

Identification and expression analysis of Jr4CLs gene family based on transcriptome and physiological data in Walnut (*Juglans regia*)

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Research Article

Keywords: Walnut, Shoot shriveling, Transcriptomics, Lignin metabolism, Jr4CL44, Drought

Posted Date: January 29th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-3871772/v1>

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Abstract

Shoot shriveling severely threat growth and development of deciduous trees in northern hemisphere, its essence is imbalance of water absorption and evaporation in the branches. In this study, the physiological characteristics of 'Xiangling' and 'Liaohe (Liaoning No. 4)' during the overwintering process were studied, and key overwintering periods were selected for transcriptome analysis. The results showed that plant hormone metabolism, wax metabolism and lignin metabolism were significantly enriched during the overwintering process. Combined with *Jr4CL* family analysis, the high-expression gene (*Jr4CL44*) under drought stress was selected by real-time fluorescence quantitative screening for functional verification. Overexpression of *Jr4CL44* can effectively remove the reactive oxygen species produced by drought stress, increase lignin content and up-regulate the expression of related genes to improve the drought resistance of *Arabidopsis thaliana*. These results indicate that *Jr4CL44* plays an important role in plant resistance to drought stress, which laies a foundation for further study on the functions in practice.

Introduction

Walnut (*Juglans regia*) not only contains rich nutritional value and economic value of cultivation (Waqar et al. 2020), but also with the implementation of the project of returning farmland to forest, the planting areas of walnut have increased significantly, which have been become an important pillar industry for farmers in mountainous areas to get rid of poverty (Almario et al. 2001). Shoot shriveling caused by strong evaporation in early spring seriously threat fruit production in the dry and cold areas of northern China, it was found that early spring fruit trees such as *Malus domestica* (Erturk et al. 2020), *Pyrus* species (Yin et al. 2002) and *Prunus persica* (L.) Batsc (Lin et al. 2004) were all affected by shoot shriveling in wintering, and gradually intensified with global climate change. Therefore, shooting shriveling has become an important factor limiting the safe winter and large-scale development of walnuts.

Most researchers believes that the essence of the shoot shriveling is the physiological drought of the tree caused by the weak overwintering ability of the branches and the water consumption of the branches greater than the water absorption of the roots (Adrian et al. 2009). Studies had shown that soluble sugar, as a low-molecular-weight compound, played an important role in maintaining the stability of cell membranes and improving the cold resistance of plants (Kwon et al. 2022). In higher plants, the enzymes closely related to sucrose metabolism mainly include sucrose phosphate synthase (Wang et al. 2022), sucrose synthase (Banzouzi et al. 2022) and invertase (He et al. 2022) play an important role in the transportation, metabolism and accumulation of sugar. Plant hormones play a crucial part in abiotic stress they can improve the adaptability of plants to the external environment by regulating endogenous hormones (Waadt et al. 2022). As an important storage material in plants, the content of soluble starch was closely related to the cold resistance of plants under different low temperature stress (Yang et al. 2013). And the content of soluble starch will decrease when plants were subjected to salt stress, it is because the low temperature enhanced the activity of starch hydrolyzing enzymes, accelerating the decomposition of starch (Boriboonkaset et al. 2013).

Lignin is a complex macromolecular organic compound formed from p-coumarol, sinapol and coniferol (Dong et al. 2022), it is one of the components of the secondary wall, which provides a guarantee for the plant to resist the invasion of various adverse environments and transport the water necessary for growth and development (Qu et al. 2023). Recent studies had shown that drought stress can increase the expression of HCT, CCoAOMT and POD in phenylpropane metabolism pathway, thus increasing the lignin content (Gu et al. 2020). AtCCoAOMT1 gene positively responded to salt stress and its expression level was significantly up-regulated, and CCoAOMT1 can also respond to drought stress through ROS and ABA signals (Chun et al. 2021). Although lignin plays an essential role in plant growth and development, only a few cells accumulate large amounts of lignin under normal growth mechanisms (Chen et al. 2022). Therefore, the study of lignin biosynthesis mechanism and the regulation of lignin from molecular level have been a hot issue at home and abroad, which have great practical significance and industrial application prospect in actual production.

In recent years, transcriptome analysis has become an effective method to generate a large number of gene sequences and construct expression profiles, and plays an important role in the mining of plant resistance genes and stress response mechanisms. At present, the researches on shoot shriveling were only limited to the physiological basis, and the mechanism had not been systematically explored. Therefore, in this study, we conducted transcriptomic analysis of two kinds of walnut with different overwintering performance, the key gene 4CL of lignin metabolism was screened and the whole genome bioinformatics analysis was performed. The differentially expressed gene (*Jr4CL44*) was screened by real-time fluorescence quantitative analysis, and the expression of *Jr4CL44* under drought stress was identified by bioinformatics analysis and genetic transformation, which will provide a new way to analyze the regulation mechanism of walnut responded to drought.

Materials and Methods

Material handling and index determination

The annual walnut branches of 'Xiangling' and 'Liaohe (Liaoning No. 4)' from Bahutai Rongsheng Farm, Xinzhuang Town, Honggu District, Lanzhou City were used as experimental materials. The annual branches separately in November 25, 2020 (4 °C / -4 °C), December 24, 2020 (4 °C / -10 °C), January 25, 2021 (5 °C / -9 °C), March 25 (15°C/-1°C) and March 25, 2021 (18°C/5°C) were collected, which were named as initial dormancy, middle dormancy, deep dormancy, freeze-thaw alternate and budding period according to their characterist.

The content of soluble sugar was determined by anthrone colorimetric method (Depuydt et al. 2011). The content of soluble starch was determined by iodine color development (Shibasaki et al. 2009). The sucrose phosphate synthase (SPS) activity was measured by high performance liquid chromatography (Irving et al. 1968). The plant sucrose invertase (Inv) activity was measured by spectrophotometry (Jeon et al. 2010). Amylase (Amy) activity was determined by iodine-starch colorimetry (Xing et al. 2015). Coomassie brilliant blue staining (G-250) method was used to determine SP (soluble protein) content (Deborah et al. 2015).

Transcriptome assay

According to the previous research on physiological and biochemical indicators, three periods with significant differences were screened out (initial dormancy, deep dormancy and freeze-thaw alternate phases). The branches of three periods were delivered to Shanghai Ouyi Biomedical Technology Co., Ltd. for transcriptome analysis. To ensure data reliability, three replicates of each sample were sequenced.

The total RNA of each sample was extracted by Trizol method, and 1% agarose gel electrophoresis was used for preliminary analysis of RNA degradation. Nanodrop and Agilent 2100 were used to test the concentration and integrity of RNA. After passing the quality inspection, a cDNA library was constructed and sequenced using Illumina HiSeq 2000. The platform performed the high-throughput sequencing work of this experiment. Clean reads were obtained after removing the reads with adapters and low quality from the raw transcriptome data. Clean reads were assembled and assembled using Trinity software to obtain transcripts (Transcripts) and genes (Unigenes) of different sizes. FPKM values were used to estimate gene expression levels, and Cufflinks software was used to analyze the FPKM values obtained by sequencing. The DEGs were compared with databases such as PFAM (Protein family), KEGG (Kyoto Encyclopedia of Genes and Genomes), and GO (Gene Ontology) to obtain functional annotation information of DEGs, and GO and KEGG enrichment analysis was performed on DEGs.

Identification and bioanalysis of Jr4CLs in Walnut

Jr4CL family genes protein sequences were obtained from walnut genomic data (<https://iris.angers.inra.fr/gddh13/>). The hidden Markov model (PF01151) was downloaded from the website Pfam (<http://pfam.xfam.org/>), and the candidate gene family obtained by preliminary screening was verified by the online software SMART (<https://smart.embl-heidelberg.de/>). ExPASy Proteomics Server (<http://web.expasy.org/protparam>) was used to analyze the molecular weight, length, isoelectric point and hydrophilicity of *Jr4CLs* amino acid sequence. The online software MEME (<http://meme-suite.org/tools/meme>) was used to predict the conserved motifs of the *Jr4CLs*, and the maximum number of conserved sequences was set to 10. The gene structure and conserved motif of *Jr4CLs* in walnut were mapped by TBtools. The phylogenetic tree of the family members was analyzed using MEGA 7.0 software. Chromosome mapping of *Jr4CLs* in walnut was performed using MapChart2.2 software and TBtools. The cis-acting elements of 2000 bp upstream of the *Jr4CLs* promoter were analyzed by PlantCARE, and then the number of hormone induction, environmental adaptation and stress-induced expression elements were counted.

Quantitative expression analysis of Jr4CLs

The four-week-old 'Liaohe' was used as the material in the tissue culture room of the Department of Fruit Science, Horticulture College, Gansu Agricultural University. Walnut test-tube seedlings with robust and consistent growth were treated with 20% (w) polyethylene glycol (PEG) for 48 hours respectively, and the normal growth test-tube seedlings were used as control with three biological repeats. The leaves of the tested seedlings were collected and frozen with liquid nitrogen, and then stored at -80 °C for subsequent RNA extraction.

The total RNA was isolated by TRIzol kit (Invitrogen, Carlsbad, CA, USA), cDNA was synthesized from total RNA using a PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time; TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. The qRT-PCR assays were performed with SYBR Green PCR Master Mix (Takara). The primers used for real-time PCR are shown in Table 1. The reaction system is: 10 μL of TB Green , 2 μL of upstream and downstream mixed primers, 6 μL of ddH₂O, 2 μL of cDNA, and a total volume of 20 μL. The reaction conditions are: 95°C predenaturation for 3 minutes; 95°C denaturation for 5 s, 58°C annealing for 30s, 72°C extension for 30s, 40 cycles. The qRT-PCR analysis of each sample was calculated from three biological replicates. And use $2^{-\Delta\Delta CT}$ to calculate the relative expression of genes, and use SPSS software's single factor analysis method to analyze the significance of differences.

Table 1
Primers used in this study

Gene ID	Gene name	Primer sequence (F)	Primer sequence (R)	Purpose
JreChr01G10261	Jr4CL1	CATTTCCACTCTCCGCACCTTCTC	TTCTTCTTCGTCTCTTCGTCTTCTTG	qPCR
JreChr01G10401	Jr4CL2	CGGTTCTTGATCTCACTGGCTTCC	GCATAATTCCAATTCGACCCGTTGAC	
JreChr01G10828	Jr4CL3	AGGAACAGCACAAACCCTCGT	CGCTGGCTGAGCTTCTCTCT	
JreChr01G10888	Jr4CL4	TGCTGTACTCACCTTACCTTCAACG	TTCTTCTTCCTTCTTGTTTCACCTCTCC	
JreChr01G11687	Jr4CL5	GTGTGAGAGAGTGAAAGAAGCAAGAATG	ACATCCAGAGAAGCCAGTTCAATTACC	
JreChr01G12667	Jr4CL6	ACAGCACAAACCCTCGTCTTTCAG	TTGAATCTCCCAGAACAGAAGCCTTG	
JreChr01G13040	Jr4CL7	TGCTGTACTCACCTTACCTTCAACG	TTCTTCTTCCTTCTTGTTTCACCTCTCC	
JreChr01G13626	Jr4CL8	CGGTTCTTGATCTCACTGGCTTCC	GCATAATTCCAATTCGACCCGTTGAC	
JreChr02G10700	Jr4CL9	GTAGGTGGTTGTGGTGTGGAAGTG	CATTCATCAGTCTAGCATACGGGTAGTC	
JreChr02G11270	Jr4CL10	CTGGTGGGACTCATCGTCAAGAAC	CAACTATGTCAGATTGCCATCCAAAGC	
JreChr02G11899	Jr4CL11	CATTTCCACTCTCCGCACCTTCTC	TTCTTCTTCGTCTCTTCGTCTTCTTG	
JreChr03G10018	Jr4CL12	TTGGAGAATACGGAGAACATGAATCGG	GAGGACATAGAATCGGTGGTTGAGC	
JreChr03G12827	Jr4CL13	GAGTACCTTGCTGTCTCAGATCATTG	TGCTCCTCTTGTTGCTGTTTCATGG	
JreChr03G13128	Jr4CL14	CTGACATAGAAGTGACAATGGTGGAGAG	ACAACATTGAGATGGAGAACGGTAAGG	
JreChr03G13375	Jr4CL15	CGAATACGATGAGTTTGGACCACCTAG	TTAAGCCGTTTCCCATCACAATCCC	
JreChr03G13461	Jr4CL16	ACAAACTCAGCCGCCTCCAA	GGGTGCAATGGACCAACTGC	
JreChr04G11976	Jr4CL17	GCAGCAGCAGCAACTAAATCAGAAG	TGAGTGGTTGGAGGCATTGGTAAAG	
JreChr05G11706	Jr4CL18	CGAATACGATGAGTTTGGACCACCTAG	TTAAGCCGTTTCCCATCACAATCCC	
JreChr05G11730	Jr4CL19	TCCGACCTCCTACCTGACCTAATTG	ATCTTGGTCTGCGGCAACGATAAG	
JreChr05G12314	Jr4CL20	CTGACATAGAAGTGACAATGGTGGAGAG	ACAACATTGAGATGGAGAACGGTAAGG	
JreChr05G12962	Jr4CL21	GAGTACCTTGCTGTCTCAGATCATTG	TGCTCCTCTTGTTGCTGTTTCATGG	
JreChr06G11268	Jr4CL22	ACCCTTCATCTTCTTCTCCTCCCAATTC	TAGACTTCTTGATTGACGGTGGTGTAC	
JreChr06G11338	Jr4CL23	CGAGCCACCCAAGCAAGACTATATC	ACTTTCATCCTTTCGCTGATCTTCTCTC	
JreChr06G11877	Jr4CL24	CAGCCATACCAACGACTCGGATTC	TCCCTGAATACCCTCCCTGTGAAC	
JreChr07G11075	Jr4CL25	TCCGACCTCCTACCTGACCTAATTG	TGATCTTTATCTGCGGCAACAATAAGC	
JreChr07G11306	Jr4CL26	GAAGCAGCCGAATAGCCAACAATTAC	CCACGAGCCTCATGTCCAAATCC	
JreChr07G11325	Jr4CL27	CAATCTCCAAGCCAACCACCTCTC	AAGCAGTGTGAGTCTTCCCAAACG	
JreChr07G11607	Jr4CL28	AGAGCTTGACAGTTGGGATTTGGATG	CTCTCAGACACAACATTCTTGGAGGAG	
JreChr07G11893	Jr4CL29	AGTTTGGCAGAAAGGGTAAGAAGAGAG	TGACTGAACGTAGCTGATAACCACATC	
JreChr07G11894	Jr4CL30	CAGTGGCAGTGTGGAAGAGGAAC	GACTAGGGAACGCAGAGCAAAGAG	
JreChr07G11896	Jr4CL31	CAAGCTGGTCACATCAATTCAACTCTG	AAGAGGAGGAGGAGGAGGAGGAG	
JreChr08G10014	Jr4CL32	CACAGCCGTCCACAACCTCATC	AGGTGTTGGGTGGGAAGCAGAG	
JreChr08G11472	Jr4CL33	CAACCTCAACCTCCTCCTCCTCTC	GCAAACAGATAATCCAGAAGGGGTAGG	
JreChr08G11573	Jr4CL34	ATCTTCTTCCATCTCTACCCTCCCATC	TTCTGTTTCATGTTCAATTTGTGCATCCC	

Gene ID	Gene name	Primer sequence (F)	Primer sequence (R)	Purpose
JreChr08G11599	Jr4CL35	CCCAGCAAAGGATGAGGAAGATAGTG	AGCAAGAACATCTCCATATCAGTGACC	
JreChr08G12150	Jr4CL36	AAGAAGCAACCGAGTGACCAACAG	ACATCTCCCTGATCCACCAACCTC	
JreChr09G10404	Jr4CL37	CTTTCCACCCTTTGACACCCACTC	CGTCCTTCTCGCTGCCTAACTTC	
JreChr10G10412	Jr4CL38	GGCTCTCAAATCATTTCCGCTCTTG	TTCCTCGGCACTAAGTTGGCATTC	
JreChr10G10423	Jr4CL39	GCTTCGGCTCCTTCTTCTACTCAAC	GCTTCGGCTCCTTCTTCTACTCAAC	
JreChr10G10964	Jr4CL40	CTGGTTTCTTAGGTGACGGCATCC	GCTTCAAGACCCTCCTCGCTTTC	
JreChr11G10166	Jr4CL41	ACACCATCATCGTCATCACACTCATC	TCCAAGCTCTTCTTCACCAGTTGC	
JreChr11G10952	Jr4CL42	TAAGGGTTGGCTATCTCGGGGAAG	GGCGTGTGGCTCAAGAACTCTATAG	
JreChr11G11200	Jr4CL43	CTGGTTTCTTAGGTGACGGCATCC	GCTTCAAGACCCTCCTCGCTTTC	
JreChr11G11369	Jr4CL44	TGGGCGTCAGGAAAGGTGAC	GTCACCTTTCCTGACGCCCA	
JreChr12G10316	Jr4CL45	CTGGTTTCTTAGGTGACGGCATCC	GCTTCAAGACCCTCCTCGCTTTC	
JreChr12G10871	Jr4CL46	GAAGTATTGGAAAGCTCGGAAACATC	GCGAGTTCAACGGAGTGCTGTAG	
JreChr12G11007	Jr4CL47	ACTCCTCCCAAATCGTCTCTCCTG	TTGTGGCACTGTGTGCTGTATTG	
JreChr13G10323	Jr4CL48	GGATGGGTGGATTCAAGCCTTCTG	AGCCTCTTCAGCCTCCTGTGTAG	
JreChr13G11269	Jr4CL49	CCAGCGGGAAGACGACAAAGG	CGGTAAACGGCAGGACGAGTG	
JreChr14G10240	Jr4CL50	AGGCTCCTTCAAGTATGTGCTGTTC	TGGTGAATTACGGCTCGAAGTATGC	
JreChr14G11032	Jr4CL51	TGTTGTGCTGAGGGAGATGAGAGG	CAGTGAAAGAGGTGCTTGGAGAGAG	
JreChr16G11309	Jr4CL52	ACACCATCATCGTCATCACACTCATC	TCCAAGCTCTTCTTCACCAGTTGC	
	18S	ACACGGGGAGGTAGTGACAA	CCTCCAATGGATCCTCGTTA	Reference gene
JreChr11G11369	Jr4CL44	ATGACACGTACGGAGAGCGAA	TCAAAGTTTGGAGGTGGTAGTAAGTT	Gene clone

Cloning and bioinformatics analysis of *Jr4CL44*

qRT-PCR analysis showed that the members of *Jr4CL* family could be significantly induced by drought stress, among which *Jr4CL44* displayed the highest expression level. Therefore, *Jr4CL44* was selected for gene cloning and functional verification. Leaf RNA was extracted by Trizol method for reverse transcription and concentration determination. cDNA with high concentration was selected as template for target gene cloning. After that, the glue recovery product of the target gene was connected to pMD19-T and transferred to *Escherichia coli* for testing. The biogenic analysis of *Jr4CL44* gene was the same as above.

Acquisition of transgenic *Arabidopsis thaliana* and determination of physiological indexes

Transgenic *Arabidopsis* seeds were obtained by infusing inflorescence. The seeds were disinfected with 75% alcohol for 5min, then with 2.6% sodium hypochlorite for 10min, and finally washed with deionized water 4–5 times. The plants were seeded on MS medium containing 30 mg/L kanamycin, and the resistant plants were screened by PCR to obtain hybrid transgenic plants. After three consecutive screening, T3 generation pure and seed transgenic plants were obtained.

Wild type and transgenic lines were selected for normal culture for 5–6 weeks, and 20 leaves of each line were taken and dried at 65°C overnight, finally mixed and ground into powder with liquid nitrogen. The lignin content of each sample was determined by TGA method (Dampanaboina et al. 2021). The flavonoid content was determined by referring to the method of Böttner (Böttner et al. 2020). In order to investigate the effect of *Jr4CL44* on 4CL gene expression downstream of lignin synthesis pathway, *Arabidopsis Thaliana* of WT and OE-*Jr4CL44* were cultured for 6 weeks, the effect of qRT-PCR on the gene expression of 4CL downstream lignin synthesis

pathway in two types *Arabidopsis thaliana* was analyzed. 18s gene was used as the reference and three times was repeated. Primers used in this study were shown below.

Data processing

Parameters were statistically tested by analyses of variance and comparisons of means were performed with a Duncan test ($P < 0.05$). Statistical analyses were performed with SPSS, version 22.0 (IBM, Armonk, NY, USA). Figures were prepared using the Origin 8.0 software (OriginLab, Hampton, MA, USA).

Results

Analysis of physiological characteristics of overwintering branches of Walnut

The most intuitive manifestation of the shoot shriveling is that the epidermis of the branch shrinks due to water loss. According to the phenotype in the figure below, the epidermal shrinkage of 'Liaohe' was more obvious than that of 'Xiangling'. As we can see from Fig.1-a, the soluble starch of the two branches increased first and then decreased in the whole overwintering stage, and the content of soluble starch in 'Liaohe' was higher than that of 'Xiangling'. The Fig.1-b showed the change trend of soluble sugar content in 'Xiangling' and 'Liaohe' were basically the same (with a gradual decline from P1 to P5), and the soluble sugar content in 'Liaohe' is always higher than that of 'Xiangling' during the whole overwintering period. The soluble sugar content of 'Xiangling' and 'Liaohe' reach the highest value in P1 (6.585% and 7.347%), while it drops to the lowest in P5 (4.848% and 5.798%). As can be seen from the sucrose content (Fig.1-c), the sucrose content of 'Xiangling' showed a trend of first increasing and then decreasing, reaching the highest point in P2. The sucrose content of 'Liaohe' was higher than that of 'Xiangling', and the sucrose content was 1.25% in P1.

It is shows in Fig.1-d that the glucose content of 'Xiangling' showed a trend of gradual decrease, while that of 'Liaohe' showed a trend of first increase and then decrease. The glucose content of 'Liaohe' was always higher than that of 'Xiangling' from P2. As we can see from Fig.1-e that the sucrose phosphorylase in two walnut varieties generally increased first and then decreased with the extension of overwintering time, and reach the maximum value of 0.0101 mg/g ('Xiangling') and 0.0129 mg/g ('Liaohe') at the same time. It can be seen from Fig.1-f that the amylase activity of the branches of 'Xiangling' and 'Liaohe' increased first and then decreased during the overwintering period, and from P1 to P5, the amylase activity of 'Xiangling' was higher than that of 'Liaohe' in the same period, and the amylase activity of the two branches reach the highest in P4 and P3, respectively, where the amylase activity of 'Xiangling' was 0.1830mg/g, while the activity of amylase in 'Liaohe' is 0.1660mg/g. The activity of sucrose synthase in 'Xiangling' decreases during the overwintering process, while that in 'Liaohe' increase first and then decrease (Fig.1-g). The sucrose invertase activity of 'Liaohe' was higher than that of 'Xiangling' from P1 to P3, and reach a maximum value of 25.485mg/g in P3, while the sucrose invertase activity of 'Liaohe' was always lower than that of 'Xiangling' from P4 to P5 (Fig.1-h).

Transcriptomic analysis of overwintering branches of Walnut

In order to further explore the molecular and related metabolic pathways of shoot shriveling in overwintering, three key periods were selected for transcriptomic analysis. The clustering diagram of sequencing samples obtained according to gene expression is shown in Fig.2-a, and the correlation between the same biological repeats are better. The density distribution of FPKM reflects the expression patterns of protein-coding genes in each sample. It could be seen that 'Liaohe' and 'Xiangling' showed similar change curves during the three overwintering periods, showing non-standard normal distribution (Fig.2-b). In order to clarify the repeatability and overall change trend of transcriptome samples of 'Liaohe' and 'Xiangling' after overwintering stress, intersample principal component analysis (PCA) was conducted using the FPKM values of all annotated genes in six transcriptomic samples as parameters. The results showed that the horizontal, vertical and vertical coordinates could explain 78.59% (PC1), 6.12% (PC2) and 6.37% (PC3) of the total sample variation, the explainable overall variation rate is 91.08%, and there is a significant aggregation trend among samples, indicating that the samples have good repeatability and could be used for subsequent difference analysis (Fig.2-c).

DEGs involved in the plant hormone, cutin and wax biosynthesis pathways

According to KEGG analysis, phytohormone metabolism, cutin and wax play an important role in the overwintering process of plants (Fig.3-a,c). The metabolism of abscisic acid mainly involves PYR, PP2C, SnRK2, and ABF, which can inhibit growth and accelerate aging. XP-035550417 was downregulated in both 'Liaohe' and 'Xiangling', while XP-0188085889 was not significantly different in 'Liaohe', and was up-regulated first and then downregulated in 'Xiangling'. The expression of genes such as AUX1, SUAR and ARF in

the auxin pathway regulated cell division and plant growth, and they all showed different trends during overwintering. The metabolism of cytokinin mainly involves CRE1, AHP, B-ARR and A-ARR which reach shoot initiation and cell division, the expression of these genes showed diversity, and XP-018808887 was up-regulated in 'Liaohe' and 'Xiangling', while XP-018819058 showed a trend of rising first and then falling in 'Liaohe', and continue to rise in 'Xiangling' (Fig.3-b).

The formation of long-chain acyl-coA from acetyl-coA can be derived into other fatty compounds through three processes (initialization, elongation, and termination) through three pathways. Acetyl-coA forms long chain acyl-coA by 2.3.1.85 and 6.2.1.3 gene, in which XP-018815548 in 'Liaohe' and 'Xiangling' were progressively down-regulated, while XP-018808861 and XP-018809212 had no significant changes. The other two pathways are more complex and involve multiple genes. For example, acetyl-coA forms 3-Oxoacyl-acp through 2.3.1.180 or Malonyl-CoA through 2.3.1.39, and finally through 3.1.2.14 forms a Long-chain acyl-coA, in with a rising trend in 3.1.2.14 (Fig.3-d).

In the process of lignin biosynthesis, many genes participate in the formation of different types of lignin, among which *4CL* gene plays an important role. XP-018812460.2, a gene essential for the formation of P-Coumaroyl CoA from P-Coumaric acid, showed different expression trends in 'Liaohe' and 'Xiangling', suggesting that the expression of lignin genes may affect lignin biosynthesis to regulate plant stress adaptability (Fig.3-e).

qRT-PCR validation of differentially expressed genes

In order to further verify the reliability of transcriptome data, major differential genes involved in plant hormone signal transduction, lignin and wax biosynthesis were selected for qRT-PCR analysis (Fig.4). Transcriptomic analysis revealed that plant overwintering was related to lignin and flavonoid metabolic pathways. *4CL*, as a functional gene with both properties, showed a gradual decline in 'Xiangling', while an increase in 'Liaohe' during the overwintering process. In plant signal transduction, SnRK2 and other genes play an important role and the expression trend changed significantly during overwintering. The B-ARR of 'Liaohe' and 'Xiangling' decreased gradually during overwintering, while the change was not obvious in qRT-PCR. CYP86, 1.2.1.84, CER1 and other genes played an important role in fatty acid metabolism and can regulate overwintering. CYP86 of 'Liaohe' increased gradually with the extension of overwintering time, while that of 'Xiangling' decreased gradually. In general, the expression trend of most genes in RNA-seq were more obvious than that in qRT-PCR, but the expression patterns of the two genes are similar, which further proves the reliability of sequencing results.

Identification and analysis of physicochemical properties of *Jr4CL* family members

AMP-binding-C (PF13193.9) hidden Markov model was used to search and screen walnut *4CL* family members, and the analysis was carried out in combination with SMART, NCBI CDD and pfam, and the final retained sequences were identified as members of the walnut *Jr4CL* gene family, designated as *Jr4CL1* to *Jr4CL52* according to their chromosomal locations (Table 2). The physicochemical property analysis of the protein sequence showed that the amino acid length is between 268 and 1219 aa using the ProtParam tool. *Jr4CL45* has the smallest molecular mass of 29.4 kD. *Jr4CL1* has the largest molecular weight of 135.7 kD, the theoretical isoelectric point is between 5.39 and 9.26. The total average hydrophilicity of the protein is 0.120 - 0.295.

Table. 2 Analysis of physicochemical properties of *Jr4CL* gene family

Gene name	Gene ID	Amino acid number	Molecular weight/D	Isoelectri point	Instability index	GRAVY
Jr4CL1	JreChr01G10261	1219	134709.30	6.04	43.12	0.087
Jr4CL2	JreChr01G10401	565	61938.44	6.79	35.70	-0.027
Jr4CL3	JreChr01G10828	603	66935.68	6.34	45.64	-0.156
Jr4CL4	JreChr01G10888	653	72914.57	6.28	45.57	-0.153
Jr4CL5	JreChr01G11687	694	76587.12	6.91	31.00	-0.069
Jr4CL6	JreChr01G12667	554	60772.60	5.63	37.18	-0.154
Jr4CL7	JreChr01G13040	573	62260.32	5.86	41.61	-0.020
Jr4CL8	JreChr01G13626	544	59499.91	6.06	41.19	-0.013
Jr4CL9	JreChr02G10700	800	88525.12	8.70	31.41	-0.245
Jr4CL10	JreChr02G11270	573	62514.69	6.31	34.47	-0.157
Jr4CL11	JreChr02G11899	394	42854.74	5.39	36.17	0.047
Jr4CL12	JreChr03G10018	832	92312.08	6.26	36.28	-0.203
Jr4CL13	JreChr03G12827	730	80196.55	8.56	30.20	-0.062
Jr4CL14	JreChr03G13128	582	64270.92	7.60	34.50	-0.136
Jr4CL15	JreChr03G13375	555	60992.05	6.83	38.14	-0.087
Jr4CL16	JreChr03G13461	523	56465.37	5.64	32.84	-0.025
Jr4CL17	JreChr04G11976	587	65052.87	8.17	32.67	-0.147
Jr4CL18	JreChr05G11706	572	62357.74	5.54	37.10	0.067
Jr4CL19	JreChr05G11730	697	76624.15	6.91	31.28	-0.054
Jr4CL20	JreChr05G12314	574	62574.92	5.42	33.61	0.014
Jr4CL21	JreChr05G12962	558	60999.74	5.81	36.82	0.023
Jr4CL22	JreChr06G11268	661	73865.53	5.94	40.28	-0.164
Jr4CL23	JreChr06G11338	573	62384.45	7.16	35.08	0.084
Jr4CL24	JreChr06G11877	697	76527.42	6.85	29.46	0.006
Jr4CL25	JreChr07G11075	660	74219.24	6.39	30.63	-0.208
Jr4CL26	JreChr07G11306	563	61944.21	8.39	32.91	-0.132
Jr4CL27	JreChr07G11325	550	59855.19	8.63	34.35	0.044
Jr4CL28	JreChr07G11607	1106	122555.35	9.04	39.53	-0.095
Jr4CL29	JreChr07G11893	550	60700.08	5.86	33.68	-0.011
Jr4CL30	JreChr07G11894	542	59598.00	5.53	30.86	-0.009
Jr4CL31	JreChr07G11896	542	59548.01	5.70	30.09	0.007
Jr4CL32	JreChr08G10014	712	79988.84	6.11	31.42	-0.220
Jr4CL33	JreChr08G11472	694	76403.81	6.81	38.21	-0.068
Jr4CL34	JreChr08G11573	590	65128.47	6.80	33.78	-0.162
Jr4CL35	JreChr08G11599	546	59807.53	6.34	32.74	-0.110

Jr4CL36	JreChr08G12150	534	57489.92	8.41	39.25	-0.050
Jr4CL37	JreChr09G10404	577	63214.78	8.94	43.99	-0.058
Jr4CL38	JreChr10G10412	564	62168.99	7.00	43.88	0.077
Jr4CL39	JreChr10G10423	594	65639.98	7.88	44.52	0.028
Jr4CL40	JreChr10G10964	542	59454.94	8.76	41.64	0.028
Jr4CL41	JreChr11G10166	561	61032.36	7.18	48.20	0.047
Jr4CL42	JreChr11G10952	573	62656.33	6.25	35.24	0.120
Jr4CL43	JreChr11G11200	662	74108.18	5.74	32.05	-0.196
Jr4CL44	JreChr11G11369	524	56485.44	6.36	37.46	-0.019
Jr4CL45	JreChr12G10316	268	29351.72	9.26	47.48	-0.295
Jr4CL46	JreChr12G10871	639	71566.70	6.67	34.22	-0.152
Jr4CL47	JreChr12G11007	525	56369.53	6.22	34.54	0.015
Jr4CL48	JreChr13G10323	544	59467.97	8.80	38.52	0.083
Jr4CL49	JreChr13G11269	727	80820.81	6.82	37.27	-0.116
Jr4CL50	JreChr14G10240	540	59238.40	8.22	38.87	0.066
Jr4CL51	JreChr14G11032	727	80459.38	6.97	37.88	-0.063
Jr4CL52	JreChr16G11309	545	60360.72	6.74	35.30	0.060

Bioinformatics analysis of the *Jr4CL* family

The gene structure analysis results of the members of *Jr4CLs* showed that Motif 2 and Motif 6 were common in the family. According to the number and species of Motifs, the evolutionary tree can be roughly divided into three groups, among which Group I contained Motif 1-Motif 7, Motif 9 and Motif 10, without Motif 8, and Group II and Group III do not contain Motif 8. Conservative motif analysis found that most members of the same group have similar Motif, indicating that the genes in the same group have functional similarity (Fig. 5-a). Motif analysis is performed on 52 *Jr4CL* family member genes using MEME online tool, the results show that the motif 1 and motif 2 have typical AMP-binding -c domains (Fig.5-b).

In order to study the evolutionary relationship of *Jr4CL* in walnut, phylogenetic tree analysis is performed on 52 *4CL* amino acid sequences (Fig.5-c). According to sequence similarity and topological structure, the evolutionary tree is divided into 7 *4CL* subfamilies (I, II, III, IV, V, VI, VII). Subclass I has 8 *Jr4CL* amino acid sequences, subclass II has 5 *Jr4CL* amino acid sequences, and subclass III has 7 *Jr4CL* amino acid sequences. Subclass IV has 4 *Jr4CL* amino acid sequences, Subclass V has 9 *Jr4CL* amino acid sequences, Subclass VI has 7 *Jr4CL* amino acid sequences, and subclass VII has 12 *Jr4CL* amino acid sequences.

The Fig.5-d shows the chromosome localization and gene replication of the *Jr4CL* gene family in Walnut. Members of the *4CL* gene family are unevenly distributed on 16 chromosomes, and only one gene is found on chromosomes 4, 9 and 16, namely *Jr4CL17*, *Jr4CL37* and *Jr4CL52*. Eight genes (*Jr4CL8*, *Jr4CL1*, *Jr4CL2*, *Jr4CL3*, *Jr4CL4*, *Jr4CL5*, *Jr4CL6*, *Jr4CL7*) are most distributed on chromosome 1, while the genes on chromosome 15 are least distributed. A total of 4 pairs of tandem repeats are identified, including 1 pair of tandem repeats in chromosome 8 and 10, and 2 pairs of tandem repeats in chromosome 7.

To analyze how *Jr4CL* in walnut is involved in gene expression regulation, cis-acting element analysis of the promoter region (2000 bp) of the *Jr4CL* family gene in walnut is performed using PlantCARE online (Fig.5-e,f).The results show that the genes promoter of *Jr4CL* family in walnut contain photoresponsive elements, abscisic acid, gibberellin, auxin and other hormone response elements, and the response elements related to low temperature and defense. Among the many response elements, the number of light response elements is relatively large (ACE, G-box, AE-box, I-box, AT1-motif, GA-motif, etc.), which may play an important role in the growth and development of plants, and the *Jr4CL* gene of walnut may be able to resist various stresses, speculating that the gene function is diverse.

Expression of *Jr4CLs* gene under drought stress

In order to identify the expression characteristics of *Jr4CLs* under stress, drought stress is used as the background. Fig.6 shows that drought stress can significantly induce the expression of most *Jr4CLs* genes, among which *Jr4CL44* and *Jr4CL37* have the highest expression. In addition, genes represented by *Jr4CL36* and *Jr4CL21* are down-regulated.

Bioinformatics analysis of *Jr4CL44* gene

The identification of the secondary structure of *Jr4CL44* protein shows that it mainly included Alpha helix (Hh) and random coil (Cc), in which α -helix accounted for the largest proportion (Figure .7-a). The hydrophilicity/hydrophobicity of *Jr4CL44* protein is predicted by ProtScale online software. The results shows that the whole polypeptide chain encoded by *Jr4CL44* was less than zero, and it is a hydrophilic protein (Figure .7-b).

The analysis of cis-acting elements on the *Jr4CL44* promoter (Figure .7-c,d) shows that it contained a variety of abiotic stress and hormone response elements, such as drought response elements (MBS) and anaerobic induced regulatory elements (ARE), suggesting that *Jr4CL44* may be involved in the regulation of abiotic stress tolerance. At the same time, there are several hormone-related elements such as Methyl Jasmonate (CGTCA-motif) and Absciscic acid (ABRE), which indicated that this gene could respond to various external hormone signals, and may play an important role in plant growth and development.

Cloning of *Jr4CL44* gene and identification of transgenic *Arabidopsis*

We clone *Jr4CL44* gene using *Juglans regia* L. cDNA as template, and its band size is 1734bp (Figure .8-a). In order to verify the successful transfer of *Jr4CL44* into *Arabidopsis thaliana*, RNA and DNA levels of the transformed plants are verified. RNA level verification shows that the PCR band size is about 1734bp, which is consistent with the expected results, indicating that transgenic *Arabidopsis thaliana* plants are obtained (Figure .8-b). At the DNA level, there are bands in transgenic *Arabidopsis* using 18s as a reference, but no bands in WT (Figure .8-c).

Determination of physiological indexes of transgenic *Arabidopsis thaliana* under drought stress

To determine the response ability of *Jr4CL44* to drought stress, three *Jr4CL44*-OE *A. thaliana* lines and the WT control are cultured under normal and drought stress for 20 days, respectively. As shown in Fig.9-a, WT and transgenic *A. thaliana* grow well without significant differences in the control of pouring water, while under drought stress, transgenic *A. thaliana* leaves wilted and yellowed more seriously than WT, suggesting that overexpression of *Jr4CL44* increased *Arabidopsis* tolerance to drought. After obtaining the transgenic *Arabidopsis thaliana*, we quantitatively detect the expression level of *Jr4CL44* in WT and overexpressed lines using real-time fluorescence. The results show that the expression level of *Jr4CL44* in transgenic *Arabidopsis thaliana* plants is significantly higher than that in WT (Fig.9-b).

In order to investigate the function of *Jr4CL44* in lignin synthesis pathway, the lignin content in leaves of *Arabidopsis thaliana* lines at 6 weeks and 8 weeks of age is determined by thioglycolic acid method. The results show that the lignin content of *Jr4CL44*-OE *A. thaliana* lines is significantly increased compared with the WT (the content of S-type lignin increased), indicating that *Jr4CL44* plays a positive regulatory role in lignin synthesis (Fig.9-c,d).

The lignin synthesis pathway is closely related to the flavonoid synthesis pathway. In order to study the effect of *Jr4CL44* on the flavonoid synthesis pathway, the flavonoid content in *Arabidopsis thaliana* lines growing normally for 1 month is determined according to the above method. The results show that the flavonoid content of OE-*Jr4CL44* is not significantly different from that of WT (Fig.9-e). To investigate the effect of overexpression of *Jr4CL44* on downstream gene expression of lignin synthesis pathway, we analyze the expression changes of genes encoding multiple enzymes downstream of 4CL in WT and OE-*Jr4CL44* *Arabidopsis thaliana*. The result shows that C3H, F5H, COMT and CCR genes are significantly up-regulated, indicating that overexpression of *Jr4CL44* affects the expression of some genes downstream of the lignin pathway (Fig.9-f).

Through DAB and NBT staining of *Arabidopsis* leaves, it is found that under normal growth conditions, the staining of WT and transgenic *Arabidopsis* are relatively light and have no significant difference. However, the color of overexpressed *Arabidopsis* leaves is light under drought stress (Fig.10-a), indicating that the degree of oxidative damage suffered by transgenic *Arabidopsis* is low under drought stress.

The results of chlorophyll content determination show that there is no significant difference between WT and overexpressed lines under normal conditions, but the chlorophyll content of overexpressed *Jr4CL44* is significantly higher than that of WT under drought stress. Electrolyte leakage shows that the electrolyte content of WT is higher than that of OE1, OE2 and OE3 under drought stress, indicating that overexpression of *Jr4CL44* reduces the electrolyte leakage of *Arabidopsis thaliana* under drought stress. Under normal conditions, there is no significant difference in fresh weight between WT and transgenic plants, but the fresh weight of WT and overexpressed lines decrease significantly after drought stress, and the fresh weight content of transgenic lines are higher than that of WT (Fig.10-b).

Additionally, we can know that overexpression of OE-*Jr4CL44* could increase the content of various substances to resist stress after drought stress by measuring SOD, POD of two types of *Arabidopsis thaliana*, but the difference is not significant with WT under normal condition. Therefore, overexpression of *Jr4CL44* remarkably increases tolerance to drought stress in *Arabidopsis thaliana* (Fig.10-b).

Discussion

Shoot shriveling is one of the common natural stresses in fruit tree production, and has become a research hotspot in fruit tree production and cultivation. The response of fruit trees to shoot shriveling is a complex phenomenon, which is regulated by the pleiotropic genes of many specific molecular networks, the result of the changes of many plant traits, the interaction of various physiological and biochemical effects and genes.

Accumulating osmotic regulating substances is also one of the important mechanisms for plants to adapt to adversity (Álvaro et al. 2018). Soluble sugar is an important osmoregulatory substance in plants and has been shown to be closely related to the cold tolerance of plants (Wang et al. 2022). When plants are subjected to low temperature, soluble sugars will be greatly accumulated in plant cells under low temperature exercise, and the frost resistance of plants will be enhanced (Wan et al. 2012). In this study, the content of soluble sugar and sucrose in osmotic adjustment substances in walnut overwintering period showed a trend of first increase and then decrease, both of which were always higher in 'Liaohe' than in 'Xiangling'. A large number of studies have shown that the content of soluble starch was related to plant cold resistance under different low temperature stresses (Liang et al. 2022). The content of soluble starch in this study showed a trend of low and high, which was similar to the results of Liu (Liu et al. 2018). Invertase (Inv), sucrose synthase (SS), sucrose phosphate synthase (SPS) and amylase are key enzymes in sugar metabolism and are closely related to plant growth and development (Guan et al. 2019). Inv can irreversibly catalyze the decomposition of sucrose into glucose and fructose, SPS is an important enzymes that catalyze the synthesis of sucrose from glucose and fructose (Su et al. 2021). In this study, the SPS activity of 'Liaohe' was always higher than that of 'Xiangling', and the SPS activity increased to the highest value when the temperature was the lowest. Zhang also found convertase and amylase in the study of cold resistance of sugarcane, which had a certain relationship with low temperature stress (Zhang et al. 2015).

At present, transcriptome sequencing technology has been widely used in various plants and adversity studies. The biological processes of plants and the complex regulatory networks involved in plant growth and development play an irreplaceable role in the analysis of stress (Khan et al. 2021, Zhou et al. 2017, Lin et al. 2019). In this study, the Illumina HiSeq sequencing platform was used to sequence the transcriptomes of the three periods of walnut overwintering. KEGG enrichment analysis showed that the overwintering process of walnut was enriched into a variety of biological processes (lipid metabolism, amino acid metabolism, secondary Metabolite metabolism, energy metabolism), among which the main differential genes are mainly concentrated in plant hormone signal transduction, cutin and wax metabolism. Therefore, the overwintering process of plants are a complex process involved the participation of multiple genes and the mutual influence of multiple metabolic processes. Plant hormones are the initiators of cold resistance gene expression and play an important role in the regulation of plant cold resistance (Li et al. 2017). It is generally believed that plant endogenous hormones auxin, gibberellin and cytokinin are growth-promoting hormones, while ethylene and abscisic acid are growth-suppressing hormones (Wang et al. 2017). Under normal circumstances, low temperature stress will increase the content of inhibitory hormones and reduce growth hormones (Meng et al.2016). Although the process of lignin anabolic metabolism have been clearly studied, further studies on lignin regulation of stress are still needed. *4CL* in plants mainly exist in the form of gene families, one is involved in lignin monomer synthesis, the other is involved in flavonoid biosynthesis, and both flavonoids and lignin can protect plants from stress (Tomotaka et al. 2014). In this study, 52 members were identified from the whole genome of walnut by bioinformatics method, and cis-acting element analysis showed that *4CL* family contains plant hormone response elements and several stress response elements. Combining transcriptome and drought stress experiments, and a highly expressed gene (*Jr4CL44*) is choosed. For the sake of characterize the response of *Jr4CL44* under external stress, we cloned *Jr4CL44* gene with 1734bp using

template of *Juglans regia* for subsequent study. Analysis of cis-acting elements of *Jr4CL44* was involved in several hormone response elements and drought elements.

Drought stress is carried out after obtaining transgenic *Arabidopsis thaliana*, the results showed that transgenic and WT *Arabidopsis thaliana* had different wilting degree under drought stress, but the wilting degree of WT was more serious, which indicated that *Jr4CL44* gene could effectively improve drought resistance of *Arabidopsis*. The results of lignin determination showed that the lignin content of *Arabidopsis thaliana* with overexpression of *Jr4CL44* was significantly higher than that of the WT, while the result of flavonoid determination showed that the overexpression of *Jr4CL44* did not affect the synthesis of flavonoids in *Arabidopsis thaliana*, indicating that *Jr4CL44* was mainly involved in lignin biosynthesis, but did not affect the synthesis of flavonoids. AtC3H, AtF5H and AtHCT have important regulatory functions on the phenylpropane pathway and growth of *Arabidopsis* (Mondal et al. 2018). The expression analysis of 4CL lignin-synthesis-related genes in *Arabidopsis thaliana* showed that overexpression of *Jr4CL44* significantly increased the expressions of C3H, F5H and COMT genes. These results suggested that *Jr4CL44* may promote the lignin deposition in transgenic *Arabidopsis thaliana* lines by affecting the expression of some downstream genes, and indirectly affected the growth and development of transgenic plants (Wang et al. 2023).

Leaf wilting can seriously affect chlorophyll synthesis, which in turn affects a series of physiological activities of plants (Ahmed et al. 2021). In this study, the chlorophyll content and fresh weight of *Arabidopsis* decreased under drought stress, and the content of transgenic *Jr4CL44* was higher than that of WT, which was consistent with the results of Doron's study (Doron et al. 2020). A large amount of reactive oxygen species (ROS) is produced in plants under abiotic stress (Zhanassova et al. 2021). A small amount of ROS can trigger the signaling pathway in adaptive response, while a high concentration of ROS will cause damage to large molecules such as DNA, proteins and membrane lipids (Melicher et al. 2022). NBT can react with superoxide anion to form blue formosa particles which are insoluble in water and precipitate on the surface of leaves, the lighter the blue color, the stronger the antioxidant capacity of the plant (Li et al. 2022). In this study, the staining degree of transgenic *Arabidopsis thaliana* was lighter than that of WT, indicating that transgenic strains could better remove superoxide anions and improve the drought resistance of plants. DAB can react with H_2O_2 to produce yellowish-brown insoluble precipitation, which accumulates on the surface of leaves, the darker the color, the higher the H_2O_2 content (Kim et al. 2019). In this study, the staining of non-stressed *Arabidopsis* was relatively light, and the degree of reddish-brown staining of leaves was gradually deepened after drought stress. Plants themselves will activate two types of protective systems, enzymatic and non-enzymatic, to reduce or eliminate the damage of reactive oxygen species under abiotic stress (Samia et al. 2021). This study showed that drought stress reduced the activities of SOD and POD, and the activities of SOD and POD in transgenic *Arabidopsis thaliana* were higher than those in WT, indicating that *Jr4CL44* can increase the scavenging ability of ROS to a certain extent, which was consistent with the results of Geng in sweet oranges (Geng et al. 2019).

Conclusion

The overexpression of *Jr4CL44* in *Arabidopsis thaliana* have better growth under drought conditions compared to WT, and can effectively clear more reactive oxygen species (ROS). Meanwhile, the expression of genes related to lignin synthesis was up-regulated. Therefore, *Jr4CL44* gene plays an important role in drought resistance, which points out the direction for further research on other functions of *Jr4CL44*, and provide a theoretical basis for the improvement of stress resistance of fruit trees.

Declarations

Contributions

XLM and XYW designed the research. XLM and YLG performed the experiments. YLG and ZXZ performed the data analysis and interpretation. XLM and ZXZ prepared the figures and tables. XLM wrote the manuscript. All authors read, commented on and approved the manuscript.

Declaration of Interest Statement

The authors declare that they have no competing interests.

Funding Information

This work was supported by Special Fund for National Natural Science Foundation of China (Project Number 32160696).

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Figures

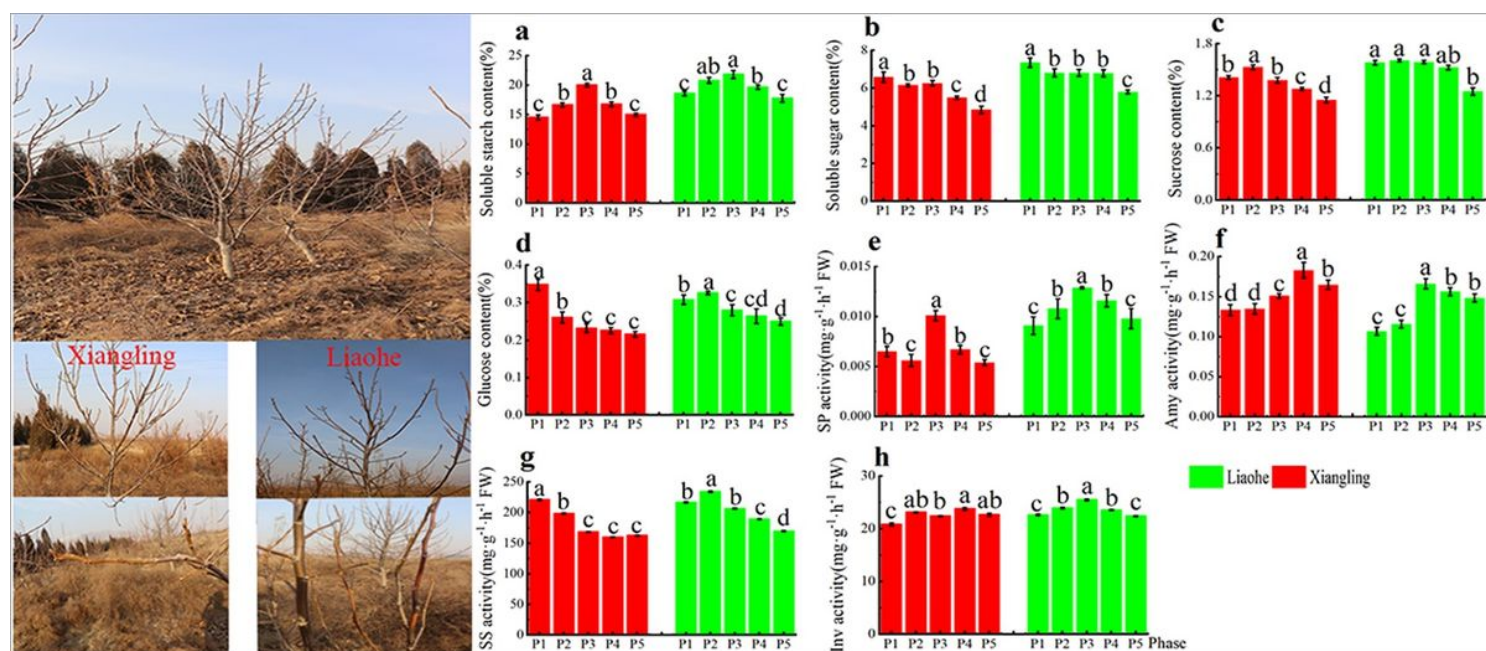


Figure 1

Physiological characteristics analysis of overwintering walnut branches

Note: P1-P5 represents the initial dormant period, middle dormant period, deep dormant period, freeze-thaw alternation period and germination period respectively, different lowercase letters represent significant differences between different periods, the same as below.

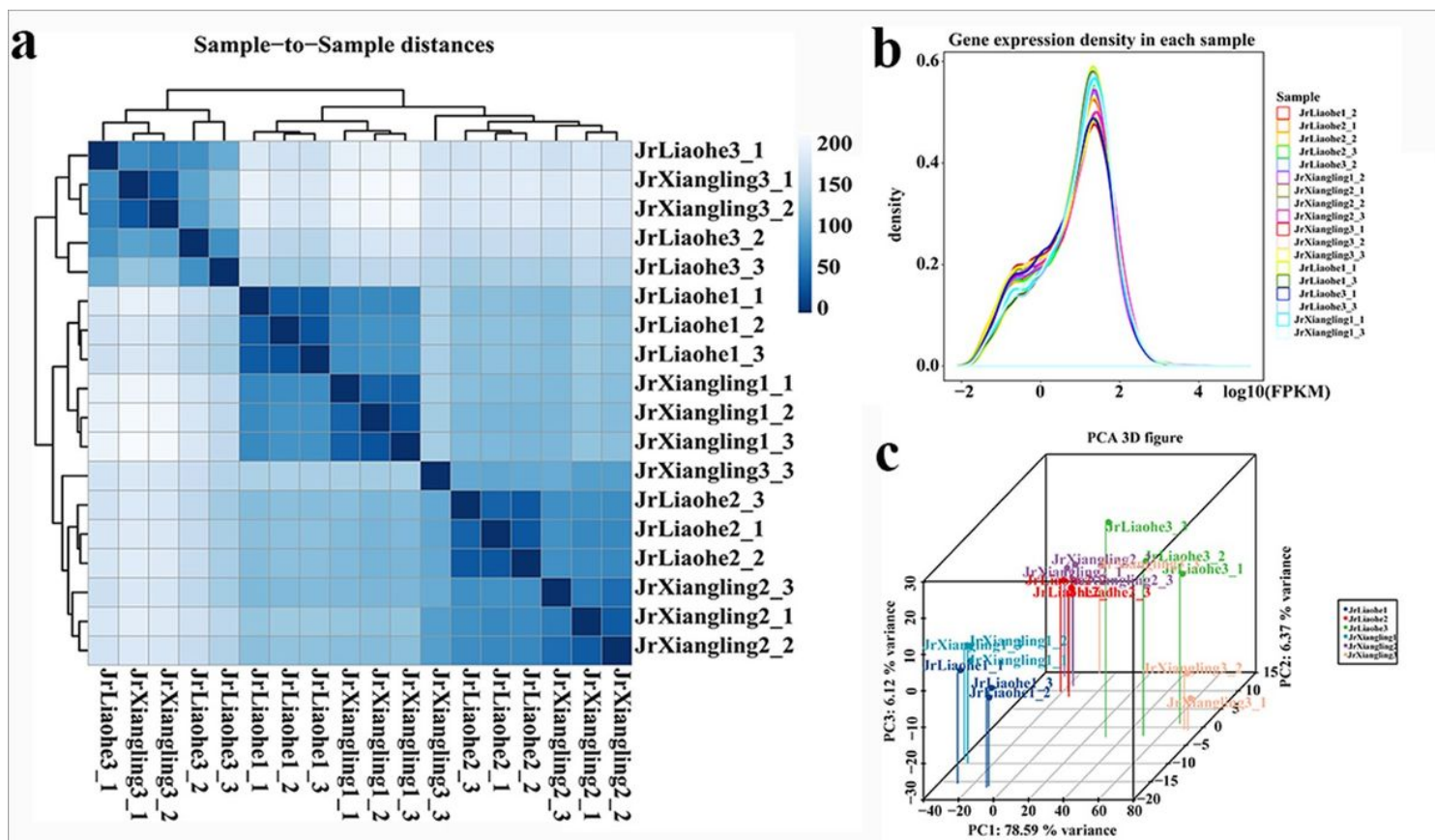


Figure 2

Transcriptomic information analysis of overwintering branches

Note: The first 1-3 in the figure represent initial dormancy, deep dormancy, and freeze-thaw alternate phases respectively, and the second 1-3 represent the number of three repeats respectively.

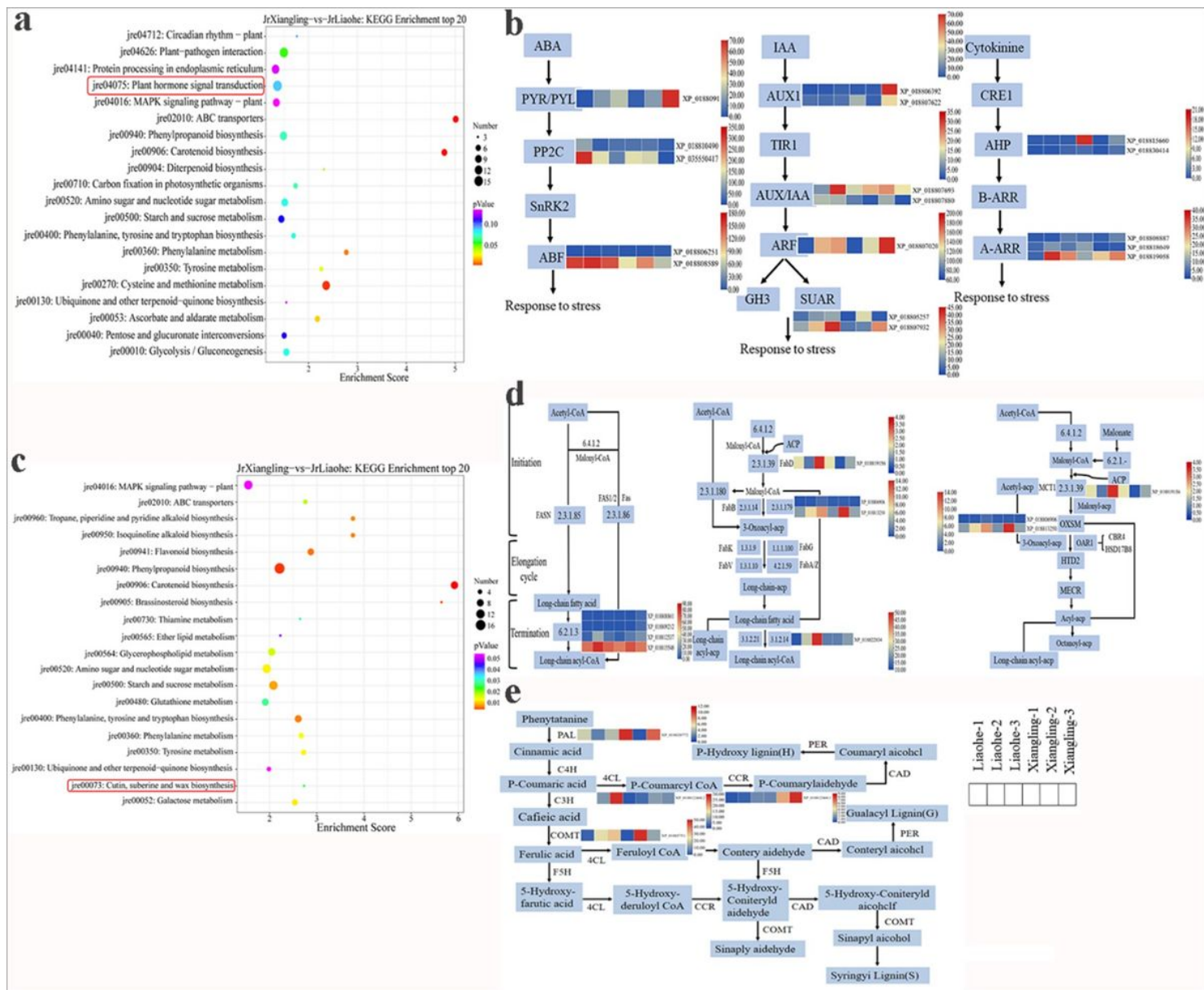


Figure 3

DEGs involved in KEGG analysis

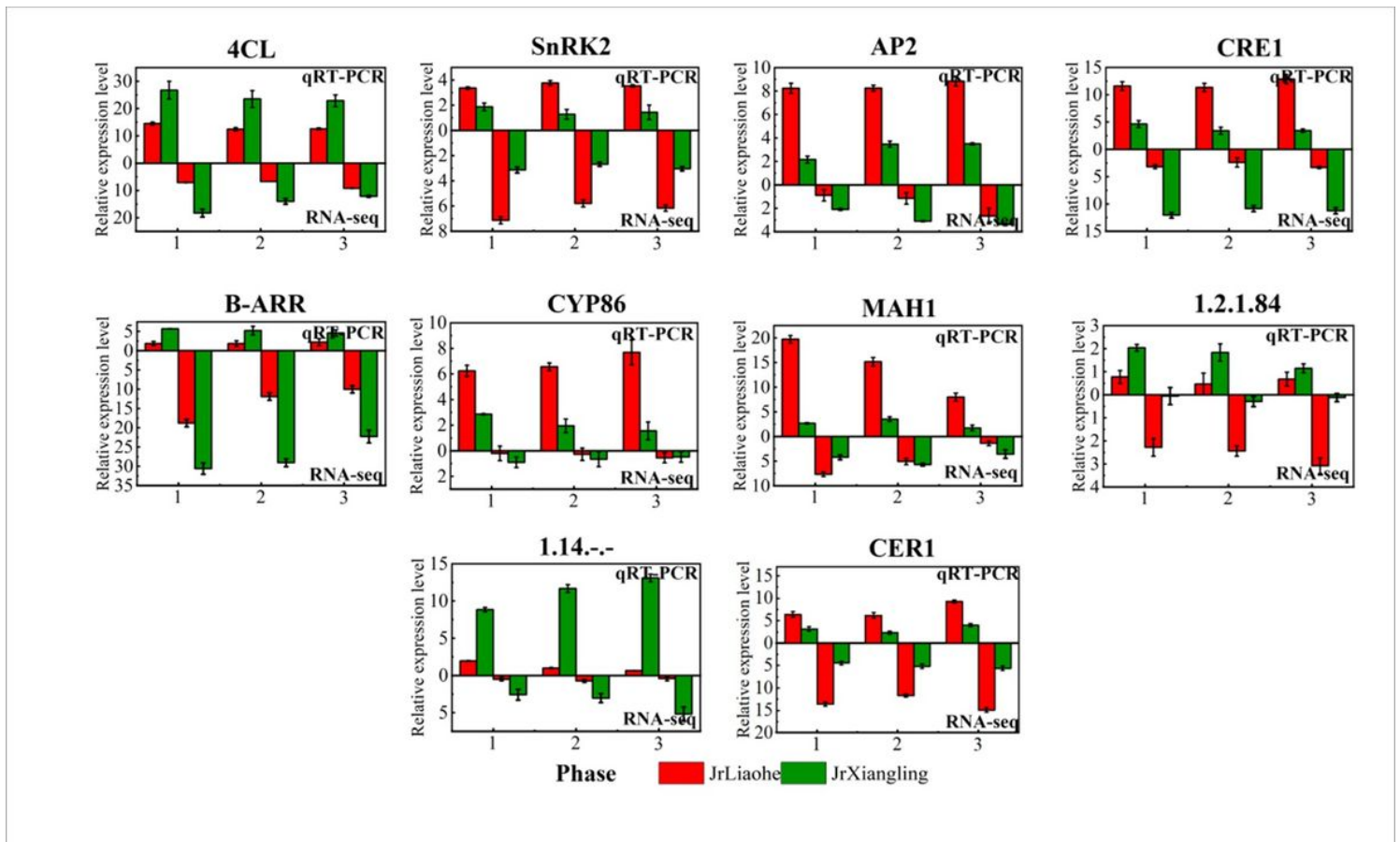


Figure 4

qRT-PCR of differentially expressed genes.

Note: 1-3 represent different periods of apple overwintering, namely initial dormancy, deep dormancy and freeze-thaw alternate phases.



Figure 5

Bioinformatics analysis of the *Jr4CLs*

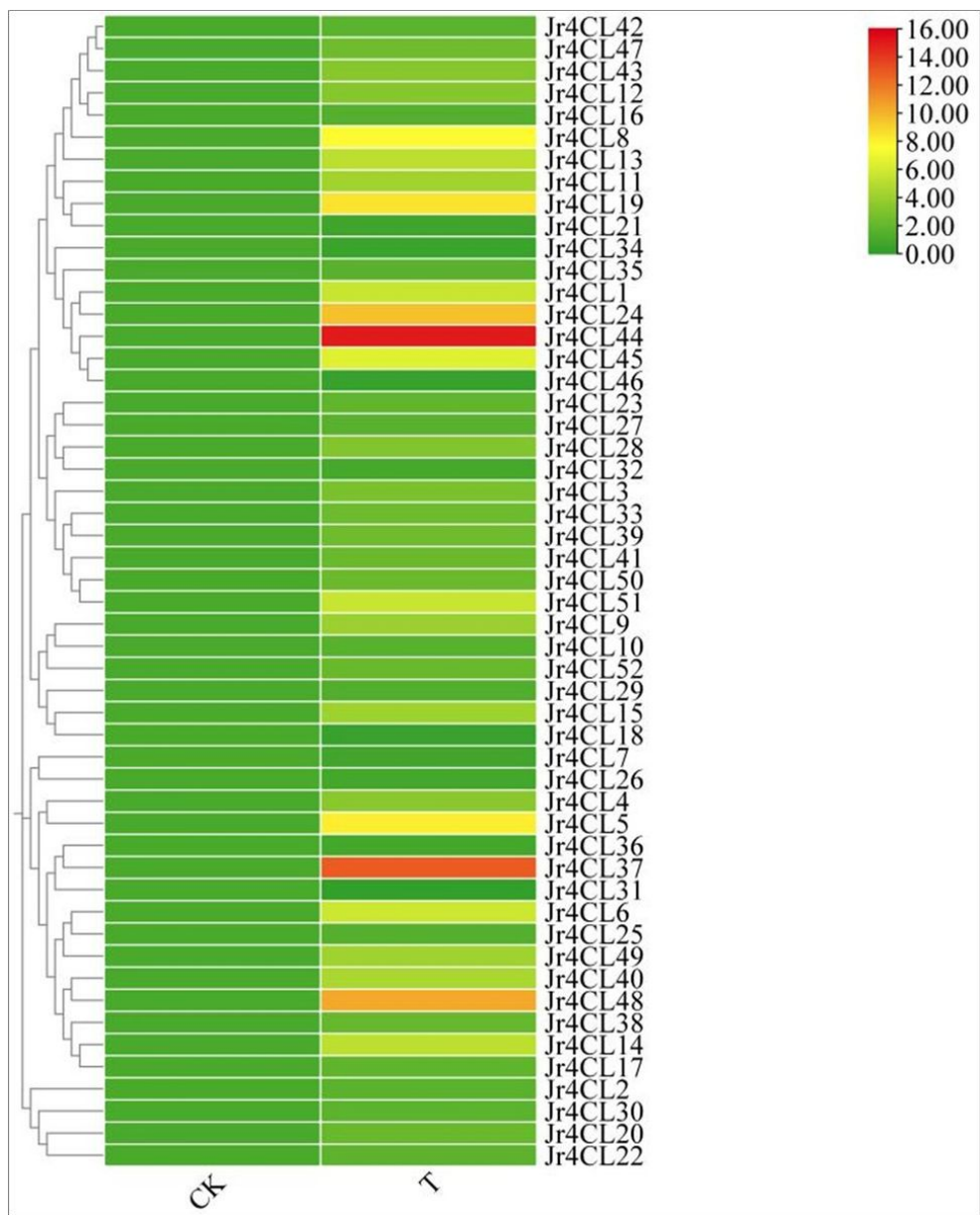


Figure 6

Heat map of Jr4CLs family under drought stress

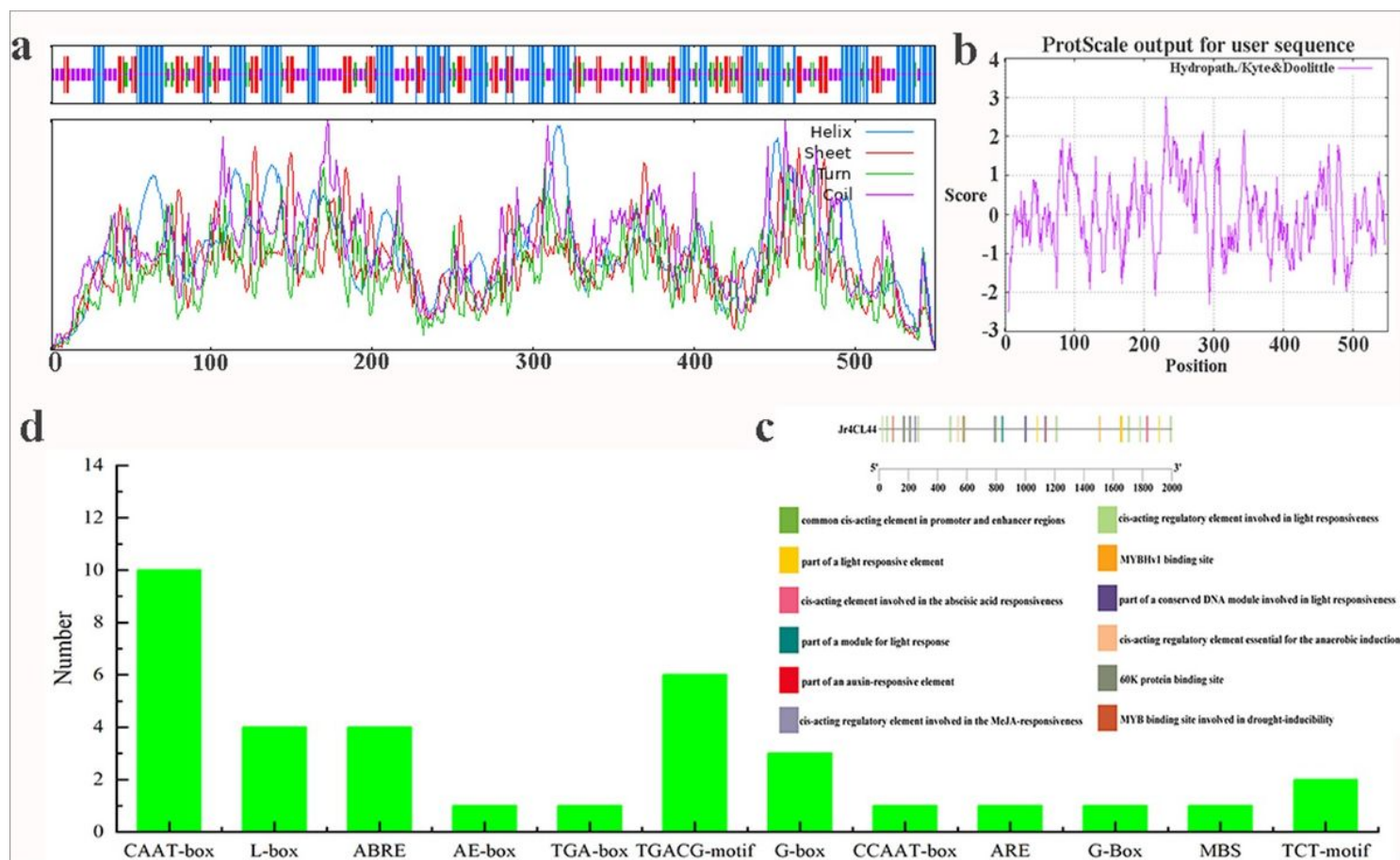


Figure 7

Bioinformatics analysis of *Jr4CL44*

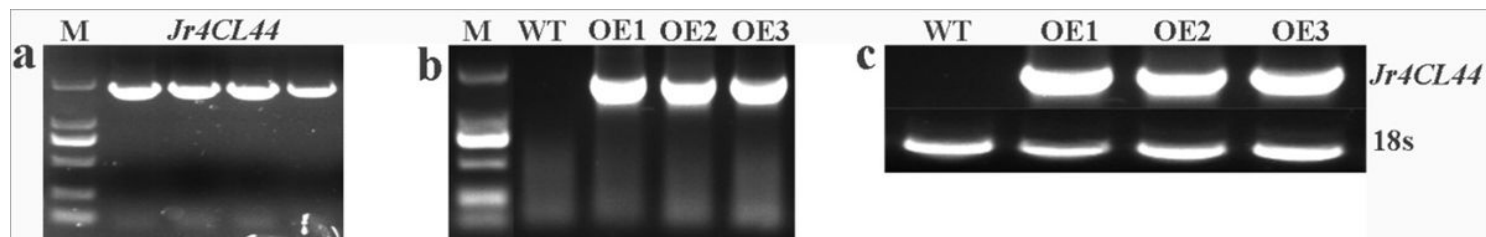


Figure 8

Cloning of *Jr4CL44* and identification of transgenic Arabidopsis

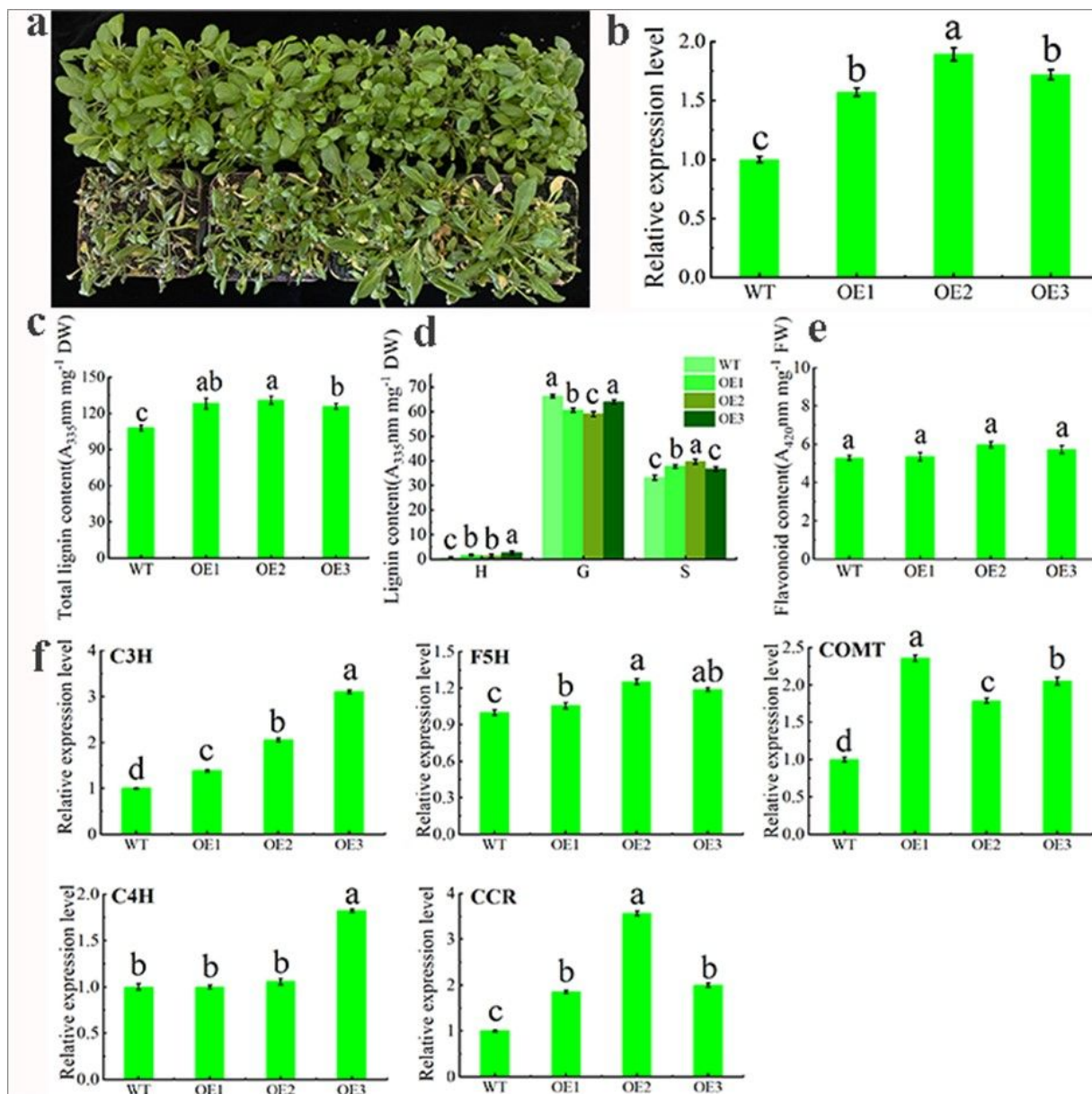


Figure 9

Phenotypic observation and lignin-related index determination of *Arabidopsis thaliana* under drought and normal conditions

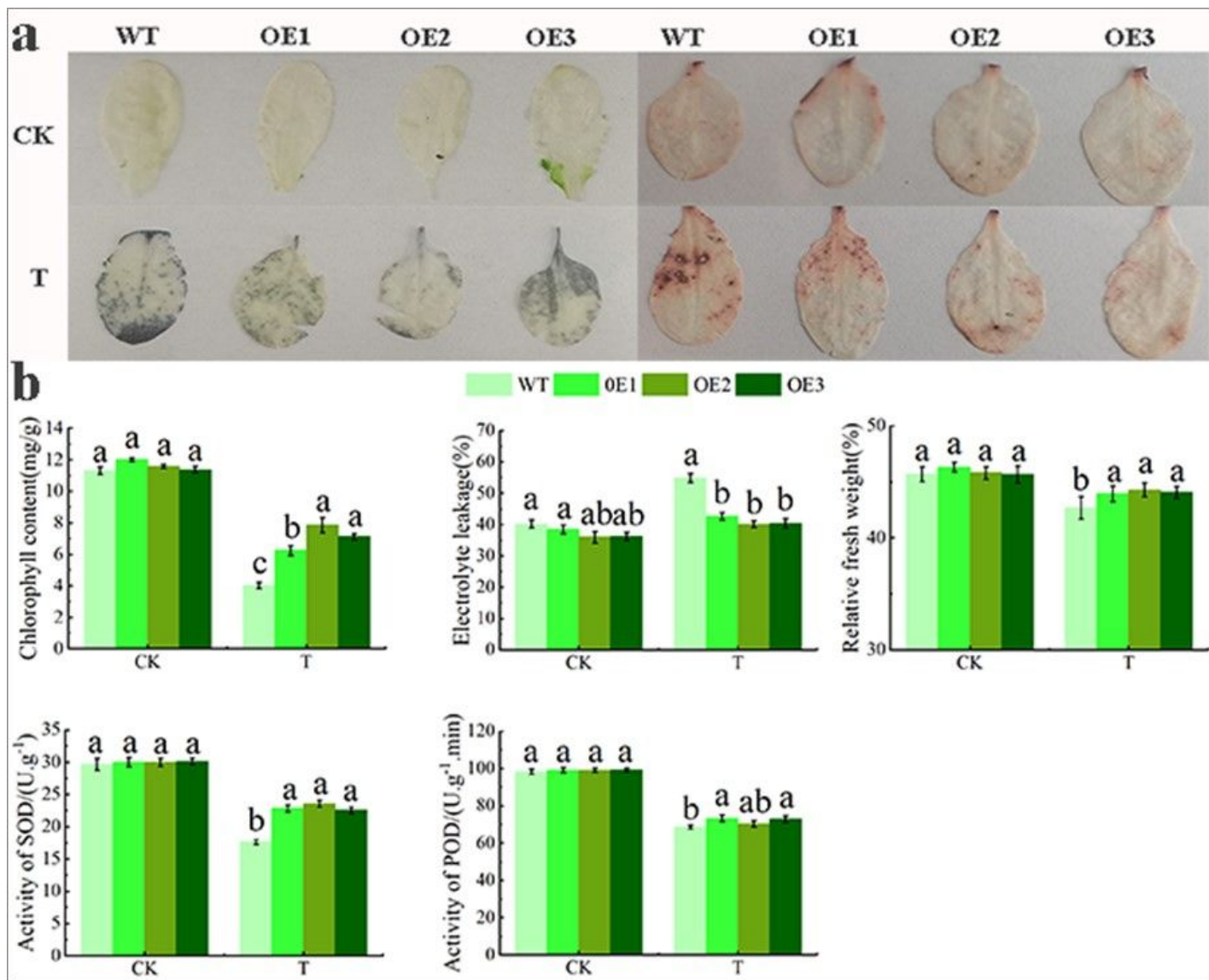


Figure 10

NBT and DAB staining and index determination of wild type and transgenic *Arabidopsis thaliana*

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