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Genome-Wide Analysis of the *CDPK* Gene Family in *Populus tomentosa* and Their Expressions in Response to Arsenic Stress and Arbuscular Mycorrhizal Fungi Colonization

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Abstract

Calcium-dependent protein kinases (CDPKs) are crucial regulators in calcium-mediated signal transduction pathways, playing a pivotal role in plant response to abiotic stresses. However, there is still limited knowledge regarding the genes of the *Populus tomentosa CDPK* family and their underlying functions in response to arsenic (As) stress and arbuscular mycorrhizal fungi (AMF) colonization. In our study, 20 *PtCDPKs* were identified in the *P. tomentosa* genome. Phylogenetic analysis categorized these *PtCDPK* genes into four subgroups based on sequence homology. Motif analysis revealed that *PtCDPK* genes within the same group share a similar exon–intron structure, conserved domains, and composition. The promoters of *PtCDPK* genes were found to contain a multitude of cis-acting elements, including light-response elements, phytohormone-response elements, and stress-response elements. The analysis of genes provided insights into the evolutionary dynamics and expansion of the *PtCDPK* gene family within *P. tomentosa*. The *PtCDPK* genes exhibited a strong collinear relationship with the *CDPK* genes of two model plants, namely, *Arabidopsis thaliana* and *Oryza sativa* L. Specifically, 10 gene pairs showed collinearity with *Arabidopsis*; in contrast, 14 gene pairs were collinear with rice. Transcriptome analysis of gene expression levels in *P. tomentosa* roots under both As stress and arbuscular mycorrhizal fungi (AMF) colonization conditions revealed that 20 *PtCDPK* genes had differential expression patterns. Under As stress, AMF inoculation led to the upregulation of 11 P*tCDPK* genes (*PtCDPKSK5*, *X2*, *1-3*, *20-1*, *24*, *26-X1-1*, *26-X1-2*, *29-1*, *29-2*, *32*, and *32-X1*) and the downregulation of 8 *PtCDPK* genes, including *PtCDPK1-1*, *1-2*, *8-X1*, *10-X4*, *13*, *20-2*, *26-X2*, and *26-X3*. The RT-qPCR results for 10 *PtCDPK* genes were consistent with the transcriptome data, indicating that AMF symbiosis plays a regulatory role in modulating the expression of *PtCDPK* genes in response to As stress. The principal findings of this study were that *PtCDPK* genes showed differential expression patterns under As stress and AMF colonization, with AMF regulating *PtCDPK* gene expression in response to As stress. Our study contributes to developing a deeper understanding of the function of *PtCDPKs* in the Ca^{2+} signaling pathway of *P. tomentosa* under As stress and AMF inoculation, which is pivotal for elucidating the molecular mechanisms underlying As tolerance in AMF-inoculated *P. tomentosa*.

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Keywords: *Populus tomentosa*; CDPK; expression patterns; arsenic stress; gene structure; phylogenetic analysis

1. Introduction

Calcium ions (Ca^{2+}) are known as a second messenger in the signal transduction of eukaryotic cells, regulating physiological processes in response to environmental stimulation [1,2]. Calcium-dependent protein kinases (CDPKs or CPKs) function as "sensor responders", directly decoding Ca^{2+} signatures into phosphorylation-mediated signaling cascades [3,4]. *CDPK* genes have been evolutionarily conserved across land plants, with the gene family expanding due to segmental duplications and functional diversification [5]. The CDPK protein has four functional domains, including an N-terminal myristoylation motif, a serine/threonine catalytic domain, an autoregulatory/autoinhibitory region, and a C-terminal calmodulin-like domain [6]. The C-terminal calmodulin-like domain contained a pair of EF-hand motifs with a helix–loop–helix configuration, and binding Ca^{2+} ions to the EF-hand motifs induces conformational changes in CDPKs, which promotes the protein kinase activity of CDPKs, alleviates autoinhibition, and activates downstream signaling cascades [1,7]. The activated CDPK protein are able to phosphorylate their substrate proteins to regulate Ca^{2+} signaling, which regulate the carbon/nitrogen metabolism, cytoskeleton, stomatal movement, and growth, while also enhancing plant resistance against biotic and abiotic stresses such as light, hormones, drought, heavy metal, salinity, and pathogen attacks [7,8].

Genome-wide investigation in model plants (such as *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays*) has revealed the functional diversity of *CDPKs* families. *A. thaliana* possesses 34 *CDPK* genes grouped into four subfamilies (I–IV), with Group I and II predominantly regulating biotic and abiotic stress responses [9]. Overexpression of *AtCPK3/4/6/11/10/23* (*CDPKs*, also termed *CPKs*) in the guard cells regulates stomatal closure via ABA signaling, which improves the drought resistance of transgenic plants [10,11]. Upregulated *AtCPK1* expression activates NADPH oxidase and phosphorylate phenylalanine ammonia lyase, ultimately leading to ABA accumulation [12]. *OsCPK13*-overexpressing transgenic rice exhibits enhanced tolerance against cold, drought, and salt stresses [13]. The upregulated expression of *OsCPK9* significantly increases the drought tolerance of rice by improving stomatal aperture regulation and osmotic adjustment capacity, thereby minimizing water loss under drought stress [14]. In maize, *ZmCPK11* exhibits dual regulation at both the enzymatic and transcriptional levels through the contribution of linolenic acid (LA) and methyl jasmonate (MeJA) to the early-phase activation of both local and systemic defense mechanisms in the touch- and wound-induced signaling pathways [15]. The ectopic expression of *ZmCPK4* in *Arabidopsis* increases the tolerance of drought stress by regulating ABA- and Ca^{2+}-dependent signaling pathways [16]. Up-regulated *CDPK* genes in *Triticum aestivum* have been detected under drought and high-salinity stress [17]. *Vitis amurensis* has been found to elevate the expression level of *CDPK* genes when subjected to cold and osmotic stresses [18]. Additionally, *CDPKs* mediate plant–microbe interactions; *Citrus sinensis CDPK* genes are expressed in citrus roots, which are related to symbiosis with arbuscular mycorrhizal fungi (AMF), and seventeen of the *CsCDPK* genes were upregulated. At the same time, *CsCDPK3*, *CsCDPK7*, and *CsCDPK28* are downregulated by *Funneliformis mosseae* inoculation under well-watered conditions; additionally, *F. mosseae* inoculation downregulates the expression of *CsCDPK20* and *CsCDPK22* under well-watered conditions but upregulates the expression under drought stress [19]. Although *CDPK* gene

families in various plants have increasingly been investigated, a comprehensive systematic analysis of the *CDPK* gene family in poplar species has not been conducted.

Arsenic (As) is a pervasive environmental metalloid contaminant, and As-exposed plants exhibit disruptions in multiple morphological, physiological, and biochemical processes, including induced calcium oscillations, oxidative damage, and inhibited nutrient uptake and plant growth [4,20]. Exposure to As also activates gene expression related to abiotic stress tolerance, detoxification processes, and the biosynthesis of secondary metabolites [21]. *GmCDPK32/68* are upregulated by As stress, correlating with enhanced superoxide dismutase (SOD) activity in soybean [2]. Upregulated *OsCPK12* in rice enhances arsenic (As) tolerance by phosphorylating vacuolar transporters to sequester As ions [4]. Four *CDPK* genes (*OsCPK4*, *-13*, *-20*, and *-21*) in rice roots have been shown to be upregulated in response to As (V) treatment [21].

Plants employ multiple strategies to mitigate As toxicity, including chelation, compartmentalization, and symbiotic associations with arbuscular mycorrhizal fungi (AMF) [8,22]. AMF are endosymbiotic fungi that have the ability to establish stable symbiotic associations within the roots of most terrestrial higher plants [23]. The mycorrhizal structures formed through the symbiosis between AMF and plant roots can enhance the growth, development, nutrient acquisition, and stress tolerance of the host plants [24,25]. AMF symbiosis activate Ca^{2+} signaling pathways in root cells, a signal perceived by *CDPKs* to regulate symbiosis-related genes [26]. However, the available literature on different *CDPK* expressions in plant responses to AMF colonization and As stress is scarce, which has limited our understanding of their roles in As tolerance and AMF symbiosis.

The genus *Populus*, belonging to the Salicaceae family, includes approximately 30–40 species widely distributed across temperate and boreal regions of the Northern Hemisphere. *Populus* species are economically valuable for timber, bioenergy production, and phytoremediation due to their rapid growth and stress-tolerant traits [27,28]. Among these, *Populus tomentosa* Carr. (Chinese white poplar) is an indigenous species of significant ecological and agricultural importance in East Asia, and this species is diploid (2n = 38), with a genome size of approximately 480 Mb, making it a tractable model for genomic studies while retaining the evolutionary complexity of perennial trees [27]. Its deep root systems and high biomass production make it an ideal candidate for phytoremediation in As-contaminated environments [29,30]. Although genome-wide identification and functional characterization of *CDPKs* have been conducted in *Arabidopsis*, *Oryza sativa*, *Zea mays*, and other plant species [10,12–14], there is little information on how the *CDPK* gene family in *Populus* species respond to As stress and AMF colonization.

In our study, we propose that AMF symbiosis will modulate the expression of PtCDPK genes, thereby enhancing the plant's tolerance to As stress. This modulation is expected to be reflected in the differential expression patterns of PtCDPK genes under As stress and AMF inoculation conditions. In the present study, members of the *P. tomentosa CDPKs* gene family were identified using bioinformatics tools, and their phylogenetic relationships, gene structures, cis-regulatory elements, correlation analysis, chromosome localization, and protein structures were systematically described by integrating various datasets. Additionally, the expression dynamics of *PtCDPK* genes under As stress and AMF colonization conditions were characterized using transcriptomic and RT-qPCR analysis. We aimed to investigate the expression dynamics of *PtCDPK* genes under both As stress and AMF colonization conditions, implicating their roles in stress adaptation and symbiotic signaling. Our results provide an essential theoretical basis for the function of *PtCDPK* in enhancing the ecological resistance of *Populus* associated with AMF symbiosis.

2. Materials and Methods

2.1. Identification of the CDPK Family Genes in Populus tomentosa

The complete genome assembly of *Populus tomentose* was retrieved from the NCBI Genome Database (https://www.ncbi.nlm.nih.gov/datasets/, accessed on 1 January 2025). The *Populus alba*, *Populus nigra*, and *Populus euphratica* sequences were also obtained from the NCBI Genome Database. *Arabidopsis thaliana CDPKs* (*AtCDPKs*) sequences were obtained from the *Arabidopsis* Information Resource (TAIR) (https://www.arabidopsis.org, accessed on 3 January 2025). The *oryza sativa* protein sequences (OsCDPKs) were downloaded from phytozome (https://phytozome-next.jgi.doe.gov, accessed on 5 January 2025). Using the *A. thaliana* CDPK protein sequence as a reference, the *P. tomentosa CDPK* (*PtCDPKs*) genes were identified through homology-based searches. Local BLASTP alignments were performed using TBtools-II (V2.210) to retrieve putative *CDPK* sequences. To validate functional domains, all candidate protein sequences were analyzed through the NCBI Conserved Domain Database (CDD) Batch Web CD-Search tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi, accessed on 8 February 2025) and the SMART database (http://smart.embl-heidelberg.de/, accessed on 10 February 2025), ensuring the presence of characteristic CDPK kinase and EF-hand calcium-binding domains. Non-redundant sequences were subsequently manually curated to eliminate entries with non-significant E-values ($\geq 1 \times 10^{-5}$) or incomplete domain architectures, retaining only high-confidence *CDPK* family members. The molecular weight (MW), isoelectric point (pI), and protein length of the protein sequences were analyzed by Expasy (https://www.expasy.org/, accessed on 15 February 2025). For all identified potential CDPK proteins, the presence of conserved EF-hand was confirmed through the NCBI Batch CD-Search tool. The subcellular localization of CDPK proteins in *Populus tomentosa* was predicted using the online tool WOLF PSORT (https://www.genscript.com/wolf-psort.html, accessed on 20 February 2025). Finally, all members of the *CDPKs* gene family were designated names based on transcriptome data of *P. tomentosa.*

2.2. Sequence Alignment and Phylogenetic Analysis

The full-length CDPK protein sequences derived from *Arabidopsis thaliana*, *Oryza sativa*, *P. alba*, *P. euphratica*, *P. nigra*, and *P. tomentosa* were aligned using the CLustalW algorithm implemented in MEGA (version 12.0) with default parameter settings. The neighbor-joining (NJ) method was employed to construct a phylogenetic tree, and nodal support was determined using 1000 bootstrap replicates. The resulting phylogenetic relationships were visualized and annotated using Adobe Illustrator (version 2024), where the tree topology was refined for enhanced graphical representation and publication-quality presentation.

2.3. Conserved Motifs, Gene Structure, and Cis-Acting Elements

Conserved protein motifs were identified using the MEME Suite (version 5.5.8; http://meme-suite.org/tools/meme, accessed on 3 February 2025) using optimized search parameters: zero or one occurrence of each motif per sequence, setting a maximum of 15 motifs, and defining an optimal motif width range of 6–50 amino acid residues. All other parameters retained default configurations. The exon–intron structures of *PtCDPK* genes were elucidated using TBtools by aligning their coding sequences (CDS) with the corresponding genomic DNA sequences, allowing for the visualization of structural features such as exon positions and intron phase. The 2000 bp regions upstream of the transcription initiation sites of the candidate genes were retrieved from the genome sequences of *P. tomentosa*. These sequences were subsequently analyzed using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 20 February 2025)

to identify cis-regulatory elements. After filtering and removing redundant information, the results were visualized in TBtools-II.

2.4. Chromosome Location, Duplication Events, and Collinearity Analysis

The information on the chromosome physical location of *PtCDPK* genes was retrieved from the genome annotation file of *P. tomentosa*. Based on this data, a chromosome physical location map for the *PtCDPK* genes was subsequently generated using MapChart (version 2.2) and visualized using TBtools-II. A gene replication event that contains two or more family members within a 200 kb region of the same chromosome was called a gene replication event and was examined using multiple collinear scanning toolkits (TBtools-II-MCScanX). The collinearity of the *CDPK* genes between *P. tomentosa* and four other plant species (*Arabidopsis*, *Oryza sativa* L., *Populus nigra*, and *Populus euphratica*) was analyzed using the Dual Synteny Plotter [31].

2.5. Plant Materials and Treatments

One-year-old dormant twigs of *P. tomentosa* were collected on 20 February 2024. Cuttings of 25 cm in length were soaked in water for 3 days and were then transplanted into plastic containers. The arbuscular mycorrhizal fungi (AMF) strain, *Rhizophagus irregularis* (Schenck and Smith, strain *BGC BJ09*), was obtained from the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China. *P. tomentosa* seedlings were cultivated in a greenhouse from March to May 2024, where the temperature was maintained within the range of 15−35 °C, and the relative humidity fluctuated between 70% and 90%. Each pot containing the seedlings received a weekly supplement of 100 mL of Hoagland's solution (2.0 mmol/L) each week. A two-factor, completely randomized block experiment was conducted, involving different As stress levels and AMF inoculation treatments. The poplar cutting strips were cultivated under arsenic (As) stress and/or *R. irregularis* inoculation conditions, resulting in four treatment groups: (1) a control group with neither As addition nor AMF inoculation (CK0); (2) a group exposed to As but without AMF inoculation (CK100); (3) a group not exposed to As but inoculated with AMF (Ri0); and (4) a group subjected to both As exposure and AMF inoculation (Ri100). For the AMF inoculation treatments, 30 g of *Rhizophagus irregularis* inoculum was added to each pot, positioned 3 cm beneath the soil surface. For the non-AMF inoculation treatments, the pots received 50 mL suspensions prepared from 30 g of unsterilized *R. irregularis* inoculum that had been filtered through a 10 μm ultrafiltration membrane. To ensure a homogeneous distribution of As within the soil, a solution of $Na_3AsO_4 \cdot 12H_2O$ containing As^{5+} at a concentration of 100 $mg \cdot kg^{-1}$ (based on dry soil weight) was applied and thoroughly mixed with the soil samples. Each of the four treatments was replicated three times biologically, with each replicate consisting of 12 pots (one seedling per pot). The seeds germinated and reached a length of approximately 2 cm. Each seedling was carefully transplanted into a plastic container measuring 15 cm in diameter and 15 cm in depth. The container was filled with a 2 kg mixture of soil and sand (v/v, 1:1).

2.6. PtCDPK Expression in Response to AMF Colonization and as Stress

The *P. tomentosa* roots were sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd. for sequencing and transcriptome analysis. Twelve libraries of the seedling roots were sequenced on the Illumina NovaSeq 6000 system (Illumina, San Diego, CA, USA). To obtain uncontaminated sequences based on the raw data, the low-quality reads (where over 50% of the bases had a quality score of 5 or below), those containing adapter sequences, and more than 10% unknown nucleotides were all removed. The uncontaminated sequences were mapped onto the *Populus tomentosa* genome for annotation, the gene expression was analyzed using the transcriptome data, and the transcript abundance of the *PtCDPKs* was

quantified with the Cufflinks package (version 2.2.1; http://bioconda.github.io/recipes/cufflinks/README.html, accessed on 5 March 2025). Differential gene expression analysis was performed using the EdgeR package within the R statistical software (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html, accessed on 10 March 2025).

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using three distinct biological samples, with each sample subjected to three technical replicates. A total of twelve genes were chosen for validation through RNA sequencing (RNA-seq), and the primers used for RT-qPCR were summarized in the Additional file (Table S1). Total plant RNA was extracted according to the instructions of the TaKaRa MiniBEST Plant RNA Extraction Kit (9769, TaKaRa Biomedical Technology (Beijing) Co., Ltd., Beijing, China), and cDNA was synthesized with the RNA reverse transcription kit (RR037A, TaKaRa Biomedical Technology (Beijing) Co., Ltd.). RT-qPCR was performed on a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the TB Green Premix Ex Taq II qPCR Kit (RR820A, TaKaRa Biomedical Technology (Beijing) Co., Ltd.). A 20 μL reaction mixture was prepared following the manufacturer's instructions for the fluorescence quantitative PCR kit. RT-qPCR was conducted with an initial denaturation step at 94 °C for 2 min, followed by 40 cycles consisting of denaturation at 94 °C for 5 s and annealing/extension at 60 °C for 40 s. The relative gene expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method [32], and β-actin served as the internal reference gene. To identify significant differences among various treatments, Tukey's multiple range tests were conducted at a significance level of $p = 0.05$ by using the SPSS software package (version 13.0 for Windows, SPSS Inc., Chicago, IL, USA). Different letters indicate significant differences.

3. Results

3.1. Identification of CDPK Family Genes in Populus tomentosa

To comprehensively elucidate the evolutionary history of the CDPK gene family in *P. tomentosa* and assess its significance in response to abiotic stress, a total of 20 CDPK genes were successfully identified from the *P. tomentosa* genome through multiple sequence alignment. The molecular characteristics of the 20 PtCDPK, including molecular weight (MW), theoretical isoelectric point (pI), subcellular localization, and other physicochemical properties, were also analyzed (Table 1). Both PtCDPK26-X1-1 and PtCDPK26-X1-2 were the largest, with 613 amino acids, and the smallest was PtCDPKSK5, with 503 amino acids. The molecular weight results indicated that PtCDPK26-X1-2 was the largest (69.03 kD) and PtCDPKSK5 the smallest (57.34 kD). The theoretical isoelectric point (PI) varied from 5.23 (PtCDPK20-2) to 6.56 (PtCDPK8-X1), with the majority of pI < 7, suggesting that the PtCDPK gene family exhibited a bias towards acidic amino acids. Our bioinformatic analysis revealed that none of the CDPK protein sequences had a transmembrane domain, 7 CDPK sequences (PtCDPK1-1, -X2, 10, 10-X4, 24, 29-1, and 29-2) were predicted to contain N-myristoylation sites, and each of all 20 PtCDPK proteins possessed S-palmitoylation sites and five EF-hand motifs. Subcellular localization prediction revealed that nine CDPK proteins (PtCDPK-X2, 8-X1, 10-X4, 13, 24, 26-X1-2, 26-X3, 32-X1, and SK5) were located in the cytoplasm, eight CDPK proteins (PtCDPK1-1, 1-2, 1-3, 20-1, 20-2, 26-X1-1, 29-1 and 29-1) were located in the chloroplast stroma, PtCDPK26-X2 and PtCDPK32 proteins were located in the nucleus, and only PtCDPK10 protein was located in the endoplasmic reticulum.

Table 1. Characteristics of the *PtCDPK* in *Populus tomentosa*.

Gene	Original ID	Length (aa)	MW (kDa)	pI	N-Yristoylation	S-Almitoylation	EF-Hand	Subcellular Location
PtCDPK1-1	LOC7458208	521	58.84	6.14	Yes	Yes	5	CS
PtCDPK1-2	LOC7490877	579	64.67	5.59	No	Yes	5	CS
PtCDPK1-3	LOC7469509	579	65.12	5.36	No	Yes	5	CS
PtCDPK-X2	LOC7479055	532	60.09	6.03	Yes	Yes	5	C
PtCDPK8-X1	LOC7482325	533	59.99	6.56	No	Yes	5	C
PtCDPK10	LOC7484759	555	63.16	5.96	Yes	Yes	5	ER
PtCDPK10-X4	LOC7465832	520	57.34	5.99	Yes	Yes	5	C
PtCDPK13	LOC7466075	528	59.71	5.87	No	Yes	5	C
PtCDPK20-1	LOC7469924	599	66.35	5.49	No	Yes	5	Cs
PtCDPK20-2	LOC7479492	598	66.49	5.23	No	Yes	5	Cs
PtCDPK24	LOC7469597	534	60.71	5.78	Yes	Yes	5	C
PtCDPK26-X1-1	LOC7469926	613	68.51	5.97	No	Yes	5	Cs
PtCDPK26-X1-2	LOC7455608	613	69.03	5.84	No	Yes	5	C
PtCDPK26-X2	LOC7454472	526	57.83	5.72	No	Yes	5	N
PtCDPK26-X3	LOC18098161	560	62.53	5.38	No	Yes	5	C
PtCDPK29-1	LOC7493997	513	57.63	5.86	Yes	Yes	5	Cs
PtCDPK29-2	LOC18096084	542	60.81	5.51	Yes	Yes	5	Cs
PtCDPK32	LOC7473631	528	59.85	6.12	No	Yes	5	N
PtCDPK32-X1	LOC7468973	532	60.46	6.32	No	Yes	5	C
PtCDPKSK5	LOC7465891	503	56.61	5.54	No	Yes	5	C

Abbreviations: aa, amino acid; MW, molecular weight; pI, isoelectric point. N, nucleus; C, cytoplasm; CS, chloroplast stroma; ER, endoplasmic reticulum.

3.2. Phylogenetic Analysis of PtCDPK Genes

To investigate the evolutionary relationships among the CDPK proteins, a phylogenetic tree was constructed using the amino acid sequences of 20 putative PtCDPKs from *Populus tomentosa*, 11 AtCDPKs from *Arabidopsis*, 5 OsCDPKs from *Oryza sativa*, 7 PaCDPKs from *Populus alba*, 8 PeCDPKs from *Populus euphratica*, and 13 PnCDPKs from *Populus nigra* (Figure 1). Based on the results of phylogenetic tree clustering, 84 *CDPK* genes were categorized into four subfamilies (Groups A, B, C, and D). The clustering analysis further revealed that homologous gene pairs displayed similar gene structures. The groups were distinguished based on their conserved domain and exon–intron structural characteristics. The number of Group D genes was the largest, including eight *PtCDPK* genes (*PtCDPK SK5*, *1-2*, *1-3*, *20-1*, *20-2*, *26-X1-1*, *26-X1-2*, and *26-X3*). The expansion of Group D genes provided insights into the evolutionary trajectory of *P. tomentosa*. The *PtCDPK1-1*, *8-X1*, *13*, *32-X1*, and *32* belonged to Group A; *PtCDPK-X2*, *10*, *24*, *29-1*, and *29-2* clustered into Group C; and Group B possessed the fewest genes, including only two genes (*PtCDPK10-X4*, and *26-X2*), indicating that both were far from the other 18 *CDPK* members. Clade A consisted of only *CDPK* genes from *P. tomentosa* and rice, totaling six members. The B branch contained only the *CDPK* genes of *Populus sinensis*, i.e., silver populus and black populus, with a total of six members. Branch C had the largest number of members, with 27, and Branch D had 25.

3.3. Exon–Intron Structure, Conservative Domains, and Motifs Analysis of the PtCDPK Genes

The gene family of *PtCDPK* was divided into four groups (A, B, C, and D), and the results revealed that these *CDPK* genes within the same subgroup exhibited similar patterns of gene structure distribution and conserved motifs. A total of 15 conserved motifs were found, and each motif was present (Figure 2B–D). Within the same group, *CDPK* genes exhibited similar numbers, types, and spatial distributions of motifs, indicating that they shared similar functions. Significant differences were observed in the conserved motifs among different clades. In contrast, motifs within the same clade exhibited a higher degree of conservation, which was consistent with the change rule of gene structure. In Groups

A, C, and D, some motif sequences, namely, 3, 8, 2, 9, 1, and 4, were found in all clades. Motif 12 was present only in Groups C and D, and motif 15 only appeared in Group A, which was related to the differentiation of gene functions. Additionally, a significant association was evident between the phylogenetic tree and the exon–intron structure of the *PtCDPK* genes. To elucidate the structural composition of *PtCDPK* genes, a comprehensive structural map was constructed using *P. tomentosa* genome sequences, including the untranslated region (UTR), coding sequence (CDS), CDPK structural domains, and introns (Figure 2B,C). A comparative analysis of the quantity and distribution of exons and introns showed that all 20 *PtCDPK* genes contained different numbers of exons, UTRs, and CDPK structural domains. Except *PtCDPK1-1* in Group A, which had nine exons, most *CDPKs* of the three groups (A, C, and D) showed seven to eight exons (10 *PtCDPK* genes with seven exons and 7 with eight exons). However, *PtCDPK26-X2* and *PtCDPK10-X4* in Group B had only three and four exons, respectively, indicating that exon loss and exon gain events occurred simultaneously during the evolution of the *PtCDPK* gene family. This phenomenon could potentially lead to the emergence of functional diversity among closely related genes. To further investigate the structural domains of PtCDPKs, the PtCDPK24 protein sequence contained both STKc-CAMK and FRQ1 domains, PtCDPK26-X2 had only the STKc-CAMK domain, and the remaining 18 *CDPK* genes all had both PTZ00184 and STKc-CAMK domains.

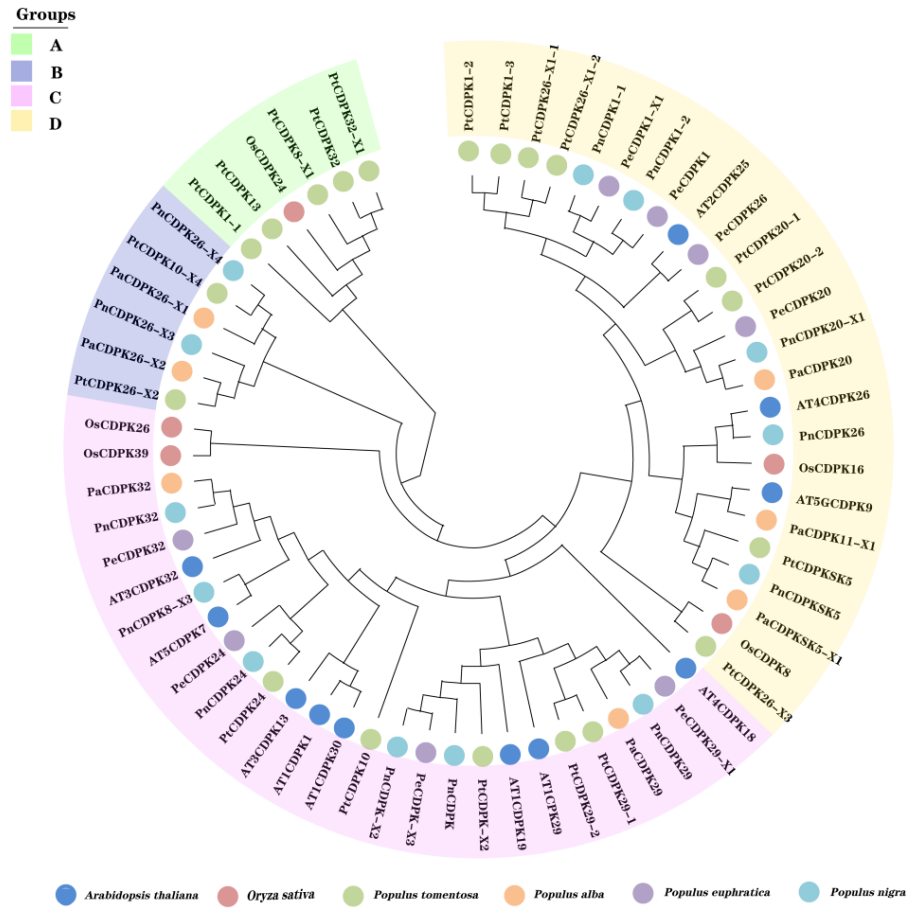


Figure 1. Phylogenetic relationship of CDPK genes in *Arabidopsis thaliana*, *Oryza sativa*, *Populus tomentosa*, *Populus alba*, *Populus euphratica*, and *Populus nigra*.

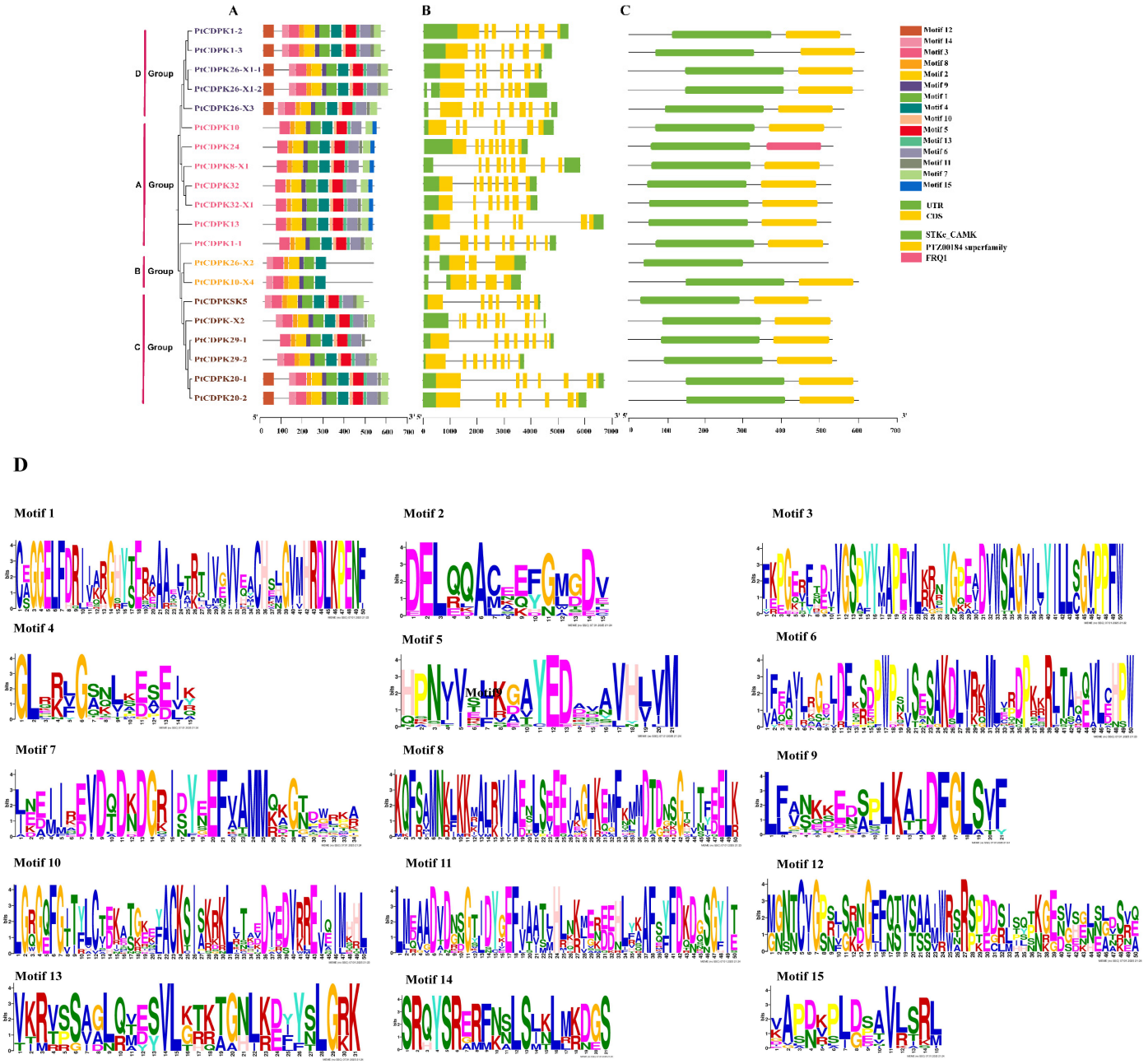


Figure 2. Phylogenetic relationships, conserved protein motifs, and gene structures of the 20 *PtCDPK* genes: (**A**) the phylogenetic tree of PtCDPK proteins; (**B**) distribution of motifs in the PtCDPK proteins; (**C**) exon–intron structures of the *PtCDPK* genes (black lines indicate introns); (**D**) sequence logo of the PtCDPK proteins motifs. The height of each amino acid represented its relative frequency at that specific position.

3.4. Cis-Element Analysis of the PtCDPK Genes

To investigate the regulatory mechanisms of *PtCDPK* genes, the 2000 bp upstream sequences from the transcriptional start site were retrieved and analyzed to identify cis-acting elements within their promoter region. This analysis revealed a conserved promoter structure across all *PtCDPK* genes, as well as the presence of over 18 distinct cis-regulatory elements. The *PtCDPK* promoter contained numerous cis-acting elements, including phytohormone-response elements, light-response elements, and stress-response elements (Figure 3). A total of 20 *PtCDPK* genes exhibited 240 light-responsive elements. The cis-responsive elements associated with plant hormone responsiveness include those for auxin, gibberellin, salicylic acid, abscisic acid (ABA), and methyl jasmonate (MeJA).

Among these, abscisic acid (65, the number of cis-elements) and methyl jasmonate (MeJA) (44) responsiveness elements were the most common. The stress responsiveness elements mainly included drought stress (9), low temperature stress (8), anaerobic induction (54), and defense and stress responsiveness (16). The optical response elements included TCT-motif, TCCC-motif, I-box, GT1-motif, and BoxII. Hormone responsiveness elements included the TGACG-motif, TCA-element, p-box, CGTCA-motif, and ABRE. In addition, several stress responsiveness elements were observed, such as cis-acting element TC-rich repeats, and anaerobic induction element (ARE), low temperature responsiveness element (LTR), drought induction element (MBS), and so on. There were also some cis-acting regulatory elements related to growth and development; for example, the O2-site participated in zein metabolism regulation, and GCN4-motif was implicated in endosperm expression.

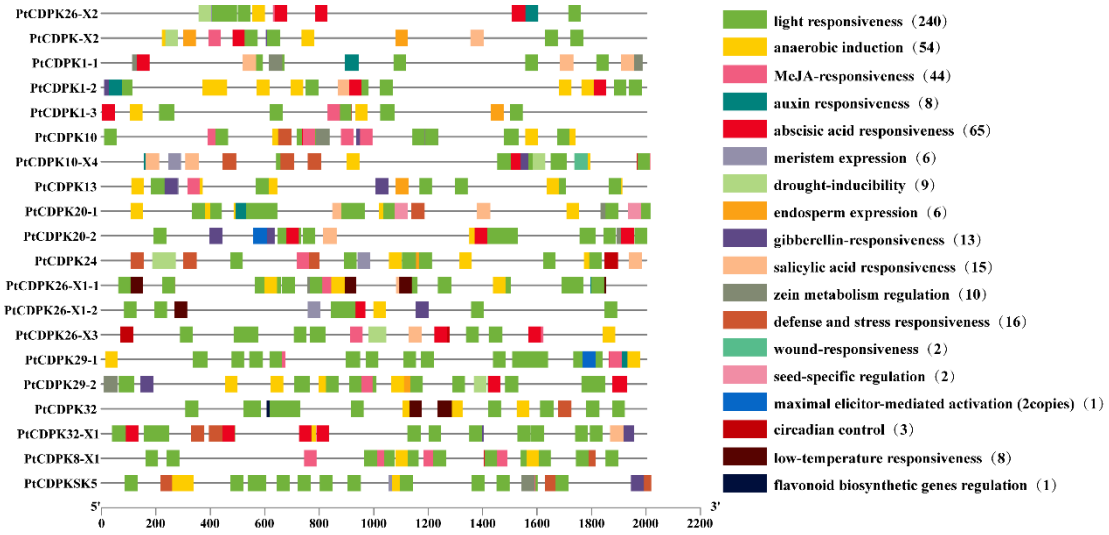


Figure 3. Cis-elements in the promoter regions of 20 *PtCDPKs*. The black line denotes the promoter length of the *PtCDPKs* genes.

3.5. Chromosome Distribution and Gene Replication of the PtCDPKs

To clarify the positions of 20 *PtCDPK* genes in the genome of *Populus*, a distribution map of *PtCDPK* genes on chromosomes was created using MapChart software. Chromosome 15 was the longest and chromosome 13 was the shortest, and the results showed no correlation between chromosome length and gene distribution. In the chromosomes of the *Populus* genome, 20 members of the *PtCDPK* family were distributed across all 13 chromosomes. The *PtCDPK* genes presented an uneven distribution, and no tandem repeat genes were observed (Figure 4). Gene duplication and divergence played crucial roles in the expansion of gene family, as well as in the emergence of new functions during evolution. Our study revealed two gene duplication events in chromosomes 5 (*PtCDPK20-2* and *PtCDPK26-X1-2*) and 12 (*PtCDPK20-1* and *PtCDPK26-X1-1*). At the same time, there were two *PtCDPK* genes distributed in chromosome 12 (*PtCDPK10-X4* and *PtCDPK1-1*) and chromosome 3 (*PtCDPK-X2* and *PtCDPK26-X3*), respectively. Chromosomes 1, 4, 6, 7, 8, 9, 10, 11, and 13 had only one *PtCDPK* gene. The largest number of *PtCDPK* genes (four genes) were identified on chromosome 12, followed by chromosome 5 (three genes). These gene clusters likely originated from tandem duplication events, indicating the expansion of the gene family during evolutionary processes.

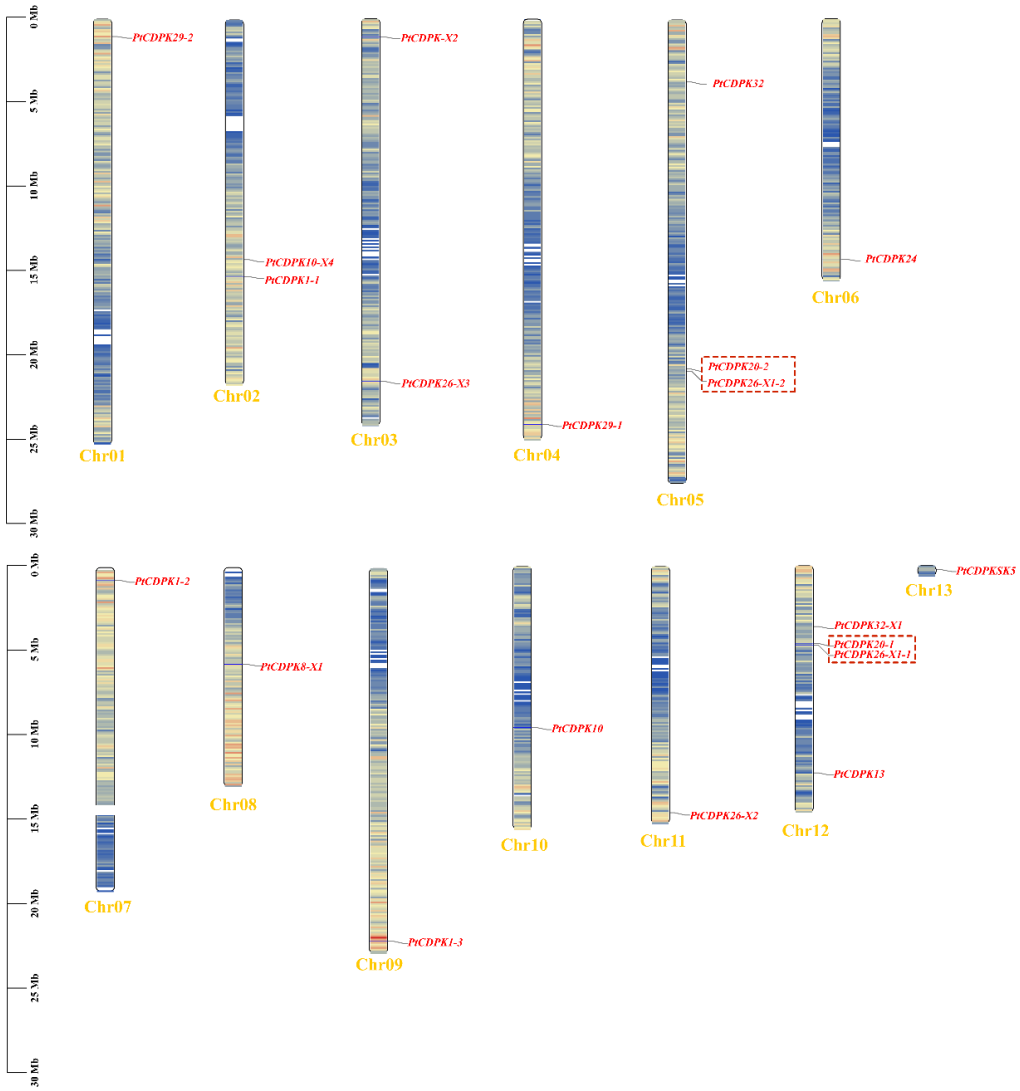


Figure 4. Chromosomal distribution of the *PtCDPK* genes. The scale on the left represents the length (Mb, mega base) of *Populus tomentosa* chromosomes. The vertical bars are used to mark the chromosomes of *P. tomentosa*, with the chromosome number indicated at the base of each chromosome. Red rectangles frame the tandem genes.

3.6. Synteny Analysis of the PtCDPK Genes

An analysis of gene collinearity within the *PtCDPK* gene family identified 12 segmental duplication events, involving 21 AP2/ERFs. These duplication events constituted 18% of the total gene family (Figure 5). To gain deeper insights into the genetic differentiation, gene duplication events, and evolutionary patterns among the *CDPK* gene families of *Populus tomentosa*, *Arabidopsis*, *Oryza sativa*, *Populus nigra*, and *Populus euphratica*, synteny analysis of the *CDPK* gene in *Populus tomentosa* and four other plants were investigated. The *PtCDPK* gene family exhibited a strong collinear relationship with *CDPK* genes in Populus plants, with the highest collinear relationship observed in *P. nigra* (33 pairs) and followed by *P. euphratica* (30 pairs). The *PtCDPK* genes shared a few genes with the two model plants, including 10 pairs of genes with *Arabidopsis* and 14 with *Oryza sativa*, respectively. *PtCDPK8-X1*, *PtCDPK26-X3*, and *PtCDPK-X2* had no colocalized genes with any of the four species, suggesting that these genes may have formed after plant differentiation (Figure 6).

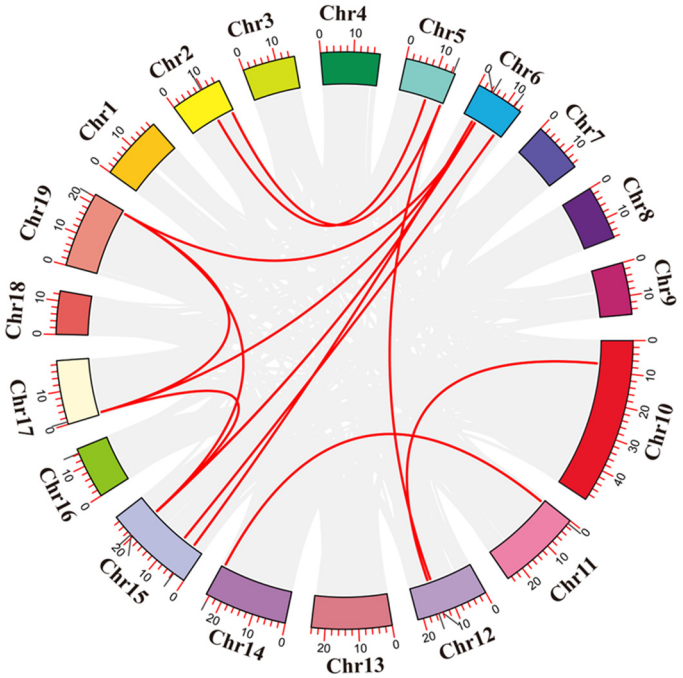


Figure 5. Schematic representations of the interchromosomal relationships of the *PtCDPK* genes. Gray represents genome-wide interspecies collinearity, and the red line represents collinearity within the *CDPK* family.

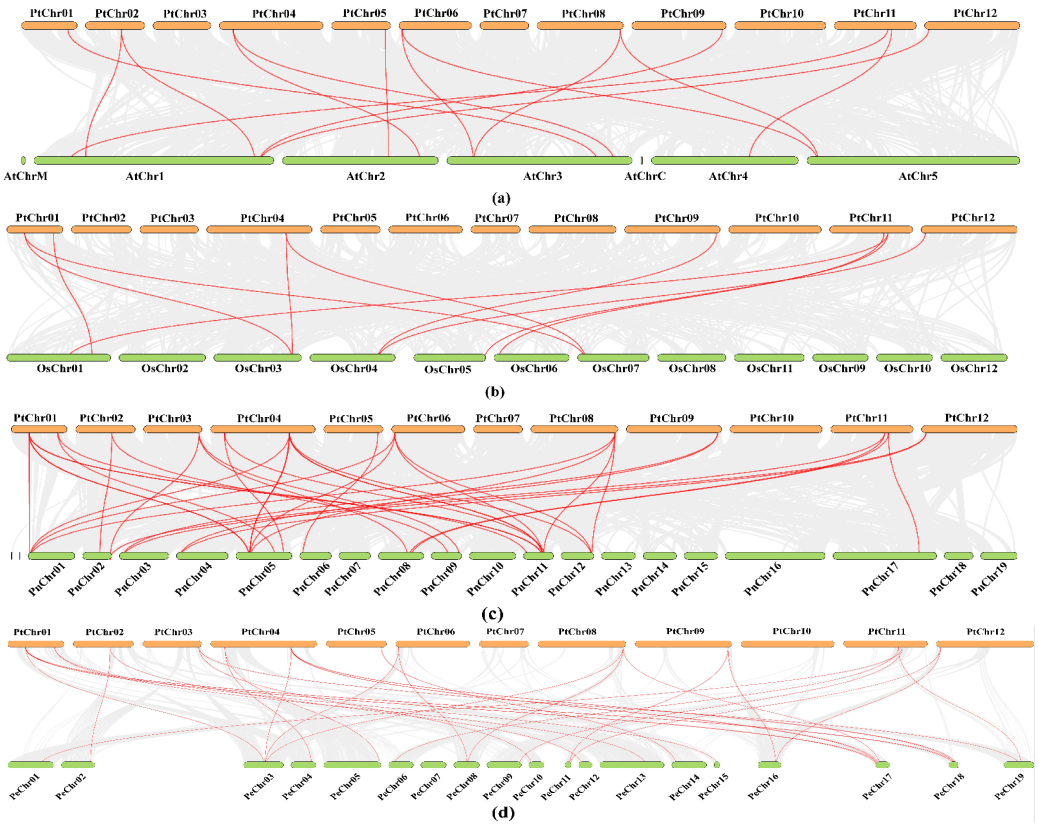


Figure 6. Synteny analysis of the *CDPK* gene in *Populus tomentosa* and four other plants. Grey lines indicate collinear blocks within the *P. tomentosa* genome and other plant genomes, and the red curve indicates *CDPK* genes with collinearity. PtChr stands for *Populus tomentosa* chromosome; AtChr stands for *Arabidopsis* chromosome (**a**); OsChr stands for *Oryza sativa* L. chromosome (**b**); PnChr stands for *P. nigra* chromosome (**c**); and PeChr stands for *P. euphratica* chromosome (**d**).

3.7. Expression Analysis of the PtCDPK Genes in Response to as Stress and AMF Colonization

Transcriptome analysis of gene expression levels revealed that 20 *PtCDPK* genes were variably expressed in *P. tomentosa* roots (Figure 7). Without AMF inoculation, As stress downregulated eight *CDPK* genes (*PtCDPK1-1*, *1-2*, *10*, *26-X1-1*, *26-X2*, *26-X3*, *32*, and *32-X1*) and promoted the expression of ten *PtCDPK* genes, including *PtCDPKSK5*, *X2*, *1-3*, *10-X4*, *13*, *20-1*, *20-2*, *24*, *26-X1-2*, and *29-2*. Conversely, under *R. irregularis* inoculation, As stress induced the expression of *PtCDPKSK5*, *24*, *26-X3 1-3*, *26-X1-1*, *26-X1-2*, *32*, and *32-X1*, while inhibiting ten *PtCDPK* genes (*PtCDPK X2*,*1-1*, *8-X1*, *10*, *13*, *20-1*,*20-2*, *26-X2*, *29-1*, and *29-2*). Under non-As stress conditions, *R. irregularis* inoculation downregulated the expression of *PtCDPK1-1*, *1-2*, *8-X1*, *10*, *10-X4*, *26-X1-1*, *26-X1-2*, *26-X2*, *26-X3*, *32*, and *32-X1*, while upregulating nine CDPK genes: *PtCDPKSK5*, *X2*, *1-3*, *13*, *20-1*, *20-2*, *24*, *29-X1*, and *29-2*. Under As stress, *R. irregularis* inoculation further improved the expression of 11 *PtCDPK* genes (*PtCDPKSK5*, *X2*, *1-3*, *20*-1, *24*, *26-X1-1*, *26-X1-2*, *29-1*, *29-2*, *32*, and *32-X1*) and suppressed *PtCDPK1-1*, *1-2*, *8-X1*, *10-X4*, *13*, *20-2*, *26-X2*, and *26-X3*.

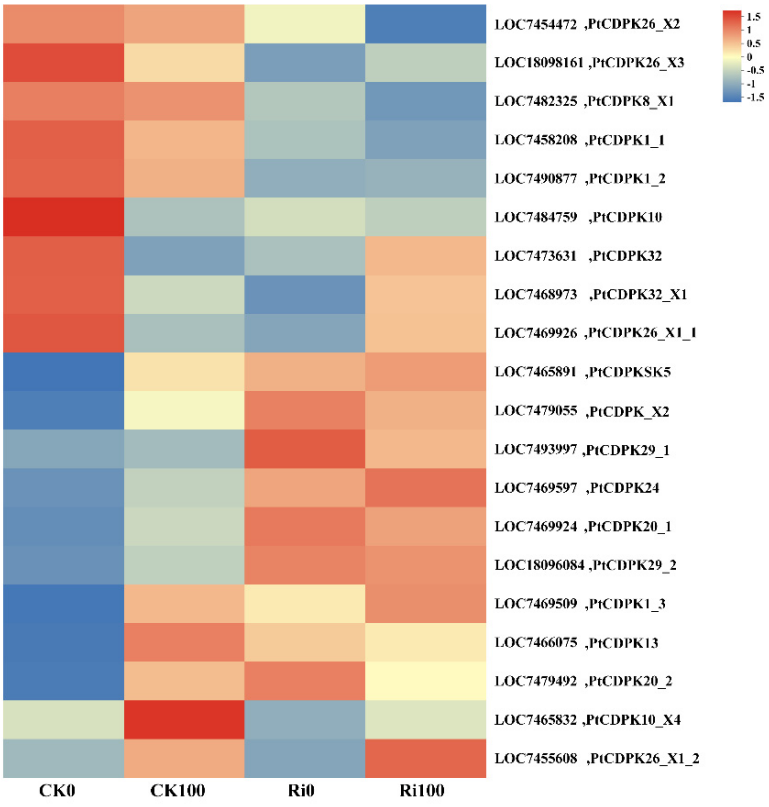


Figure 7. Expression analysis of the *PtCDPKs* genes in response to As stress and AMF colonization. CK0: No As stress with no AMF inoculation; CK100: As stress (100 mg As kg^{-1} soil) with no AMF inoculation; Ri0: No As stress with AMF inoculation; Ri100: As stress (100 mg As kg^{-1} soil) with AMF inoculation.

To validate the expression patterns derived from transcriptome data under both As stress and AMF inoculation conditions, 10 *PtCDPK* genes were chosen from the transcriptome dataset for RT-qPCR analysis. The results were consistent with the transcriptome data; they indicated that PtCDPKs played more significant roles in regulating As stress and AMF inoculation responses (Figure 8).

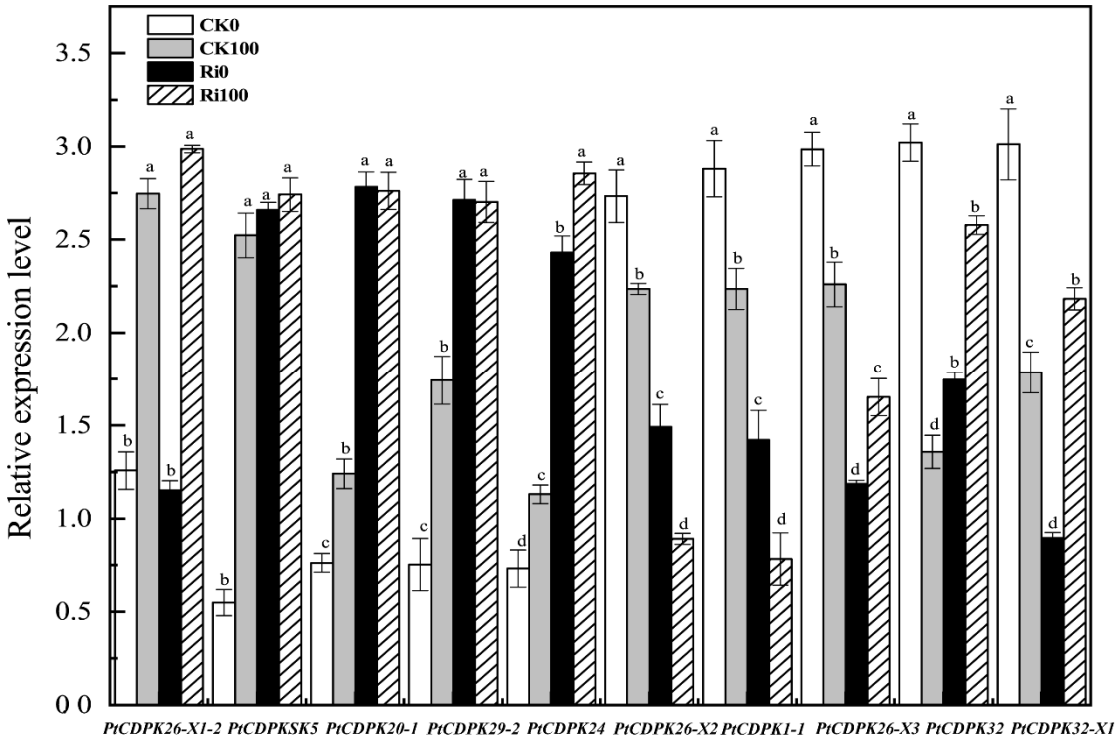


Figure 8. *PtCDPK* gene expression with AMF colonization and/or As stress treatment by qRT-PCR. Note: The relative gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method, with β-actin used as the reference gene. Significant differences among four treatments were analyzed by Duncan's Multiple Range Tests at $p < 0.05$. Distinct letters indicate statistically significant differences. CK0: No As stress with no AMF inoculation; CK100: As stress (100 mg As kg^{-1} soil) with no AMF inoculation; Ri0: No As stress with AMF inoculation; Ri100: As stress (100 mg As kg^{-1} soil) with AMF inoculation.

4. Discussion

The calcium-dependent protein kinase (*CDPK*/*CPK*) gene family represent an essential component of calcium signaling networks in plants, functioning as pivotal molecular transducers that decode transient Ca^{2+} fluxes into phosphorylation-mediated cellular responses. This evolutionary conserved kinase system bridges environmental stimuli with intracellular regulation, enabling precise control of physiological processes through reversible protein modification [7]. The genome-wide identification and functional characterization of *CDPKs* in numerous plant species significantly deepened our understanding of their roles in abiotic and biotic stress responses, developmental regulation, and symbiotic interactions [5]. The present study systematically identified and characterized the *CDPK* gene family in *Populus tomentosa* and examined their expression patterns in response to arsenic (As) stress and arbuscular mycorrhizal fungi (AMF) colonization.

The *CDPK* gene family exhibited remarkable structural conservation throughout its evolutionary history from mosses to angiosperms. There are several *CDPK* gene families in different plant species, including 34 *CDPK* genes in *Arabidopsis* [1], 40 in *Zea mays* L. [33], 20 in *Triticum aestivum* [34], 29 in *Solanum lycopersicum* [29], and 19 *CDPK* genes in *Cucumis sativus* [7], where the numbers of CDPK proteins vary among plant species. In this study, 20 *CDPK* genes were identified in the *P. tomentosa* genome, highlighting the evolutionary significance of *CDPKs* as integral components of calcium signaling networks. The identification of *PtCDPK* genes revealed a diverse set of proteins with varying physicochemical properties. The molecular weight (MW) and isoelectric point (pI) analysis showed a range of variability, with PtCDPK26-X1-2 being the largest protein with 613 amino acids and a MW of 69.03 kDa, while PtCDPKSK5 was the smallest with 503 amino acids and a

MW of 57.34 kDa (Table 1). The acidic nature of most PtCDPK proteins was evident from their pI values below 7, which is in line with previous findings indicating the predominance of acidic residues in the *CDPK* gene family [1,2].

The bioinformatics analysis of the transmembrane domain and subcellular location revealed that most PtCDPK proteins lack transmembrane domains, suggesting a cytoplasmic or nuclear localization rather than membrane association. N-myristoylation sites were predicted in seven PtCDPK sequences, which was consistent with the findings for other species, such as *Arabidopsis* and *Z. mays* [6,15].

The phylogenetic analysis grouped the PtCDPK proteins into four major clusters (Groups A, B, C, and D), indicating a conserved evolutionary pattern similar to that observed in other poplar species [35]. It was observed that members within the same subgroup shared similarities in terms of protein sequence length, motif composition, and gene structure, indicating a strong evolutionary relationship. Consequently, it was speculated that homologous genes located on the same branch of the phylogenetic tree may have analogous biological functions in response to plant–microbe interactions and abiotic stress. Group D contained the largest number of *PtCDPK* genes, showing potential gene expansion through duplication events, which aligned with the segmental and tandem duplication patterns observed in other model species [35]. It has been widely recognized that two primary mechanisms propelled the expansion of gene families in plants, namely, gene duplications and tandem duplications [36]. The *PtCDPK* genes exhibited two gene duplication events, suggesting that the expansion of the *PtCDPK* gene family occurred a few times during evolution. The *PtCDPK* genes shared a few genes with the two model plants, including 10 pairs of genes with *Arabidopsis* and 14 with *O. sativa*, respectively. Based on the results of gene replication and collinearity analysis, our results confirmed that *PtCDPKs* were relatively conserved.

The classification of *PtCDPKs* into four subgroups (A, B, C, D) revealed conserved evolutionary patterns and functional specialization. These results were consistent with those of the *Arabidopsis* and rice *CDPK* families [6]. In this study, the similarity in exon–intron distribution and motif composition within subgroups, as indicated by the shared motifs 3, 8, 2, 9, 1, and 4 across Groups A, C, and D, indicated the role of purifying selection in maintaining core kinase and calcium-sensing functions. The exon counts and conserved domain architectures were also found in the same subgroup of *Arabidopsis CDPKs*, suggesting that these features were subject to functional constraints [35]. However, *PtCDPKs* displayed unique structural plasticity; compared with the 7–8 exon norm in other subgroups, *PtCDPK26-X2* and *PtCDPK10-X4* in Group B exhibited drastic exon reduction (3–4 exons), and this pattern indicates the evolutionary trajectory of *CDPKs* [34,37]. Similar exon loss/gain events were also reported in soybean *CDPKs*, which likely contributed to functional divergence by altering the protein-coding capacity and regulating element availability during polyploidization [38]. The FRQ1 domain was absent in most PtCDPKs, and PtCDPK24's FRQ1 domain resembled that of *Arabidopsis* AtCPK28, which used an auxiliary domain for membrane recruitment [39]. The FRQ1 domain in PtCDPK24 likely mediated scaffolding interactions, as seen in *Nicotiana tabacum* NtCDPK2, where FRQ1 facilitates immune complex formation [40].

The cis-acting element analysis of the *PtCDPK* gene promoters revealed a complex regulatory network that integrates hormone signaling, light perception, and stress adaptation. Some stress-responsive cis-elements (ABRE, MYB, and W-box) in *PtCDPKs* were identified, indicating their roles in abiotic stress signaling [1]. *PtCDPK9* and *PtCDPK22* contained multiple ABREs, which serve as binding sites for ABA-responsive transcription factors (AREB/ABF) in As tolerance [21]. MYB elements in the *PtCDPK4* and *PtCDPK12* promoters suggested regulation by drought-responsive MYB TFs, as observed in

ZmCDPK11 [16]. In addition, *PtCDPK16* and *PtCDPK25* contained fungal-elicitor-responsive elements (Box-W1), implicating their roles in AMF symbiosis by modulating AMF-induced calcium oscillations. Similar cis-acting elements in *Medicago truncatula MtCPK3* regulated mycorrhizal symbiosis [40]. Hormone-responsive elements (MeJA, SA) in *PtCDPK7* and *PtCDPK14* were associated with JA-mediated defense pathways, as reported for *LeCDPK2* in tomato [41].

Transcriptomic analysis revealed that 20 *PtCDPKs* were differentially expressed due to As stress, and 11 *PtCDPK* genes showed significant upregulation. Similar results were found in the As-induced expression of *OsCPK12* and *GmCDPK32*, which enhanced antioxidant enzyme activity [2,42]. Conversely, *PtCDPK7* and *PtCDPK18* were downregulated, possibly due to As-induced oxidative damage or feedback inhibition. Similar repression was observed in *AtCPK12* under drought stress, indicating stress-specific regulation [39].

In our study, the expressions of *PtCDPKSK5*, *X2*, *1-3*, *20-1*, *24*, *26-X1-2*, *29-1*, and *29-2* were improved, but *PtCDPK1-1*, *1-2*, *8-X1*, *26-X2*, and *26-X3* were suppressed under both As stress and AMF inoculation conditions, which suggested a trade-off strategy between stress adaptation and symbiotic investment synergistic roles [21]. The downregulation of *PtCDPK* genes (a homolog of the JA-responsive *LeCDPK2*) could suppress JA signaling and improve AMF symbiosis, and similar results were found in *Medicago MtCDPK4* [43,44]. The *MtCPK3* upregulated by mycorrhizal symbiosis facilitated the calcium spiking and symbiosis signaling [42]. AMF colonization and drought stress showed synergistic effects in *Citrus sinensis* roots. The expression levels of *CsCDPK20* and *CsCDPK22* were repressed in *Funneliformis mosseae*-inoculated citrus under well-watered conditions but induced in *F. mosseae*-inoculated citrus under drought stress conditions [45]. The defense- and stress-responsive cis-elements were identified in the promoters of seven *PtCDPK* genes (*PtCDPKSK5*, *8-X1*, *10-X4*, *20-1*, *24*, *32*, and *32-X1*), indicating that these genes may be regulated by As stress and AMF inoculation. Furthermore, the expression of *PtCDPKSK5* and *PtCDPK20-1* were induced. At the same time, *PtCDPK8-X1* were repressed by both As stress and AMF inoculation, which indicated that the expression regulation of *PtCDPK*s was not entirely consistent with the cis-elements present in their promoters. Similar results were also found in *CsCDPK* genes in *Citrus sinensis* roots, which were attributed to the gene integration of other regulatory elements such as trans-acting factors [45].

5. Conclusions

In this study, we identified 20 PtCDPK genes in the *Populus tomentosa* genome and systematically analyzed their evolutionary relationships, structural features, and expression patterns under arsenic (As) stress and arbuscular mycorrhizal fungi (AMF) colonization. Our key findings included the following: (1) phylogenetic and motif analyses revealed that PtCDPKs were evolutionarily conserved, with genes in the same group sharing similar exon–intron structures and functional domains; (2) the promoters of PtCDPK genes contained diverse cis-acting elements, suggesting their potential involvement in hormone-, light-, and stress-related signaling pathways; (3) comparative genomic analysis indicated that *PtCDPKs* exhibited strong collinearity with *CDPK* genes in *Arabidopsis* and rice, highlighting their conserved roles in plant evolution; (4) transcriptome and RT-qPCR data demonstrated that *PtCDPK* genes exhibited differential expression under As stress, with AMF symbiosis significantly modulating the expression of 19 *PtCDPK* genes. Specifically, AMF inoculation upregulated 11 *PtCDPKs* and downregulated 8 *PtCDPKs* under As stress, indicating a regulatory role of AMF in *PtCDPK*-mediated stress responses. These findings provided novel insights into the genetic basis and regulatory mechanisms of *PtCDPK* genes in *P. tomentosa*, particularly their response to As stress and AMF symbiiosis. Future

work should prioritize the mechanistic dissection of *PtCDPK*-AMF interactions, which may unveil novel strategies for improving plant tolerance to environmental stressors.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15071655/s1: Table S1: Primer sequence.

Author Contributions: Conceptualization, Q.Z. and X.C.; methodology, M.G. and J.S.; validation, S.W., Y.W. and W.W.; formal analysis, S.W. and W.W.; writing—original draft preparation, M.G., J.S. and Q.Z.; writing—review and editing, M.G. and X.C.; visualization, Y.W. and J.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data reported in this study are available in the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CDPKs	Calcium-Dependent Protein Kinases
AMF	Arbuscular Mycorrhizal Fungi
TAIR	The Arabidopsis Information Resource
ABA	Abscisic Acid
MeJA	Methyl Jasmonate

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