Genome-wide identification of LcC2DPs gene family in Lotus corniculatus provides insights into regulatory network in response to abiotic stresses

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Article

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Abstract

Low temperatures and drought reduce forage yield and quality, with protein kinases crucial for plant stress response. This study examines the LcC2DPs protein kinase family in *Lotus corniculatus*, identifying 90 members, with some tandemly distributed on chromosomes 2–6, and grouped into 5 subfamilies (I-V). 34 homologous gene pairs were found in *Arabidopsis thaliana*. *LcC2DP* genes promoters contain hormone and stress response elements. GO analysis highlights enrichment in hormone response and kinase activity. Transcriptomic analysis links 78 genes to environmental response and stress growth, with 10 validated by qRT-PCR after treatment with 100 μM ABA and IAA, 20% PEG6000, and 4°C. Protein interaction analysis identifies 5 core proteins (LcC2DP5, 11, 15, 38, and 58) activated by drought and cold stress. Gene analysis revealed that only LcC2DP5 and LcC2DP15 share co-expression transcription factors, with *bZIP*, *bHLH*, *WRKY*, *NAC*, *MYB_related*, *MYB*, *C3H*, and *C2H2* being prominent. These proteins are expressed under drought and cold conditions, highlighting *LcC2DP5* and *LcC2DP15* activity. *NAC* and *C2H2* are vital for drought response, while *bZIP* and *MYB_related* are important for cold response. This suggests that various *LcC2DPs* in *Lotus corniculatus* respond to hormones and stress via a TF regulatory network.

Introduction

Global climate change is increasing the frequency and severity of extreme weather, like droughts and cold spells[1–2], which threaten ecosystems, agriculture, and forage production[3–4]. These conditions disrupt water supply, hinder forage growth, thus reduce yield and quality, sometimes leading to plant death[5]. For instance, summer droughts with high temperatures can cut forage yield by about 30%[6]. In plant cells, genes and their products manage signal perception and transduction, activating downstream genes to protect against stress[7]. Calcium-dependent protein kinases (CDPKs) are vital in forage grasses' response to abiotic stress[8], acting as key signaling effectors. They detect environmental changes and trigger intracellular pathways[9]. Under drought and low temperature stress, *CDPKs* enhance forage grass adaptability by influencing stomatal movement, antioxidant enzymes, osmoprotectants synthesis, and gene expression[9–10].

Protein kinases, through phosphorylation and dephosphorylation, are crucial for plant response to abiotic stresses such as drought and low temperature[11]. Phosphorylation of protein kinases amplifies intracellular signal cascades by altering its charge, conformation, and function, while dephosphorylation removes foreign signals in plant pathways[12]. Most protein kinases are calcium-dependent, playing a role in calcium signal responses[13]. Calcium-dependent (CD) factors, such CDPK, are crucial in plant stress responses, growth, and development[14]. Studies indicate that cells detect signals via Receptor-like kinase (RLK) and membrane hardening, which then activate Ca^{2+} channels to raise cytoplasm Ca^{2+} levels[14]. Ca^{2+} receptors like Ca^{2+}-dependent protein kinase (CDPK), Calmodulin (CaM), Calmodulin-like proteins (CMLs), and Calcineurin B-like proteins (CBLs), facilitate signal transduction[13]. CDPK with a C2 domain, crucial for binding Ca^{2+}, are extensively involved in regulating cell signaling, perception, conduction, gene

expression, growth, and development[15]. Additionally, hormones, particularly Abscisic acid (ABA), are vital in plant responses to various stresses, including low temperature[16]. Research indicates that ABA boosts salt and drought resistance in *Suaeda liaotungensis* through working with jasmonic acid (JA) under cold stress[17]. MAPK protein kinase is key in the signal transduction of indole-3-acetic acid (IAA)[18], where G protein activates intracellular signals after IAA is detected by cell membrane receptors[19], can be to induce the stransduction of IAA.

Transcription factors (TFs) play a pivotal role in helping plants respond to harsh environments by binding to specific promoter and adjusting gene expression[20]. TFs generally have two domains: the DNA-binding domain (DBD) and the transcriptional activation or inhibition domain[21]. Based on DBD structure, plant TFs are categorized into families like *MYB*, *WRKY*, *GARP*, *TCP*, *bZIP*, *bHLH*, and *HSF*, etc.[22]. Among them, *bHLH*[23], *NAC*[24], *MYB_related*[25], *MYB*[26], *C3H*, and *C2H2*[27] are crucial in regulating plant stress responses to drought, low temperature, and salt.

C2 domain protein (C2DP), a class of calcium-dependent binding proteins in eukaryotes, is involved in stress response and growth regulation[28–33]. CDPK proteins with C2 domain play a crucial role in plant growth, development, and stress responses like drought[34]. While their functions have been studied in model plants like Arabidopsis (*Arabidopsis thaliana*)[28], tobacco (*Nicotiana tabacum*)[29], and rice (*Oryza sativa*)[31], they are under-researched in forage crops *Lotus corniculatus*, also known as five-leaf grass or cowhorn, is a high-quality perennial legume with strong stress resistance, widely cultivated for forage and soil conservation[35–36]. This study identified and characterized the LcC2DPs protein kinase gene family, analyzing their expression in response to ABA and IAA hormone treatments and stress conditions like drought and low temperature. The findings offer insights and potential gene candidates for enhancing stress resistance in *Lotus corniculatus* breeding.

Materials and methods

Plant materials

Lotus corniculatus was planted at a key laboratory's experimental base of Education Ministry at Guizhou University (Guiyang, Guizhou, 106° 675 'E; 26° 427 ' N).

Family members identification

The research utilized genome and protein sequences, and annotation data from a *Lotus corniculatus* database (http://www.kazusa.or.jp/lotus/) The Hidden Markov Model (HMM) profile (version 3.3) (PF00168) was used to identify C2DPs proteins by searching the Pfam database[37]. An initial domain search with an E-value < 1×10^{-10} was conducted to create a species-specific model for the *Lotus corniculatus* genome protein sequence. C2 domain protein sequences of *Lotus corniculatus* were then submitted to the Conserved Domain Database (CDD)

(https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi), and screened with SMART (http://smart.embl-heidelberg.de) and Pfam (http://pfam.xfam.org/) to exclude sequences without C2 domain[38]. This process identified high-cofidence C2 domain family proteins in *Lotus corniculatus* from all non-redundant genes.

Physicochemical and molecular analysis

Expasy (https://web.expasy.org/protparam) and WoLF PSORT (https://wolfpsort.hgc.jp) software were used to analyze theoretical molecular weight (MW)[39], isoelectric point (pI), and subcellular localization. MEME Suite 5.5.0 (https://meme-suite.org/meme/tools/meme) identified conserved motifs in *Lotus corniculatus C2DP* genes, with a maximum of 10 motifs[39]. TBtools' Visualize Gene Structure examined intron and exon regions of *LcC2DPs*, TBtools' Visualize Gene Structure examined intron and exon regions, and Gene Location Visualize from GFF mapped chromosome localization. A phylogenetic tree was created using the Neighbor-Joining (NJ) algorithm in MEGA 7.0, and final plot was visualized with iTOL (https://itol.embl.de/)[5]. Collinearity analysis was performed with MCScanX. TBtools aided in visualizing collinearity analysis. A 2000 bp upstream DNA sequence from *LcC2DPs* was used for promoter analysis, with *cis*-acting elements predicted via the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html)[40].

Function prediction

GO and KEGG analyses were conducted using the DAVID tool (https://david.ncifcrf.gov/), and figures were created with Microscopic Letter (https://bioinformatics.com.cn/). A *Lotus corniculatus* protein-protein interaction network was constructed by importing *Lotus corniculatus* C2DPs sequences into the STRING database (https://string-db.org/), saved as a TSV file, and visualized the PPI network with Cytoscape software. Using the Japonicus plant database (https://planttfdb.gao-lab.org/prediction.php)[41]. A Python script analyzed gene transcriptome FPKM values, and a coexpression network was built with DEGs and TF data Cytoscape v3.7.10 software was used for visual analysis.

Gene expression analysis

To study *LcC2DP* gene expression, 12-day-old hydroponic seedlings with healthy rhizomes were transplanted into soil for 2 weeks culture. During this time, they underwent drought, low temperature, and hormone treatment. Drought stress was simulated by applying respectively carried out during this period, 500 ml 20% PEG solution daily for 15 days. The *Lotus corniculatus* seedlings were placed in a growth incubator (Ningbo Southeast Instruments Co., Ltd.) at 4°C for 72 hours. For 15 days, 100 umol IAA and ABA were applied to the leaves and soil twice daily. Except for the low temperature group, the culture conditions were maintained at 25 ℃ with a 16-hour light and 8-hour dark cycle, 60% humidity, and 20000 Lk light intensity. Total RNA was extracted from each sample (drought and hormone, 0, 3, 6, 9,12, and 15 days; low temperature, 0, 6, 12, 24, 48, and 72 hours) using the OMEGA Plant RNA Kit (OMEGA,

Guangzhou, China), and approximately 2 μg of RNA was used for reverse transcription with a StarScript II First-strand cDNA Synthesis Mix (CenStar, Beijing, China). *LcC2DPs* and *LcUBI* detection primers were designed with Primer Premier 5.0 software. The cDNA served as templates for quantitative real-time PCR (qRT-PCR) using these primers (Table S2), with cycling conditions of 94 C for 3 min, then 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72 C for 30 s on a qTower3G System (Analytik Jena AG, Jena city, Germany). *LcUBI* gene was the endogenous control for data normalization[42], and gene expression was quantified using the comparative CT method[43]. All experiments included at least three independent biological replicates.

Statistical analysis

Data processing was performed using Microsoft Excel 2013, statistical analysis was carried out using SPSS24.0, and figures were generated using GraphPad Prism 8.4.3. In this research, all the experiments were carried out in three independent biological replicates. In each graph, error bars indicate the mean values ± standard error (SE) of three replicates. To test the statistical significance between measurements of different treatments, a multiple test selection with analysis of variance (ANOVA) and Student's t-test were used. The significance level between the various time points has been placed (*P < 0.05, **P < 0.01, and ***P < 0.001).

Results

LcC2DPs family members

Using HMMER, InterPro (PF00168), and SMART software, 90 *LcC2DPs* genes were identified after searching for sequences with typical C2 structural domains in *Lotus corniculatus* genome. Protein sequences revealed significant variation in the lengths and molecular weights of LcC2DP members. Their amino acids ranged from 60 (LcC2DP24) to 1,056 (LcC2DP3, LcC2DP27), and molecular weights varied from 6,681.80 (LcC2DP24) to 78,9062.1 (LcC2DP9) kDa. The *LcC2DPs* family included 34 basic proteins and 56 acidic members with isoelectric points below 7. Fat coefficients of the family ranged from 60.13 (LcC2DP49) to113.15 (LcC2DP61), with 87 proteins being hydrophilic. The *LcC2DPs* family consists of 42 stable and 48 unstable proteins, with LcC2DP62 having the highest instability index of 60.07. These proteins were found in various cellular locations, including the nucleus, cytoplasm, cytoplasmic membrane, chloroplast, endoplasmic reticulum, cytoskeleton, peroxisome, vesicles, and mitochondria. Specifically, 28 proteins were in the nucleus, 23 in the cytoplasm, and 14 in the plasma membrane (Table S1). This indicates that *LcC2DPs* family members are widely involved in various life processes.

Motif, intron and exon of LcC2DPs

To explore the diversity of *LcC2DPs* family members in *Lotus corniculatus*, the conserved motifs of LcC2DP proteins were analyzed using online MEME software. The analysis identified five subfamilies

within *LcC2DPs* family (Fig. 1A). Group I had at least 6 conserved motifs (motif 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) in 13 proteins. Group II primarily featured motif1 (77.8%) or motif1 + motif6 (22.2%). Group III mainly included motif1 (50%) or motif1 + motif6 (50%). Group IV, with 8 members, consisted of motif1, 4, 6, 9 (67%) and motif 1, 6 (33%). The co-occurrence of motif 1 and motif6 is crucial for Group IV members. In group V, the combination included motif1 (7.1%), motif6 (7.1%), motif1 + motif6 (75%), and motif1 + motif6 + motif7 (10.71%) (Fig. 1B). This suggests that motif1 and 6 are the most conserved in *LcC2DPs* family, indicating the diverse and special roles in plant cell. According to the method[44], the *LcC2DPs* gene structure was mapped using genome annotation, showed 1–18 exons and 1–15 introns (Fig. 1C). An exon appeared in 38 genes across Groups I-IV, while an intron was found in 7 genes from Groups I-III and V. Exons were more frequent than introns, and the size of coding sequences (CDS) after intron removal varied significantly, reflecting differences in protein molecular weight and amino acid composition among *LcC2DPs* family members.

Chromosome localization

TBtools analysis showed that *LcC2DPs* genes in *Lotus corniculatus* are distributed across chromosomes chr0 to chr6. Chr0 contains the most genes, with 34. Chr1, 2, and 4 each have 13 genes. Chr5 has the fewest, with just 1 gene, while chr6 has 9 genes. The tandem gene distribution is common, with genes *LcC2DP*39, 40, 41, and 65 on chr1; *LcC2DP44* and *LcC2DP51* on chr2; *LcC2DP82* and *LcC2DP84* on chr3; *LcC2DP71*, 72, *74*, *75*, *76*, *77* and *78* on chr4 (Fig. 2A).

Phyletic evolution

A phylogenetic tree was created using amino acid sequence alignment of C2DPs proteins from *Lotus corniculatus* and Arabidopsis (Fig. 2B), dividing them into five groups (I-V). Both species showed similarities: Group I includes 26 LcC2DPs and 11 AtC2DPs, some with only the C2 domain and others with an additional phospholipase D domain, PLDc, which produces phosphatidic acid from phosphatidylcholine, may play a role in forming transport vesicles or act as a component in signaling pathway trafficking. Group II comprises 20 LcC2DPs and 21 AtC2DPs members, some with a C2 domain and others with a C2_ArfGAP domain related to glycosylation. Group III includes 8 LcC2DPs and 16 AtC2DPs members, featuring either a C2 domain or a C2B_MCTP_PRT domain. The PRT-c domain, often found with a calcium-dependent C2 domain, is located at the C-terminus of phosphoribosyl transferases and similar proteins, and typically includes a potential transmembrane region. Group IV consists of 8 LcC2DPs and 5 AtC2DPs, with some members having only a C2 domain and others having an additional VWA domain. Group V includes 27 LcC2DPs and 22 AtC2DPs, with some members having only a C2 domain, while others have an additional GRAM domain. The GRAM domain is mainly found in membrane-associated proteins and may play a role in protein and lipid binding.

Collinearity genes

Gene duplication and divergence contribute to gene family expansion and the evolution of new functions[45–46]. A collinear gene analysis was conducted within *Lotus corniculatus* to explore the origin

and evolutionary history of the *LcC2DPs* family. The *LcC2DPs* family contains 10 pairs of homologous genes on chromosomes 1 to 6 (Fig. 2C). Collinearity analysis between *Lotus corniculatus* and Arabidopsis revealed 34 homologous relationships among 24 *Lotus corniculatus* and 25 genes Arabidopsis genes (Fig. 2D). Gene pairings vary, with some Arabidopsis genes, like *AtC2DP*22, corresponding to multiple *Lotus corniculatus* genes (*LcC2DP57*, *79*, *87)*.

Cis-acting elements of promoter

Cis-acting elements in gene promoter affect the expression patterns. To understand the function of *LcC2DPs* gene family promoters, 2000 bp upstream sequences were analyzed by bioinformatics. These promoters are mostly enriched with elements responsive to light (GT1-motif), auxin (AuxRR-core), and abscisic acid (ABRE), related to the growth and development. They also contain elements for stress regulation, including responses to cold (MYB), drought (MYC or MBS), hypoxia (ARE), and defense (TC-rich repeats) (Fig. 3).

GO and KEGG enrichment pathways

GO analysis revealed that 90 *LcC2DP* genes were categorized into 30 groups, spanning cellular components, molecular functions, and biological processes. The cellular components primarily involved endosome, cytoplasm, endoplasmic reticulum, membrane, lysosomal membrane, plasma desmosis, vesicles, and membranes. Molecular functions of the endoplasmic reticulum mainly included calcium ion binding, protein binding, calmodulin binding, enzyme regulator activity, GTPase activation, kinase activity, protein serine/threonine kinase activity, catalytic activity, organic acid and carboxylic acid binding. Biological processes covered abscisic acid response, hormone signaling pathways, cell signal transduction, growth and developmental regulation, abiotic stimulus response, oxidative stress and defense response regulation. GO analysis revealed that the pathways of hormone response, protein binding activation, and kinase activity are crucial for the biologically active of *C2DPs* family members, including responses to environmental stress, growth regulation and other functions (Fig. 4A).

KEGG enrichment analysis identified 90 *LcC2DPs* genes across 13 pathways, primarily involving material metabolism: substance metabolism and transportation, ubiquitin system, coenzyme and protein metabolism, genetic information processing, pantothenic acid biosynthesis, gene transcription. This indicates that the metabolism, ubiquitin system, and gene transcription mechanism are key pathways enriched for *LcC2DPs* genes (Fig. 4B).

Gene clustering group

Under abiotic stress, 90 *LcC2DP* genes were grouped into different clusters[47]. In stem node tissue, gene expression showed three patterns: 53 genes were up-regulated (red), 25 were down-regulated (blue), and 12 showed no change (yellow), representing 58.89%, 27.8%, and 13.33% of the total, respectively. Over 0, 3, 6, 9, and 12 days, up-regulated genes numbered 36, 28, 43, 39, and 19, while down-regulated genes were 42, 49, 39, 35, and 39. Twelve genes showed no significant expression change over 12 days

(Fig. 4C). The *LcC2DPs* family, expressed in various plant tissues, plays diverse roles in cellular stress response, mainly by regulating pathways.

Gene expression pattern

qRT-PCR analysis of 10 *LcC2DPs* genes screened in the root, stem, and leaf of *Lotus corniculatus* confirmed their expression in all these organs (Fig. 5A). The expression levels of certain genes, like *LcC2DP1*, *2*, *24*, *43*, *60*, *61*, and *62* ($p < 0.05$, $p < 0.01$ or $p < 0.001$), were notably higher in stems and leaves compared to roots. *LcC2DP8* ($p < 0.001$) and *LcC2DP*31, 36 ($p < 0.001$) were primarily expressed in stems and leaves, respectively. This suggests that *LcC2DPs* genes play a crucial roles across root, stem, and leaf.

Analysis of ABA-responsive (ABRE) and IAA-responsive (AuxRR-core) elements in *Lotus corniculatus C2DPs* family showed that treating plants with 100 μM ABA and IAA improved the growth over 15 days (Fig. 5B). Samples were collected at various growth stages for qRT-PCR analysis (Fig. 5C). Results showed that *LcC2DPs* family members respond to ABA and IAA hormones, with expression patterns varying over 0 to 15 days, forming four groups. The first group (*LcC2DP*1, 24, and 31) had higher early expression with ABA (0–9 or 0–6 days) than IAA. The second group (*LcC2DP*2, 8, 36, 43, and 61) showed opposite, higher expression trends for IAA and ABA throughout. The third group, *LcC2DP*60, exhibited notable transcription fluctuations in response to both hormones across all stages (0–6, 6–12, and 12–15 days). The fourth group, *LcC2DP*62, showed higher gene expression under ABA treatment than IAA throughout the 0–15 period.

Cis-acting elements in gene promoters can help analyze gene expression patterns and functions. To investigate the *LcC2DPs* gene family's functions, *Lotus corniculatus* was exposed to PEG6000 for drought simulation (0, 3, 6, 9, 12, and 15 days) and 4°C for cold stress (0, 6, 12, 24, 48, and 72 hours). Under drought, plant showed leaf curling, poor growth, and slow height increase (Fig. 6A-a). In cold conditions, wilting began after 12 hours, worsening in the upper leaves, and by 72 hours, the *Lotus corniculatus* drooped (Fig. 6A-b). qRT-PCR analysis of *LcC2DP* genes in young leaves under drought and low-temperature conditions revealed differences in the expression of 10 genes (Fig. 6B). Compared to the initial expression, the *LcC2DP1* gene showed fluctuating expression, being up-regulated in the early stages (3–6 days, $p < 0.001$) and down-regulated later (12–15 days, $p < 0.001$ or $p < 0.01$). *LcC2DP8* was up-regulated during the middle to later phase (6–15 days, $p < 0.01$ or $p < 0.001$), while *LcC2DP24, 31, 43*, and *60* consistently showed increased expression throughout ($p < 0.05$, $p < 0.01$ or $p < 0.001$). *LcC2DP60* and *LcC2DP61* were up-regulated in the early to middle stages (3–12 days, $p < 0.05$, $p < 0.01$ or $p < 0.001$).

Under the 4 ℃ environment, the expression of *LcC2DPs* family genes in *Lotus corniculatus* varied (Fig. 6C). *LcC2DP1* was significantly up-regulated between 12 and 48 hours ($p < 0.05$), while *LcC2DP24* showed up-regulation only at 72 hours ($p < 0.01$). *LcC2DP8* and *LcC2DP36* exhibited an ‘increase-decrease-rise’ pattern. *LcC2DP43* and *LcC2DP60* were continuously down-regulated from 6 to 72 and 12 to 72 hours, respectively. Notably, *LcC2DP61* showed no significant expression changes, indicating that

LcC2DP61 is not induced by low temperature for transcription. *LcC2DP62* in plant cell was up-regulated at 6–72 hours. Results showed that in addition to *LcC2DP61*, *9* other genes are involved in cold response in *Lotus corniculatus*.

Regulatory pathways

The C2DPs protein kinase family creates complex networks for cellular adaptation to stress[48]. To explore the cooperative or division of labor among *C2DPs* family members, they were analyzed using STRING (STRING://cn.string-db.org) for PCC statistics. Results showed that five core proteins, LcC2DP5, 11, 15, 38 and 58, primarily control the regulatory pathways among 90 family members, with 27 members interacting to varying extents with these core proteins (Fig. 7A). Results indicates that LcC2DP1, 20, and 67 proteins interact with XLG2 protein from the G-alpha superfamily, which acts as an energy-releasing engine in signal transduction[49]. The activation and conformational changes in *LcC2DPs* depend on XLG2 activation.

To confirm the response of five core genes to drought and low-temperature stress, qRT-PCR was used to measure the expression of *LcC2DP5*, *11*, *15*, *38*, and *58*. The study found that all five core genes responded to drought stress, initially increasing and then decreasing. Gene expression for *LcC2DP5* and *15* increased continuously from 0 to 9 days, *LcC2DP11* and *38* from 0 to 6 days, and *LcC2DP58* from 0 to 3 days. *LcC2DP11* showed a significant decrease at 12 days compared to day 0 ($p < 0.05$). *LcC2DP11*, *38*, and *58* had notable expression changes from 3 to 6 days ($P < 0.01$, or $P < 0.05$). *LcC2DP5* showed the most significant expression from 9 to 15 days ($p < 0.001$), while *LcC2DP15* was significantly expressed from 6–12 days ($P < 0.001$) (Fig. 7B). Five core genes also responded to low-temperature. *LcC2DP5*, *11*, and *38* continuously increased from 0 to 72 hours at 4 ℃, while *LcC2DP15* and *LcC2DP58* peaked at 72 and 24 hours, respectively. *LcC2DP5*, *11*, and *38* showed differential expression from 6 to 48 hours ($P < 0.001$, $P < 0.01$ or $P < 0.05$) and decreased after 72 hours. *LcC2DP11* and *LcC2DP15* were significantly expressed from 48 to 72 hours ($P < 0.001$). *LcC2DP58* was significantly expressed only at 24 hours ($P < 0.001$) (Fig. 7C).

Co-expression transcription factor

Cell regulates its physiological process by TFs acting as mediators[5]. To explore the abiotic stress response mechanism of *LcC2DPs* family members, a Python script was used to identify co-expressed TFs for five core proteins. Only LcC2DP5 and LcC2DP15 provided co-expressed data, suggesting they are key in regulating abiotic stress responses within the *LcC2DPs* family (Fig. 7D). This analysis was based on FPKM data from our existing transcriptome using a Python script[47], we conducted co-expression analysis of transcription factors ($r > 0.9$). The study identified 49 types TFs, totaling 134, co-expressed with *LcC2DP5* gene. The top eight TF types by member count include *bHLH* (8), *WRKY* (7), *C3H* (7), *bZIP* (7), *MYB* (5), *MYB_related* (5), *C2H2* (5), and *NAC* (5). Analysis showed the *LcC2DP15* gene is co-expressed with 38 types, totaling 125, including *bHLH* (15), *WRKY* (14), *MYB_related* (10), *NAC* (9), *bZIP* (9), *C2H2* (7), and *C3H* (7) (Table S3). These TF families are known to play roles in abiotic stress

responses[23,50–56], suggesting that *bHLH*, *WRKY*, *C3H*, *bZIP*, *MYB*, *NAC*, *C2H2*, and *MYB_related* are key pathways for *LcC2DPs* family in mediating abiotic stress regulation in plants.

Expression of transcription factors

Differential expression analysis of key abiotic stress-related TFs (*bZIP*, *bHLH*, *WRKY*, *NAC*, *MYB_related*, *MYB*, *C3H*, and *C2H2*) in *Lotus corniculatus* under PEG-induced drought showed that all eight TFs were up-regulated (Fig. 8A). The *bHLH* was significantly expressed only at the initial stage (3 days) ($P < 0.001$) before decreasing. *MYB_related* was significantly expressed during both the initial and middle stages (3–9 days) ($P < 0.001$ or $P < 0.01$), with reduced expression after 9 days ($P < 0.01$). In contrast, other TFs like *bZIP*, *WRKY*, *NAC*, *MYB*, *C3H*, and *C2H2* were up-regulated throughout the stress period (3–15 days) ($P < 0.001$, $P < 0.01$, or $P < 0.05$), with *bZIP*, *NAC*, and *C2H2* TFs showing the most significant differential expression in response to drought ($P < 0.001$). In *Lotus corniculatus* exposed to low temperature (4°C), all eight TFs (*bZIP*, *bHLH*, *WRKY*, *NAC*, *MYB_related*, *MYB*, *C3H*, and *C2H2*) co-expressed with *LcC2DPs* were induced by cold stress (Fig. 8B). *MYB_related* showed differential expression in early and middle stages (6–24 hours) ($P < 0.001$), and *MYB* in initial stage (6–12 hours), both peaking at 6 hours ($P < 0.001$). *NAC* and *C3H* were active during early and middle phases (6–48 hours) ($P < 0.01$ or $P < 0.05$).

Analysis of core TFs linked to *LcC2DPs* under drought and low temperature stress showed that *bZIP* was consistently up-regulated throughout both conditions (3–12 days for drought and 6–48 hours for low temperature) ($P < 0.001$). *bHLH* was up-regulated early in drought (3 days) ($P < 0.001$) and entire low temperature exposure (6–72 hours) but down-regulated later in both stresses (15 days for drought and 72 hours for low temperature) ($P < 0.001$ or $P < 0.05$). *WRKY* was up-regulated during 3–6 and 12–15 days of drought, and at 6 and 24–72 hours of low temperature treatment ($P < 0.001$, $P < 0.01$ or $P < 0.05$). *NAC* was consistently up-regulated during drought period (3–15 days) ($P < 0.001$) but down-regulated in later stage (72 hours) under low temperature, showing different regulatory patterns for each stress. *MYB_related* had varied expression early on, then gradually decreased. The transcription of *MYB* differed between stresses, increasing under drought and decreasing under low temperature ($P < 0.001$, $P < 0.01$, or $P < 0.05$). *C3H* and *C2H2* showed differential expression patterns under drought and low temperature stress. *C3H* was up-regulated early and mid-stress ($P < 0.001$ or $P < 0.01$), while *C2H2* was consistently up-regulated ($P < 0.001$ or $P < 0.05$). Under drought, TFs expression ranked: *NAC* > *C2H2* > *WRKY* > *bZIP* > *C3H* > *MYB* > *MYB_related* > *bHLH.* In low temperatures, it was: *bZIP* > *MYB_related* > *C2H2* > *C3H* > *NAC* > *bHLH* > *MYB* > *WRKY*. *NAC* and *C2H2* were key under drought, while *bZIP* and *MYB_related* were crucial under low temperature.

Discussion

The continuing warming of global climate has severely affected grass growth and development. Research into genes involved in stress regulation will be essential for grass molecular breeding. This paper identifies and analyzes 90 *C2DP* genes in *Lotus corniculatus* genome, which are linked to stress responses in rice (*Oryza sativa* L)[57], cotton (*Gossypium hirsutum* L)[58] and *Arabidopsis* (*Arabidopsis*

thaliana)[59] were found in of *Lotus corniculatus* (Table S1). The *LcC2DP* gene family is larger than in rice, cotton and *Arabidopsis*, which have 82, 31, and 16 *C2DP* genes, respectively. Rich functionality in a gene family is contingent upon its member numbers[60]. The complexity and diversity of *C2DPs* gene family suggest that variations among plant species may result from gene loss and duplication, key factors in gene family evolution. These processes can lead to the emergence of new genes with unique functions[61].

After identifying *LcC2DPs* family, their physicochemical properties were examined (Table S1). Subcellular localization showed that LcC2DPs proteins in the cell nucleus and cytoplasm, similar to CDPK in other plant[62]. This suggests that LcC2DP proteins activate transcription factors and regulate gene expression in response to signals, like other protein kinases. The division of labor family members is linked to their nuclear and cytoplasmic localization, affecting their role in signal transduction.

LcC2DP genes primarily cluster on chromosomes 1, 2, 3, and 4 (Fig. 2A), indicating possible tandem duplications in these regions of the *Lotus corniculatus* genome[63]. Such duplications are key in stress resistance, membrane function in rice and *Arabidopsis*, and signal transduction in legumes[64–66]. This implies that many *LcC2DP* genes play roles in stress responses and cellular signal transduction, potentially explaining the large number of family members in plants like rice and sweet potato, and suggesting interactions between chromosomes. Phylogenetic analysis based on gene structure classified 90 family members into five groups (Fig. 2B). The *C2DP* genes of *Arabidopsis* were similarly divided into five groups[59], confirming our analysis. *C2DPs* genes in *Lotus corniculatus* outnumber those in cotton and rice[57–58]. *C2DPs* were grouped into 5 in *Arabidopsis* and 7 in rice, with rice having fewer members than *Lotus corniculatus*. Gene functional redundancy has emerged during evolution. Gene duplication is a key driver of new gene functions but can cause redundancy. This redundancy helps maintain metabolic balance across environmental changes and development[67]. The study indicates that under purifying selection, duplicated genes varied expression or specialization supports plant adaptation to diverse environments[68].

Genome analysis shows that tandem duplication plays a crucial role in gene family expansion by generating new genes and clusters[69], In this study, tandem duplication analysis identified 10 pairs of collinear homologous genes within *Lotus corniculatus* and 34 pairs between *Lotus japonicas* and Arabidopsis (Fig. 2D). This suggests that tandem duplication has contributed to gene expansion in the *Lotus corniculatus* genome, potentially aiding its adaptation to complex environmental conditions and physiological functions compared to plant like potato[48]. Collinearity analysis revealed only 20 homologous gene pairs between *Cucumis melo*, *Vitis vinifera*, and *Arabidopsis thaliana*[41]. Our findings indicate a strong evolutionary relationship between the *LcC2DPs* gene family and Arabidopsis, suggesting similar functions. Overall, *Lotus corniculatus* shares a similar evolutionary process and genetic trait variation with Arabidopsis and other organisms.

Cis-acting elements in promoters can elucidate gene expression regulation, cellular signaling network, and gene-environment interactions[70]. The prediction of cis-acting elements in *LcC2DP* promoter regions identified multiple hormone (AuxRR-core and ABRE) and stress response elements (MYC, MBS, ARE, TC-rich, and MYB) were identified (Fig. 3). Evidence suggests that *AtCDPKs* is involved in external stimuli responses with ABA[71– 72]. In *Arabidopsis*, *CDPKs* have been linked to abiotic stresses like drought, cold, heat, and salinity[10, 72]. This indicates *LcC2DP* family genes may play a complex role in stress responses related to plant hormone regulation. Changes in *LcC2DPs* gene expression under hormone and abiotic stress were analyzed using qRT-PCR. This study confirms that *LcC2DPs* is involved in plant hormone and abiotic stress responses, including ABA, IAA, drought, and low temperature.

CDPK regulate ABA-mediated signaling and influence phytohormones responses, such as IAA and cytokinins (CTK)[73–74]. qRT-PCR results showed *LcC2DP*'s sensitivity to 100 μM ABA and IAA in *Lotus corniculatus* (Fig. 5C), leading to its classification into four groups based on the hormones response differences over time. Specifically, *LcC2DP1*, *24*, *31*, *LcC2DP2*, *8*, *36*, *43*, *61*, *LcC2DP60*, and *LcC2DP62* belong to groups 1, 2, 3, 4, respectively, according to RNA-seq data[47]. CDPK proteins are crucial for signal transduction, gene expression, growth, and development by binding to Ca^{2+}, and they enhance plant resilience to various stresses[75–76]. Our study highlights the diverse biological roles of protein kinases. In grape and rice[73,30], *VpCDPK16* and *OsCPK21* respond to ABA signaling, while IAA affect CDPK expression and activity, with *NtCDPK1* transcripts increasing upon IAA treatment[77]. The expression level of different *LcC2DP* genes in *Lotus corniculatus*, treated with ABA and IAA, suggest a potential role in hormone signaling regulation.

The cis-acting elements analysis and qRT-PCR assays on tender leaves exposed to PEG6000 and low temperature (4 ℃) revealed that 10 gene showed expression differences under drought and cold conditions. These genes exhibited varying expression patterns during drought treatment, categorized into four models: *LcC2DP1*, *24, 31*; *LcC2DP2, 8, 36, 43*, and *61*; *LcC2DP60*; *LcC2DP62* groups (Fig. 6B). CDPK are crucial for *Arabidopsis* under drought and other stresses[71]. For example, *GhCDPK60* enhances drought resistance[78], while *GhCDPK4* boosts tobacco's drought tolerance[79]. In conclusion, *LcC2DPs* family is closely linked to drought response and exhibits complex regulation in *Lotus corniculatus.*

CDPK proteins have diverse roles in responding to abiotic stresses like cold, salinity, and heat in plants[34, 71]. *LcC2DP1, 2, 24*, *8*, *31, 36*, *60*, and *62* genes show varied expression at different stages under low-temperatures (Fig. 6C). *LcC2DP61* showed no significant changes throughout the process and was unaffected by low temperature. *LcC2DP61*'s expression did not align with its promoter's 'defense and stress responsiveness' elements, possibly due to other regulatory like trans-acting factor[80]. Besides, *LcC2DP43* expression significantly decreased at each stage in 4 ℃ environment, indicating a unique transcription model pattern. *ShCDPK12* and *ShCDPK30* function differently in response to cold stress in *Solanum habrochaites* and Arabidopsis[34]. *CDPKs* respond to abiotic stresses like drought, heat, salinity,

and cold[71]. This study reveals that *LcC2DPs* reacts differently to low-temperature, possibly due to their abundance of stress-related cis-elements and family members.

Protein-protein interactions are crucial in biological pathways[81]. Using the online tool STRING (available at //cn.string-db.org) with Cytoscape for predicting and visualizing the interactions among *LcC2DPs* family members. Analysis revealed that this family has five core proteins (*LcC2DP5*, *11*, *15*, *38*, and *58*). *LcC2DP1*, *LcC2DP20*, and *LcC2DP67* interact with XLG2, a G-alpha superfamily member (Fig. 7A). XLG2 and G-alpha protein positively influence hormone signal transduction like IAA, ABA, and gibberellin (GA), affecting cell division, developmental, and stress response in plants[82–83]. Thus, *LcC2DP* family genes are crucial in plant hormone signaling and stress response pathways.

The TFs bind to cis-regulatory elements in target gene regions to regulate plant growth, development, and stress responses[84]. A Python script identified co-expressed TFs for five core proteins, revealing that only LcC2DP5 and LcC2DP15 provided insights with co-expressed TFs. This suggests they are the central genes in the *LcC2DPs* family involved in plant abiotic stress (Fig. 7D). Co-expression analysis identified eight TFs (*bZIP*, *bHLH*, *WRKY*, *NAC*, *MYB_related*, *MYB*, *C3H*, and *C2H2*) in the regulation network of *LcC2DP5* and *LcC2DP15* for drought and 4 ℃ responses. Among them, *bHLH* and *MYB* regulate drought, *NAC* and *MYB_related* regulate low temperature, while *bZIP*, *C2H2*, *WRKY*, *and C3H* form a common regulatory network under these stresses. The qRT-PCR analysis confirmed these findings after PEG6000 and 4 ℃ treatments.

NAC (NAM, ATAF1/2, and CUC2) and MYB proteins are important factors in the stress response[11, 85]. Our study showed that *NAC* and *MYB* expression increased after 20% PEG treatment for 15 days in *Lotus corniculatus* (Fig. 8A), highlighting their role as key stress response regulators. As a crucial regulator in heat and drought stress responses, *SNAC3*, in particular, enhances plant tolerance to heat and drought[85]. Overexpressing *TaMYB33* and *GbMYB5* genes enhances the drought tolerance in *Arabidopsis* and tobacco[86,26], indicating that *NAC* and *MYB* transcription factors are vital for drought resistance in *Lotus corniculatus*. Low temperature pose significant environmental challenges, impacting plant physiology and yield[87]. This study shows that *bHLH* and *MYB-related* are highly expressed at 4°C in *Lotus corniculatus* (Fig. 8B), with *bHLH* family members playing a key role in responding to cold stress by regulating cold -responsive genes like *COR* (cold-responsive) and *CBF* (cold-regulated binding factor)[88–90]. Simultaneously, the *MYB_related* family is crucial in responding to low temperature stress. Research shows that *AhMYB30* from peanut *MYB_related* family enhances the freezing tolerance in transgenic *Arabidopsis* via DREB/CBF and ABA pathways[50]. Our findings indicate that *bHLH* and *MYB_related* transcription factors are key in regulating *Lotus*'s response to low temperature.

Notably, high expression of *bZIP*, *WRKY, C3H*, and *C2H2* was detected in both drought and cold conditions, suggesting their role in relating these stresses in *Lotus corniculatus* (Fig. 8A, B). Various TF families, such as *bZIP*[91], *WRKY*[56] *C3H*[92] and *C2H2*[27], are crucial in regulating plant responses to stress, particularly cold and drought, by enhancing tolerance. Our findings support these roles. *bZIP*, *WRKY, C3H*,

and *C2H2* genes are activated by cold and drought stress. While *bHLH*[93], *bZIP*[94], *NAC*[54], *MYB_related*[22], *MYB*[5], and *C2H2*[95]genes are found in both the nucleus and cytoplasm, *WRKY*[96] and *C3H*[92] genes are localized in the nucleus. This study implicates that *NAC*, *C2H2*, *bHLH*, *MYB*, *bZIP*, and *MYB_related* TFs move to the nucleus after cytoplasmic activation, whereas *WRKY* and *C3H* are activated in the nucleus to regulate gene expression.

C2DP, a calcium-dependent protein kinase, is activated by Ca^{2+} during stress signal transduction, leading to phosphorylation and related TFs expression for stress response[97]. *ZmNAC84* phosphorylation activates downstream genes by CDPK to participate in ABA-induced antioxidant defense[98]. Additionally, *MYB* is involved in the ABA signaling pathway for drought tolerance, regulated by PYR/PYL/RCARs, protein phosphate 2C (PP2Cs), and SNF1-related protein kinase 2s (nRK2s) in *Arabidopsis*[60, 99]. *bHLH* plays a role in plant's response to low temperatures through CDPK phosphorylation pathways[100]. MAPK (mitogen-activated protein kinase) activation enhances the cold resistance via *MYB-related* under low temperature stress[101]. It is suggested that *LcC2PDs* phosphorylate activates *NAC*, *MYB*, and *bHLH*, *MYB-related* to improve drought and cold stress tolerance in *Lotus* plants. Additionally, *bZIP*, *WRKY*, *C3H*, and *C2H2* are involved in both drought and cold stress responses (Fig. 9). Understanding these TFs can provide insights into plant abiotic regulation, aiding in the functional identification and analysis of stress regulation genes in forage crops.

Conclusion

This study used bioinformatics and experiments to identify and analyze the LcC2DP protein kinase family in *Lotus corniculatus*, finding 90 *LcC2DP* genes divided into five subgroups. Homologous genes pairs were present in both in *Lotus corniculatus* and Arabidopsis chromosomes. The *LcC2DPs* promoters contained hormone response elements (AuxRR-core, ABRE) and stress response elements (MYB, MYC, MBS) for cold and drought. GO enrichment revealed hormone response and kinase activity functions, and 13 KEGG pathways were identified. RNA-seq analysis showed 78 *LcC2DPs* related to stress response, with 10 genes linked to ABA, IAA, drought, and low temperature through qRT-PCR. Protein-protein interaction analysis identified five core proteins, with LcCDP5 and LcC2DP15 co-expressed with various TFs, including *bZIP*, *bHLH*, *WRKY*, *NAC*, *MYB_related*, *MYB*, *C3H*, and *C2H2*, under stress conditions. *NAC* and *C2H2* primarily respond to drought stress, while *bZIP* and *MYB_related* respond to low temperature. These findings form a basis for exploring *LcC2DP* family gene mechanisms in drought and cold stress responses.

Declarations

Declaration of Competing Interest

The authors declare no competing interests.

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Author Contribution

Author contribution LS conceived and designed the experiments. GY performed the experiments. GY carried out the bioinformatics analysis. GY wrote the manuscript. YL and SC gave insightful suggestions. LS improved the manuscript errors and English language. All authors read and approved the manuscript.

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Data Availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center, Beijing Institute of Genomics (China National Center for Bioinformation), Chinese Academy of Sciences, under accession number CRA002426 that are publicly accessible at https://bigd.big.ac.cn/gsa.

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Figures

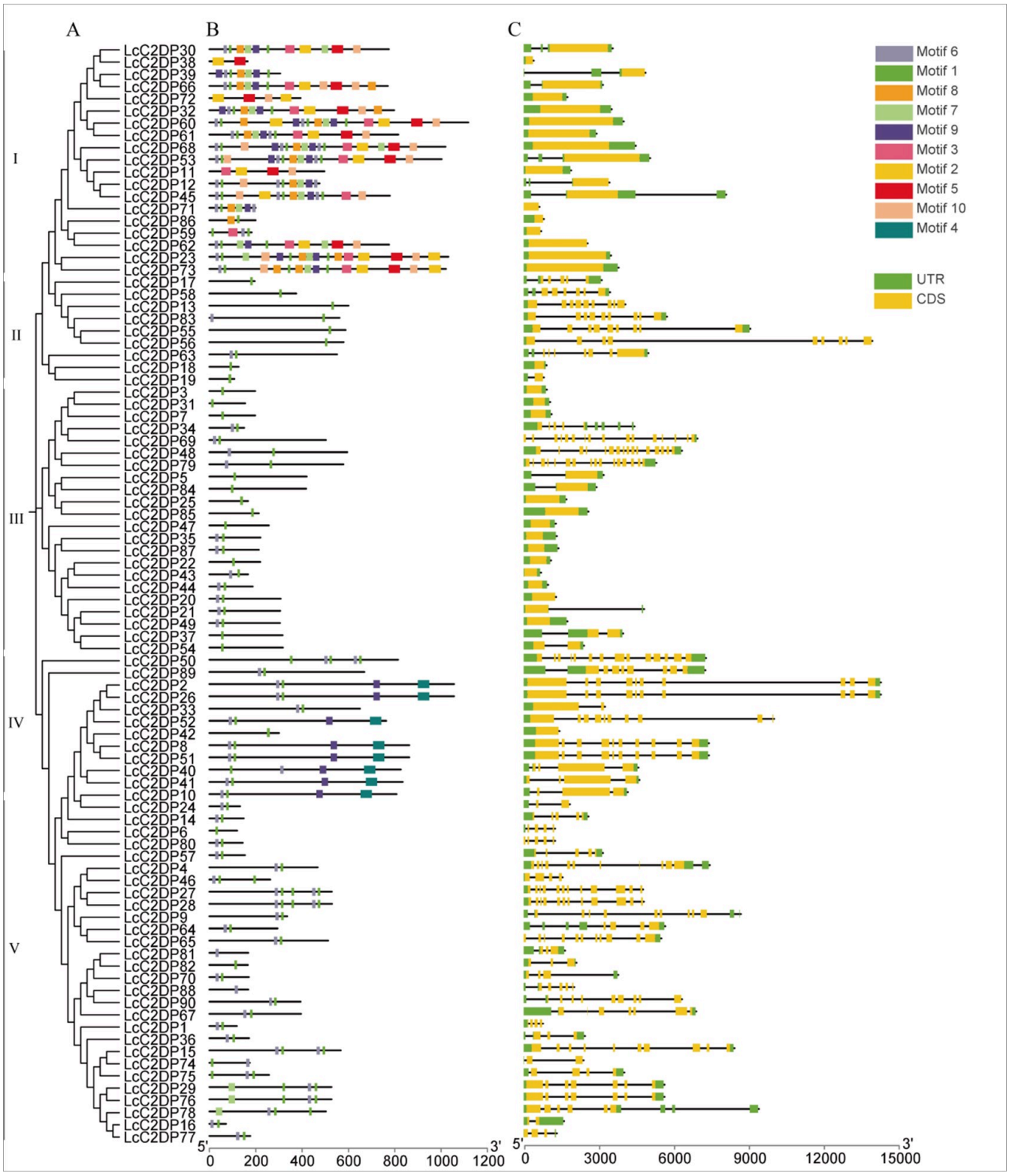

Figure 1

Phylogenetic relationship, gene structure, and motif composition of *LcC2DPs*. (A) Rootless phylogenetic tree from MEGA7.0. (B) Motifs 1-10 shown in different colors. (C) Exon-intron structure: green for UTR, yellow for CDS, black for intron.

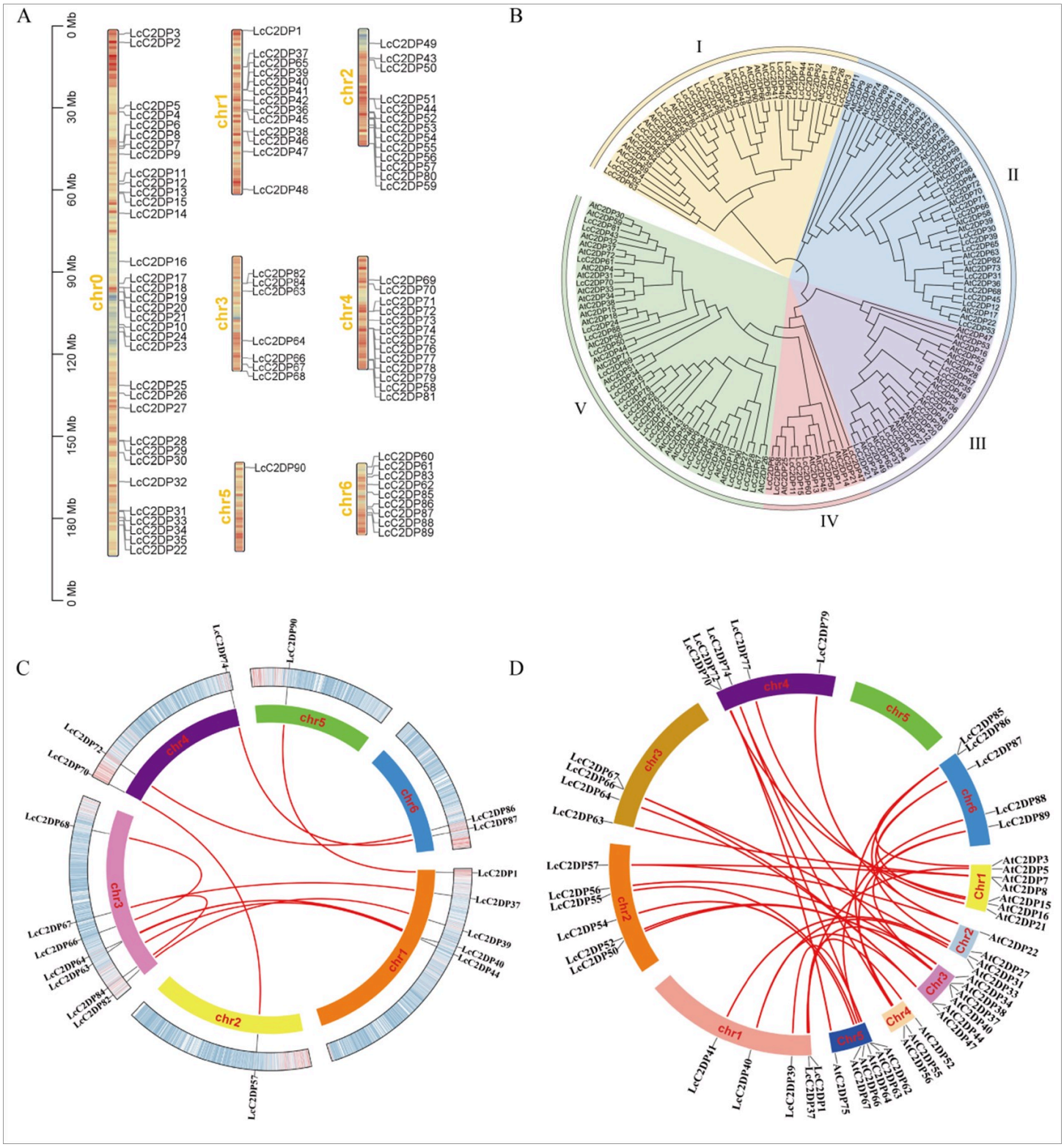

Figure 2

Chromosomal localization, phylogenetic, and collinearity analysis of *LcC2DPs*family. (A) Chromosomal positions with length scale. (B) Phylogenetic tree with colored groups using Neighbor-joining (NJ) method and 1000 Bootstrap replications. (C) Intra-specific collinearity with red lines for duplicated genes; colored rectangles for chromosomes; blue circle for gene density. (D) Inter-specific collinearity: Chr1-5 for *Arabidopsis*, chr1-6 for *Lotus*, red lines for duplicated *LcC2DPs* and *AtC2DPs*.

Figure 3

Cis-regulatory element analysis of *LcC2DPs* promoter regions.

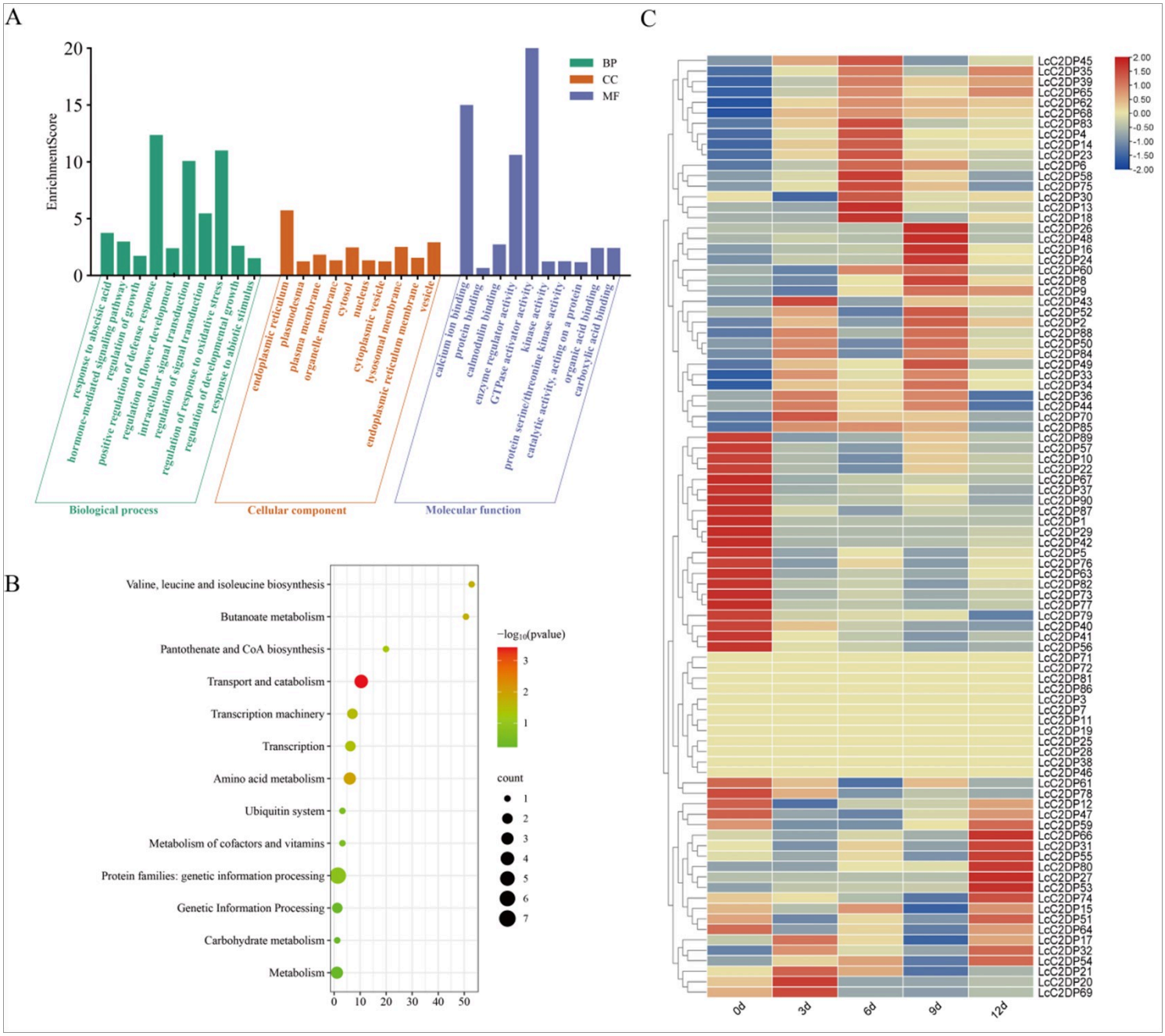

Figure 4

Analysis of *LcC2DPs*gene family with GO (A) and KEGG (B) enrichment, where dots size and color indicategene numbers and q-value. (C) Heatmap of *LcC2DPs*expression under submergence stress over time.

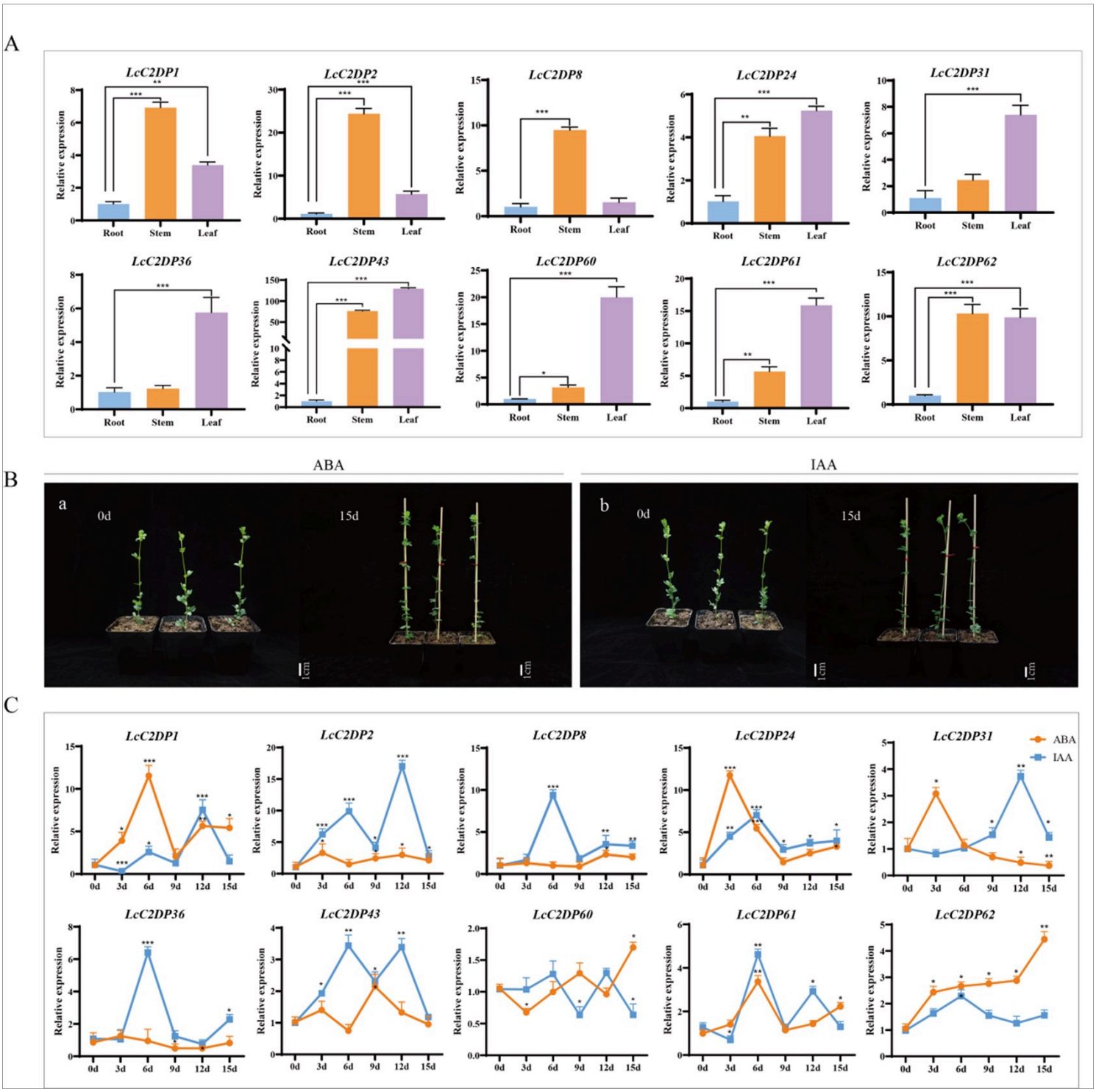

Figure 5

LcC2DPs gene expression profiles. (A) Expression in roots, stems, and leaves. (B) *Lotus* phenotype after 100 mM ABA and IAA treatment. (C) qRT-PCR expression levels post hormone treatment. Data are mean ± SD (n=3). Significance assessed by t-test. *, p<0.05; **, p<0.01; ***, p<0.001.

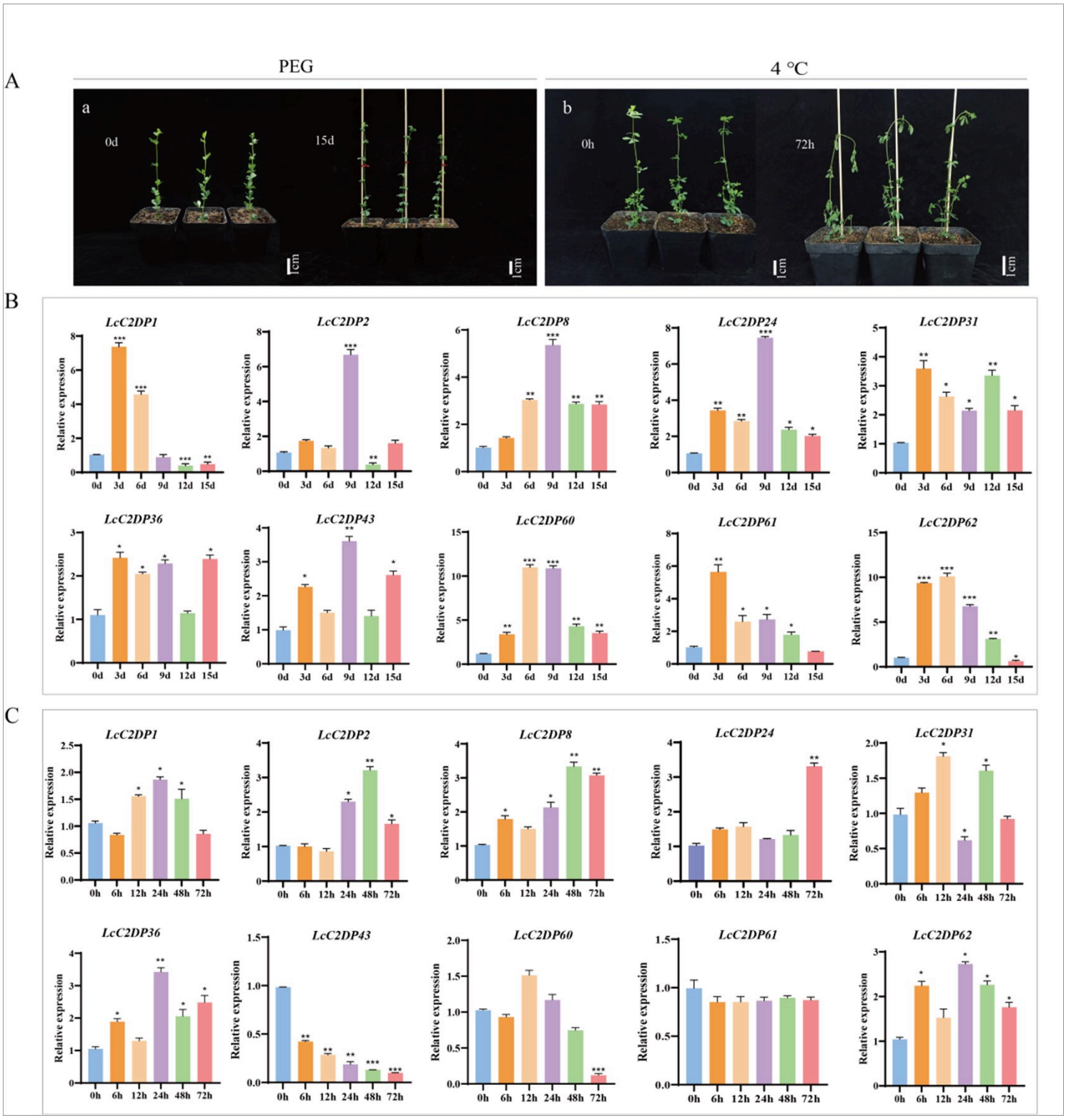

Figure 6

Plant phenotypes and *LcC2DPs* expression with 20% PEG and 4 °C treatments. (A) Phenotype comparison. (B) Expression under drought. (C) Expression in low temperatures. Data are mean ± SD (n=3). Significance assessed byt-test: *, p<0.05; **, p<0.01; ***, p<0.001.

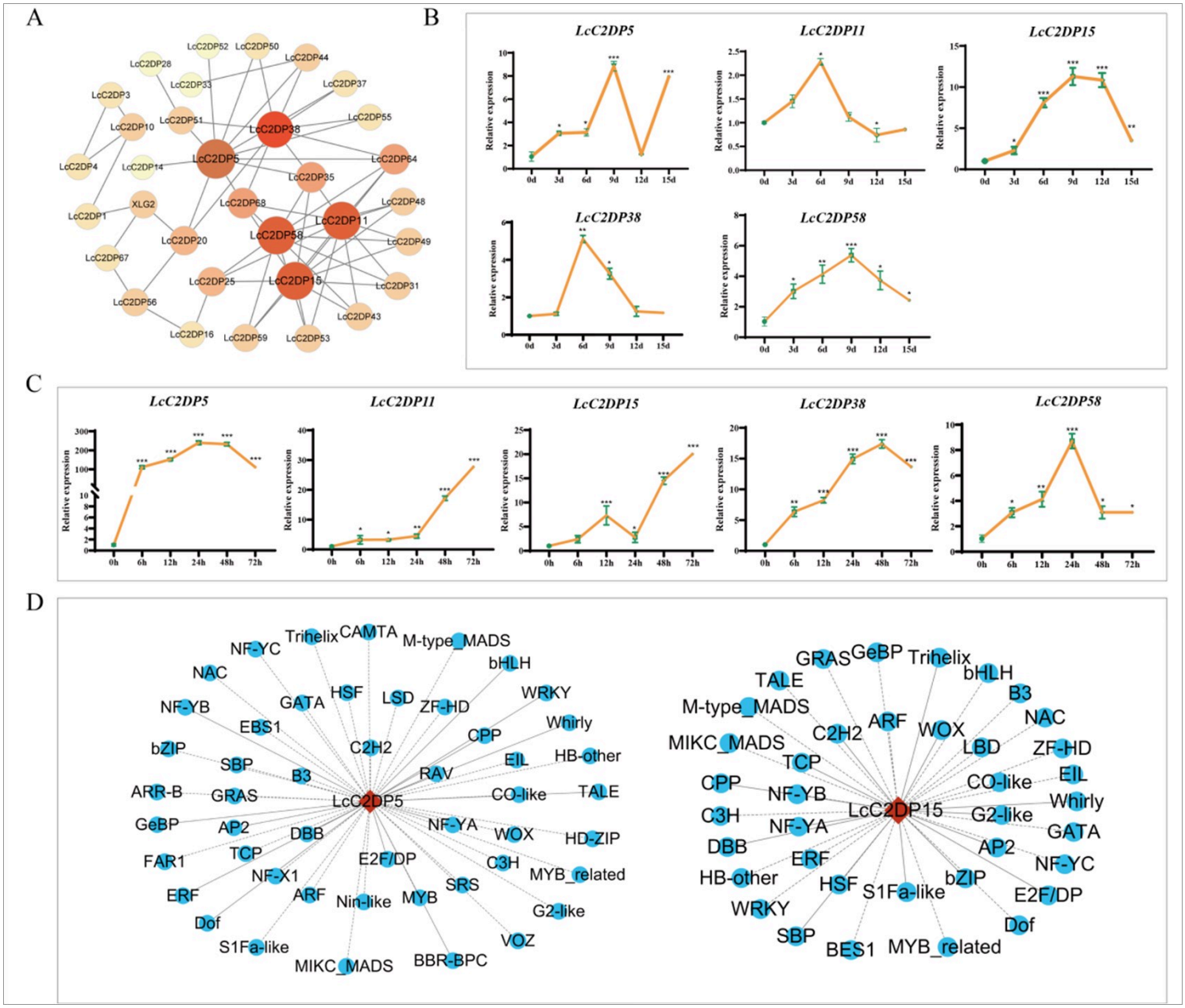

Figure 7

Analysis of protein-protein interactions, core gene expression, and co-expression. (A) Protein interactions. Expression of *LcC2DP5*, *11*, *15*, *38*, and *58* under drought (B) and low temperature (C). (D) Co-expression of *LcC2DP5* and *LcC2DP15*, with solid lines for positive and dashed for negative correlations.

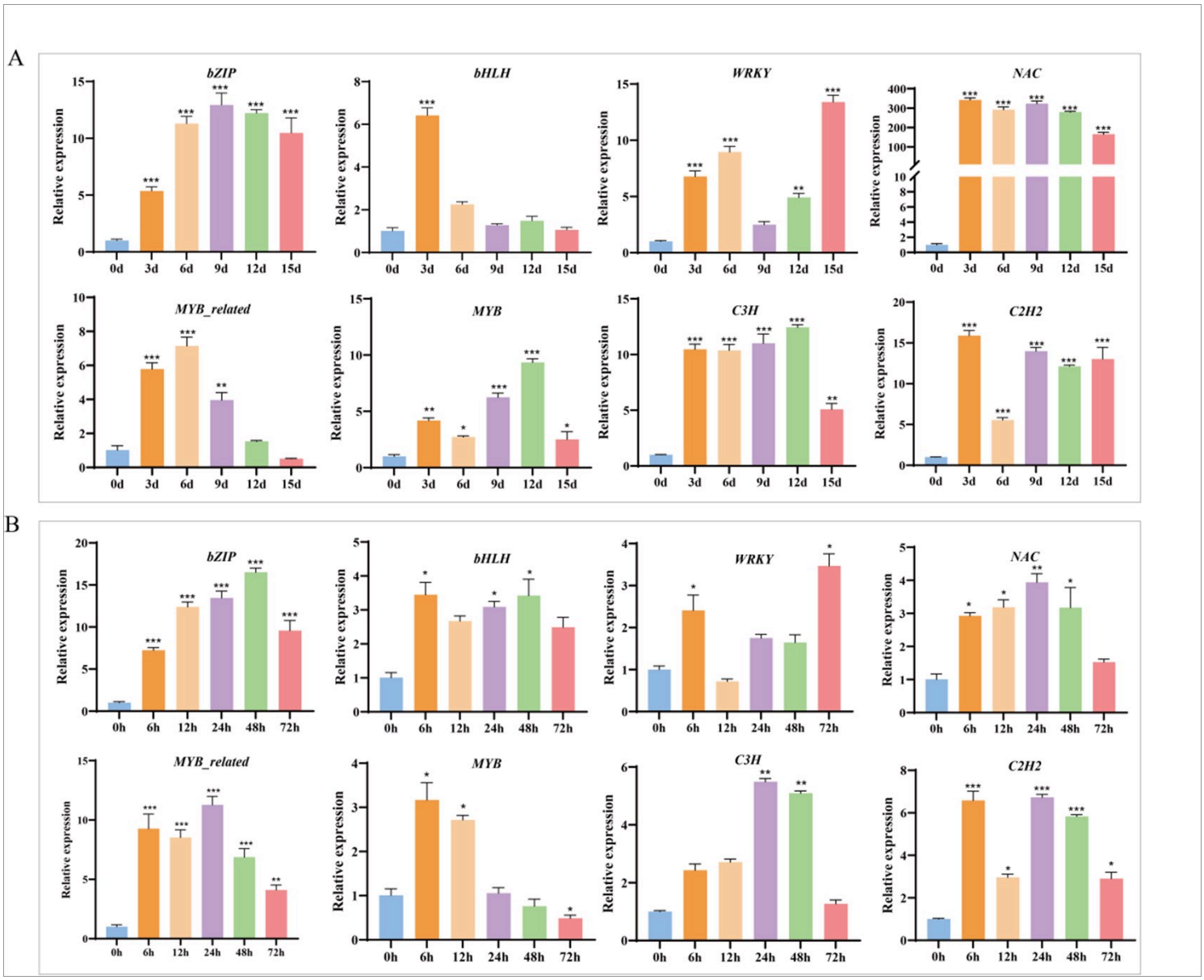

Figure 8

TF expression via qRT-PCR under drought (A) and low temperature (B), with significance assessed by T-test. Data are mean ± SD (n=3). Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

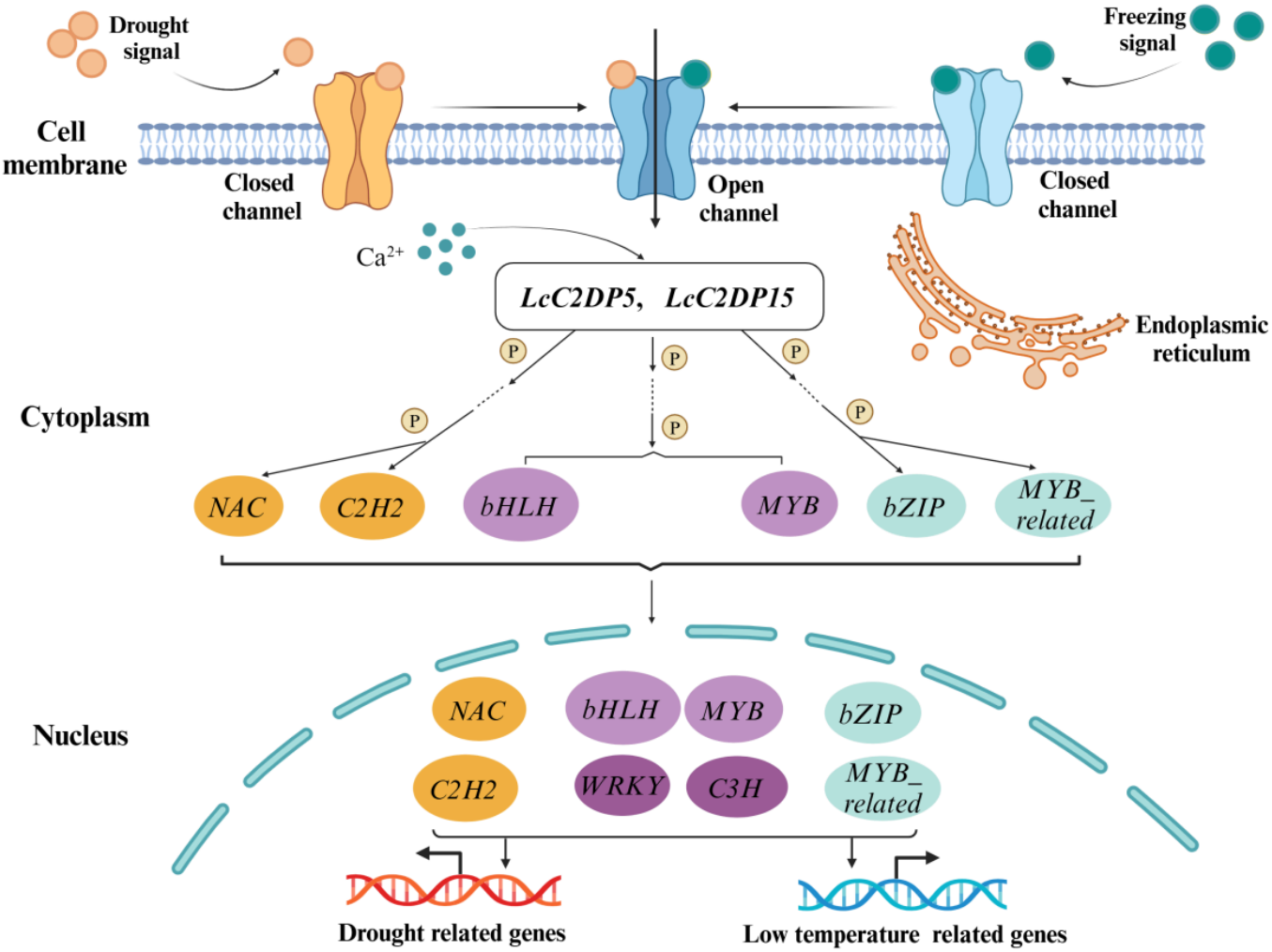

Figure 9

Transcriptional regulatory network of *LcC2DP5* and *LcC2DP15* under drought and low temperature.

[1] When drought and low-temperature signals are detected by membrane receptors, calcium ions are released into the cytoplasm, activating protein kinases *LcC2DP5* and *LcC2DP15*. This triggers phosphorylation cascades that activate transcription factors like bZIP, bHLH, WRKY, NAC, MYB-related, MYB, C3H, and C2H2, leading to the transcription of stress-related genes and enhancing plant resilience.

[2] *NAC*, *C2H2*, *bHLH*, *MYB*, *bZIP*, and *MYB_related* TFs move to the nucleus after activation, while *WRKY* and *C3H* are activated in the nucleus.

[3] *NAC* and *C2H2* are key in *LcC2DP5* and *LcC2DP15* pathways during drought, whereas *bHLH* and *MYB_related* are crucial in low temperature.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx