CKAN_00809200	2.3597	1.0000	0.6293	3.3160
CKAN_00351400	13.4457	1.0000	3.9450	2.9640
CKAN_01502300	15.6467	1.0000	26.2137	6.4123
CKAN_02148700	8.4567	1.0000	2.3507	7.1947
CKAN_02523600	3.9440	1.0000	5.8397	2.9183