The CDPK Gene Family in Mustard (Brassica juncea L.): Genome-Wide Identification and Expression Analysis under Cold Stress

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Article

The *CDPK* Gene Family in Mustard (*Brassica juncea* L.): Genome-Wide Identification and Expression Analysis under Cold Stress

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Abstract: The *CDPK* family genes play crucial roles in signal transduction pathways during plant development and stress response. In this study, we comprehensively analyzed the *CDPK* family genes in mustard (*Brassica juncea* L.), resulting in the identification of 101 genes (*BjuCDPK1–101*) located on chromosomes AA_Chr01 to BB_Chr08. RNA-seq analysis showed that most *BjuCDPK* genes were predominantly expressed in the root and flower bud, suggesting their organ-specific expression patterns. Furthermore, the expression levels of most *BjuCDPK* genes were significantly altered by cold stress. The promoter regions of *BjuCDPK* genes had abundant cis-acting elements related to hormone and stress. The interaction network analysis predicted that BjuCDPK proteins respond to abiotic stress probably via interaction with ABF1, ABF4, and/or DI19. Our findings provide valuable information for further investigation of the cold stress adaption of mustard via the CDPK signaling pathway.

Keywords: mustard (*Brassica juncea* L.); *CDPK* gene family; gene expression; cold stress

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1. Introduction

Calcium signature refers to the stimulus-specific increase in cytosolic calcium ion (Ca^{2+}), which serves as a second messenger in various signal transduction pathways and environmental stimulus responses of plants [1,2]. The intracellular modulation and cytosolic concentration of Ca^{2+} are highly responsive to different abiotic stresses, including drought, high salinity, ultraviolet light, heavy metals, waterlogging, extreme temperature, and wounding [3]. Three classes of calcium sensors, including calmodulins (CaMs) and calmodulin-like proteins (CaMLs), calcineurin B-like proteins (CBLs), and calcium-dependent protein kinases (CDPKs), modulate intracellular Ca^{2+} transductions in plant signals [4–6]. These proteins can be divided into two modes according to the mechanisms of their activation by Ca^{2+}: (1) sensor relay proteins, including CaMs, CaMLs, and CBLs, which have no intrinsic enzymatic activity and interact with sensor responder proteins to transduce signals after binding to Ca^{2+}; (2) sensor responder proteins, unique in CDPKs, which have additional kinase activities besides all the functions of sensor relay proteins [7,8].

The structure of CDPK proteins consists of four domains, including a variable N-terminal domain, a catalytic domain of serine/threonine kinase, a junction domain containing an autoinhibitory region, and a calmodulin-like regulatory domain (CaM-LD) [9]. The N-terminal of CDPKs is highly variable in length and sequence, and sometimes undergoes post-translational modifications, such as myristoylation and palmitoylation, to anchor CDPKs to the membrane of plant cells [10]. In *Arabidopsis thaliana*, 97.05% (33/34) of CDPKs have a variable N-terminal domain with a length of 25–180 amino acids (aa) that may play an essential role in substrate recognition [11]. The autoinhibitory junction domain acts as an autoinhibitor and contains a pseudosubstrate sequence interacting with the active site and then inhibiting the activity of CDPKs [12]. The catalytic domain,

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including highly conserved regions such as the ATP-binding site, Ser/Thr protein kinase active site, and 11 subdomains (I-XI subdomains), plays a central role in the function of CDPKs [13]. The C-terminal CaM-LD of CDPKs contains four elongation factor (EF) hands, a helix-loop-helix structure (about 29 aa) that senses calcium signals, and can be directly activated by free Ca^{2+} binding [14].

CDPK genes were found to distribute in various plant species, including early lower plants (such as green algae), and they were function as transcriptional regulators and play critical roles in regulating of signal transduction and stress responses [15]. For example, overexpression of *GmCDPKSK5* enhances the tolerance to high temperature and humidity stress and improves the vitality of soybean seeds [16]. Additionally, *AtCPK6* overexpression can improve drought and salt tolerance, but the mutant of *AtCPK6* showed no significant difference from the wild type, indicating redundancy of functions among *AtCPK* genes [17]. Specially, cold stress can trigger the influx of calcium ions into plant cells, leading to physical changes in cellular structure, such as membrane fluidity and cytoskeletal reorganization [18,19]. Previous studies have revealed the positive role of CDPKs in response to cold or freezing tolerance. For instance, cold-induced calcium signals could be rapidly decoded by AtCPK28 and phosphorylated NIN-LIKE PROTEIN 7 (NLP7), which controls the expression of a set of downstream *COR* genes, and thus enhance the cold tolerance of *Arabidopsis* plants [20]. In rice, OsCDPK13 is required for signal transduction in response to cold stress and gibberellin (GA) in rice seedlings [21]. OsCPK17 plays an important role in cold tolerance by affecting the activity of membrane channels and sugar and nitrogen metabolism [22]. OsCPK24 is another positive regulator of response to cold stress in rice by participating in the calcium signaling pathway and phosphorylation of OsGrx10 to maintain a higher glutathione level [23]. VaCPK20 from a wild grapevine species (*Vitis amurensis* Rupr.) was reported to be involved in cold and drought stress response pathways, as overexpression of *VaCPK20* in *Arabidopsis* could increase the expression of several stress-responsive genes [24]. Overexpression of *Populus euphratica CPK10* in *Arabidopsis* could also enhance the freezing tolerance of transgenic lines [25]. In some other cases, CDPKs can play negative roles in regulating of the cold stress response as well. For instance, ZmCPK1 acts as a negative regulator and calcium-independent kinase in response to cold stress, and can suppress the expression of the cold-induced marker gene *Zmerf3* in maize [26].

In recent years, more than a dozen of *CDPK* gene family have been comprehensively identified in various plant species. For instance, genome-wide analysis has identified 40 *CDPK* genes in maize (*Zea mays*) [27], 34 in *Arabidopsis* [28], 31 in pepper (*Capsicum annuum*) [29], 30 in each of grass (*Brachypodium distachyon*) and poplar (*Populus trichocarpa*) [30,31], 29 in each of rice (*Oryza sativa*) and foxtail millet (*Setaria italica*) [32,33], 25 in each of canola (*Brassica napus*) and legume (*Medicago truncatula*) [34,35], 19 in cucumber (*Cucumis sativus*) [36], 17 in peach (*Prunus persica*) [37], and 11 in strawberry (*Fragaria x ananassa*) [38]. Wild tomato (*Solanum habrochaites*), which is closely related to cultivated tomato and has strong resistance to cold stress, has been found to possess 33 *CDPK* genes, whereas the cultivated variety *S. lycopersicum* only comprised 29 *CDPK* genes [39,40]. To date, the genomic information and expression patterns of *CDPK* genes in mustard (*Brassica juncea*) remain unknown. In this study, we identified the *CDPK* family genes in mustard, and investigated their phylogeny, gene structure, conserved motifs, chromosomal localization, gene duplication, proteins interaction, and expression levels in different organs and under cold stress. The findings provide a foundation for future research on the roles of *CDPK* genes in the response of mustard to stresses.

2. Materials and Methods

2.1. Identification of BjuCDPK genes in the genome of Brassica juncea

For the identification of the members in the *CDPK* gene family, the genome of *Brassica juncea* cv. *tumida* T84-66 V2.0 was obtained from the Brassicaceae Database (BRAD V3.0) [41]. A Hidden Markov Model (HMM) search was conducted via the HMMER 3.0 software (E value $< 1 \times 10^{-5}$) with the Pkinase domain (PF00069) and EF-hand 7 domain (PF13499), which were retrieved from the Pfam database (http://pfam.xfam.org/ accessed on 12 December 2022). After that, all putative CDPKs were retested through the NCBI conserved domain database [42]. The ProtParam module in Biopython

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[43] was used to predict the basic physicochemical characteristics of the BjuCDPK proteins, including protein length (aa), molecular weight (MW), grand average of hydropathy (GRAVY), instability index, and theoretical isoelectric point (p*I*). The DeepLoc 2.0 online tool [44] was employed to characterize the subcellular location of each BjuCDPK protein.

2.2. Gene structure, chromosomal mapping, and gene duplication analysis

The gene information, including location of exons, introns, and untranslated region of each identified *BjuCDPK* genes, and their predicted positions mapped onto the *Brassica juncea* chromosomes was downloaded from BRAD, and visualized by TBtools [45]. The duplication events across the *BjuCDPK* genes were detected by the WGDI (v0.6.1) software [46], and visualized by shinyCircos [47]. Each of the duplicated *BjuCDPK* gene pairs was aligned by the ParaAT 2.0 [48] with MUSCLE (v3.8.31) [49] as multiple sequence aligner, and non-synonymous (Ka) and synonymous substitution rates (Ks) were estimated by KaKs_Calculator 3.0 [50] using the MA (model averaging) method.

2.3. Phylogenetic analysis, conserved motif recognition, and promoter analysis

The CDPK protein sequences of *Arabidopsis* and rice, which were obtained from the Phytozome (https://phytozome.jgi.doe.gov/ accessed on 12 December 2022, reference genome TAIR10) and Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/ accessed on 12 December 2022, reference genome MSU7), respectively, were employed to construct phylogenetic trees with sequences of mustard. CDPK protein sequences from three species (mustard, *Arabidopsis*, and rice) were aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/ accessed on 12 December 2022) and then submitted for phylogenetic analysis by MEGA 11 (v11.011). A maximum-likelihood (ML) phylogenetic tree was constructed with 1000 bootstrap replicates. The BjuCDPK protein sequences were submitted to MEME software (https://meme-suite.org/meme/tools/meme accessed on 12 December 2022) for recognition of conserved motifs by setting the number of motifs as 10. To analyze the hormone-responsive and stress-responsive *cis*-elements in the promoters, the promoter sequences (2 kb upstream of the translation start site) of the *BjuCDPK* genes were obtained and submitted to the PlantCARE database [51].

2.4. Protein-protein interaction network prediction

Protein-protein interaction network analysis for all 101 BjuCDPK protein sequences was performed by the STRING online tool (https://string-db.org/ accessed on 12 December 2022). The orthologs of *Arabidopsis* were selected as the reference.

2.5. Plant materials, cold stress treatments, and transcriptome analysis

Mustard seeds were germinated on wet filter paper at 26 °C. After that, the germinated seedlings were transplanted into growth chambers at 14 h/10 h (day/night) cycle at 26 °C. The 21-day old seedlings were exposed to cold stress at 14 h/10 h (day/night) cycle at 4 °C. Leaf samples of seedlings were harvested at 0 h before cold treatment (unstressed seedlings), and 1 h, 3 h, 6 h, 10 h and 24 h after cold stress treatment. Each time point involved three biological replicates. About three micrograms of total RNA isolated by TRIzol Reagent (Invitrogen, Waltham, MA, USA) from each sample was used to generate sequencing libraries by NEBNext® UltraTM RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). The sizes of complementary DNA (cDNA) fragments ranged from 250 to 300 bp. Universal PCR primers, Phusion High-Fidelity DNA polymerase, and Index (X) Primer (New England Biolabs, Ipswich, MA, USA) were employed for PCR amplification. Clustering of the index-coded samples was conducted on AcBot Cluster Generation System with TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA). Library sequencing was performed on an Illumina NovaSeq instrument with 150 bp pair-end reads. NGS QC Toolkit (v2.3.3) [52] was used for quality control. The clean reads with high quality were mapped to reference genome Braju_tum_V2.0 using HISAT2 (v2.2.1) [53], and relative expression levels in transcripts per million

(TPM) for each gene of each sample were calculated by StringTie (v2.2.0) [54] with default parameters.

2.6. *qRT-PCR for expression analysis*

Two micrograms of total RNA for each leaf sample of seedlings were reverse transcribed using HiScript® III Reverse Transcriptase (Vazyme Biotech Co., Ltd.). Real-time PCR was conducted on a CFX Connect™ real-time PCR detection system (Bio-Rad Laboratories, Inc.) using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.). *TIP41* (tonoplastic intrinsic protein 41) was used as the reference gene, and each sample was assessed in triplicate of technical replication. All primers used are listed in Table S1.

3. Results

3.1. *Identification and characterization of CDPK family genes in Brassica juncea*

In this study, the Hidden Markov Model (HMM) profiles of the Pkinase domain (PF00069) and EF-hand 7 domain (PF13499) were used to characterize putative CDPK proteins. A total of 101 candidate *CDPK* genes were identified in the *Brassica juncea* genome (Braju_tum_V2.0). The physicochemical properties of their encoded proteins were analyzed based on their putative amino acid sequences (Table S2). These *CDPK* genes were designated as *BjuCDPK1* to *BjuCDPK101* based on their chromosome localization (Figure 1 and Table S2). The Gene Location Visualize of TBtools was used to construct a chromosomal location map. The results showed that the 101 *BjuCDPK* genes were unevenly distributed on all chromosomes except for AA_Chr08 (Figure 1). Among them, 12 *BjuCDPK* genes were detected on BB_Chr02, 10 on AA_Chr03, eight each on of BB_Chr05 and BB_Chr08, seven on AA_Chr09, six each on of AA_Chr02, AA_Chr10, and BB_Chr03, five on each of AA_Chr06, AA_Chr07, and BB_Chr07, four each on of AA_Chr01, AA_Chr05, BB_Chr01, BB_Chr04, and BB_Chr06, and three on AA_Chr04 (Figure 1).

The length of the 101 BjuCDPK proteins ranged from 419 aa (BjuCDPK74) to 915 aa (BjuCDPK31), and the MWs ranged from 47.234 kDa (BjuCDPK74) to 104.460 kDa (BjuCDPK31). The *p*I varied from 4.83 (BjuCDPK8) to 9.34 (BjuCDPK29), with 91 BjuCDPKs being acidic proteins and the remaining being basic proteins. The predicted grand average of hydropathy (GRAVY) values of all BjuCDPK proteins were negative, suggesting that all these proteins are hydrophilic. Instability index analysis revealed that 51.49% (52/101) of BjuCDPK proteins were unstable, while the rest were stable proteins (Table S2). Subcellular localization analysis indicated that 83.17% (84/101) of BjuCDPK proteins were located at the cell membrane, while 16 proteins were located in the cytoplasm, and only one protein (BjuCDPK31) was localized in the nucleus. All BjuCDPK proteins contained EF-hand domains. Most BjuCDPK proteins (86/101) contained four EF-hand domains, while six had three EF-hand domains and nine had only two EF-hand domains. In terms of lipid modification sites, 81 BjuCDPK proteins might have myristoylation sites in their N-terminus and 82 BjuCDPK proteins might contain S-palmitoylation sites (Table S2).

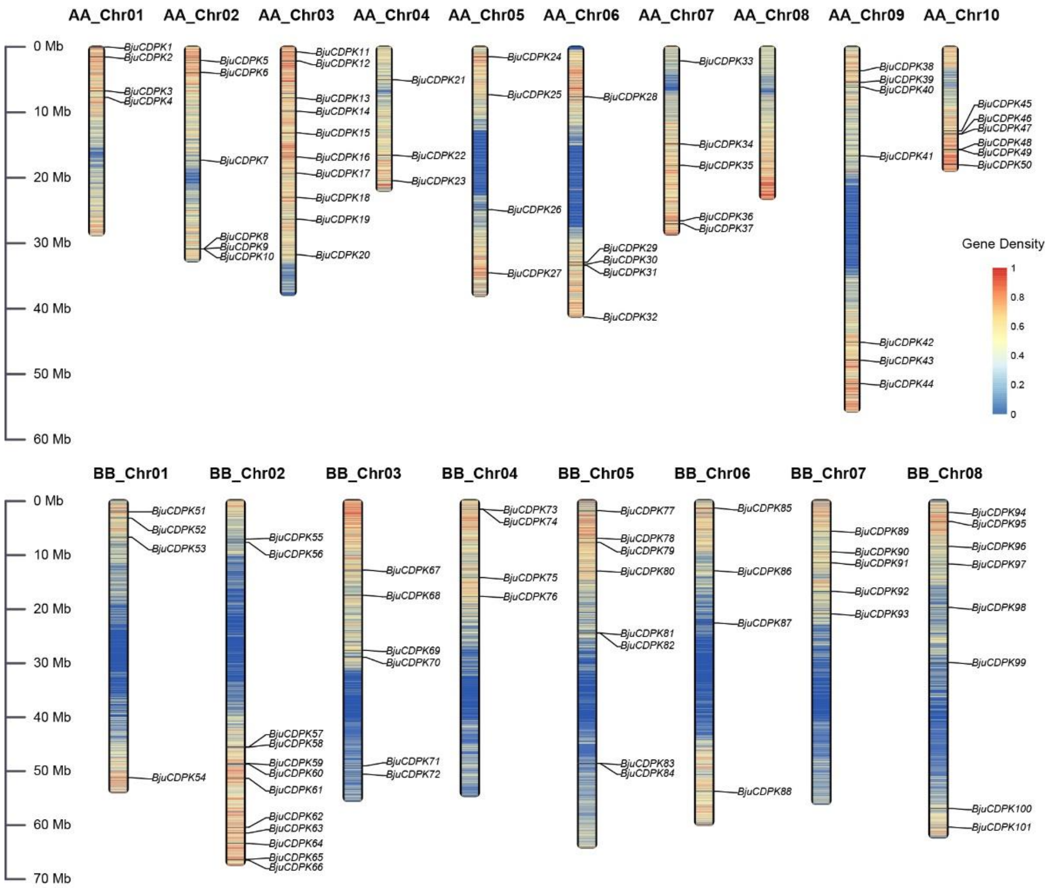

Figure 1. Distribution of 101 *BjuCDPK* genes in mustard genome. Chromosome numbers are listed above and the chromosome sizes are indicated on the left of the figure. The length of each chromosome was estimated in mega base (Mb).

3.2. Phylogenetic relationship of CDPKs in Arabidopsis, rice, and mustard

To investigate the evolutionary relationships of CDPK proteins in different plant species, a phylogenetic tree was constructed for 164 CDPKs, including 29 OsCPKs, 34 AtCPKs, and 101 BjuCDPKs (Figure 2). Based on the structural characteristics of protein sequences and previous classification of CDPK proteins in *Arabidopsis*, the 101 BjuCDPKs were clustered into four distinct groups, namely Group I to IV [26]. The largest group (Group I) contained 34 BjuCDPKs; Group II comprised 26 BjuCDPKs; Group III included 32 BjuCDPKs; while Group IV had only nine BjuCDPKs. An analysis of the evolutionary relationships indicated that the CDPK proteins in mustard have a closer relationship with those in *Arabidopsis* than with those in rice (Figure 2).

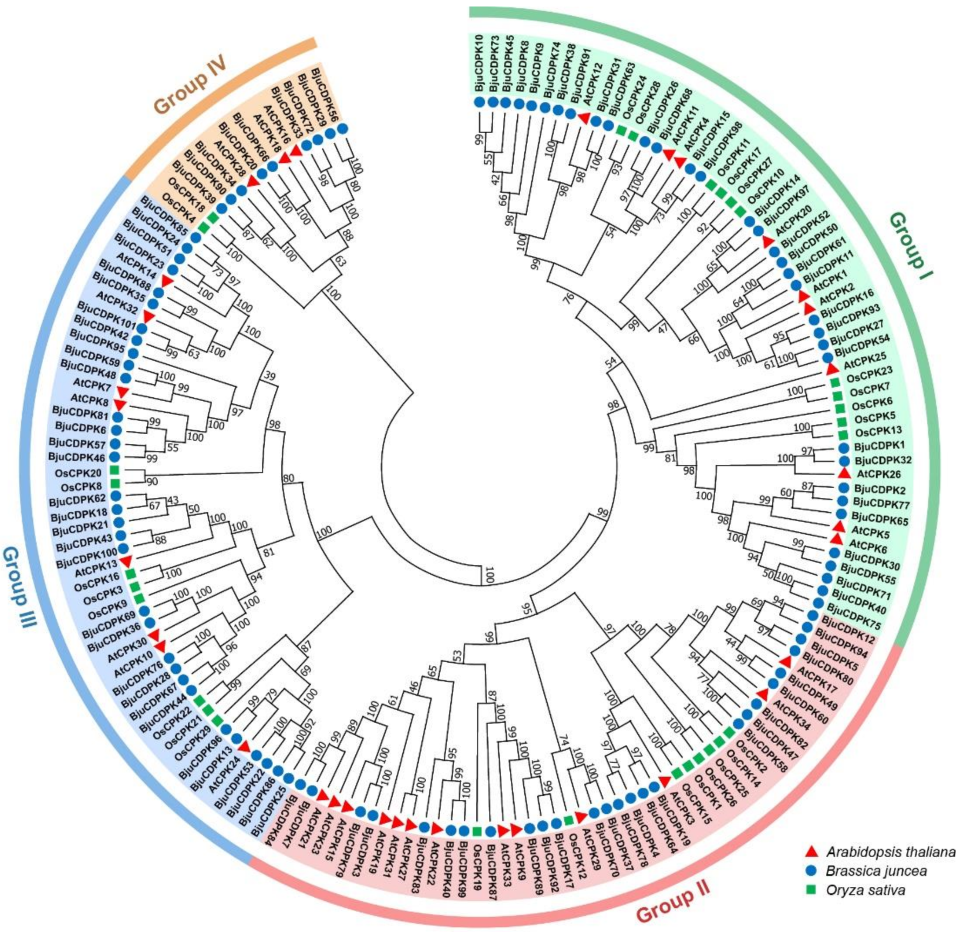

Figure 2. Phylogenetic analysis of the CDPKs in *Arabidopsis thaliana*, rice (*Oryza sativa*), and mustard (*Brassica juncea*). A total of 165 CDPKs were classified into four distinct groups (Group I to IV) based on their protein sequence structural characteristics. The red triangles, blue cycles, and green squares represent AtCPKs in *Arabidopsis*, OsCPKs in rice, and BjuCDPKs in mustard, respectively.

3.3. Conserved motif and gene structural analysis of BjuCDPKs

To investigate the structural characteristics of the mustard CDPKs, a motif analysis was performed by using the MEME server (Figure S1). A total of 10 different motifs were identified, including six protein kinase and four EF-hand domains. Most BjuCDPK proteins contained all these ten motifs, and most members in the same group contained uniform motif arrangements (Figure 3A, 3B). The exceptions included BjuCDPK74 and BjuCDPK52 in Group I, which were lack of two protein kinase domains, BjuCDPK41 and BjuCDPK99 in Group II, which were lack of three protein kinase domains, BjuCDPK83, which was lack of two protein kinase domains, as well as BjuCDPK18 and BjuCDPK100 in Group III, which were lack of one protein kinase domain. In addition, all members of Group IV contained six protein kinase domains. The number of EF-hand domains in each BjuCDPK protein was presented in Table S2.

The gene structure varied among the 101 *BjuCDPKs*. The number of exons in the coding sequence (CDS) ranged from five (*BjuCDPK5*) to 14 (*BjuCDPK41*). Group III and Group IV members exhibited a similar exon/intron structure in their encoding genes. *BjuCDPK* genes in Group III harbored seven to eight exons, and those in Group IV had 11 to 13 exons. However, there were considerable differences in the number of exons between Group I (6–11 exons) and Group II (5–14 exons) members (Figure 3A, 3C).

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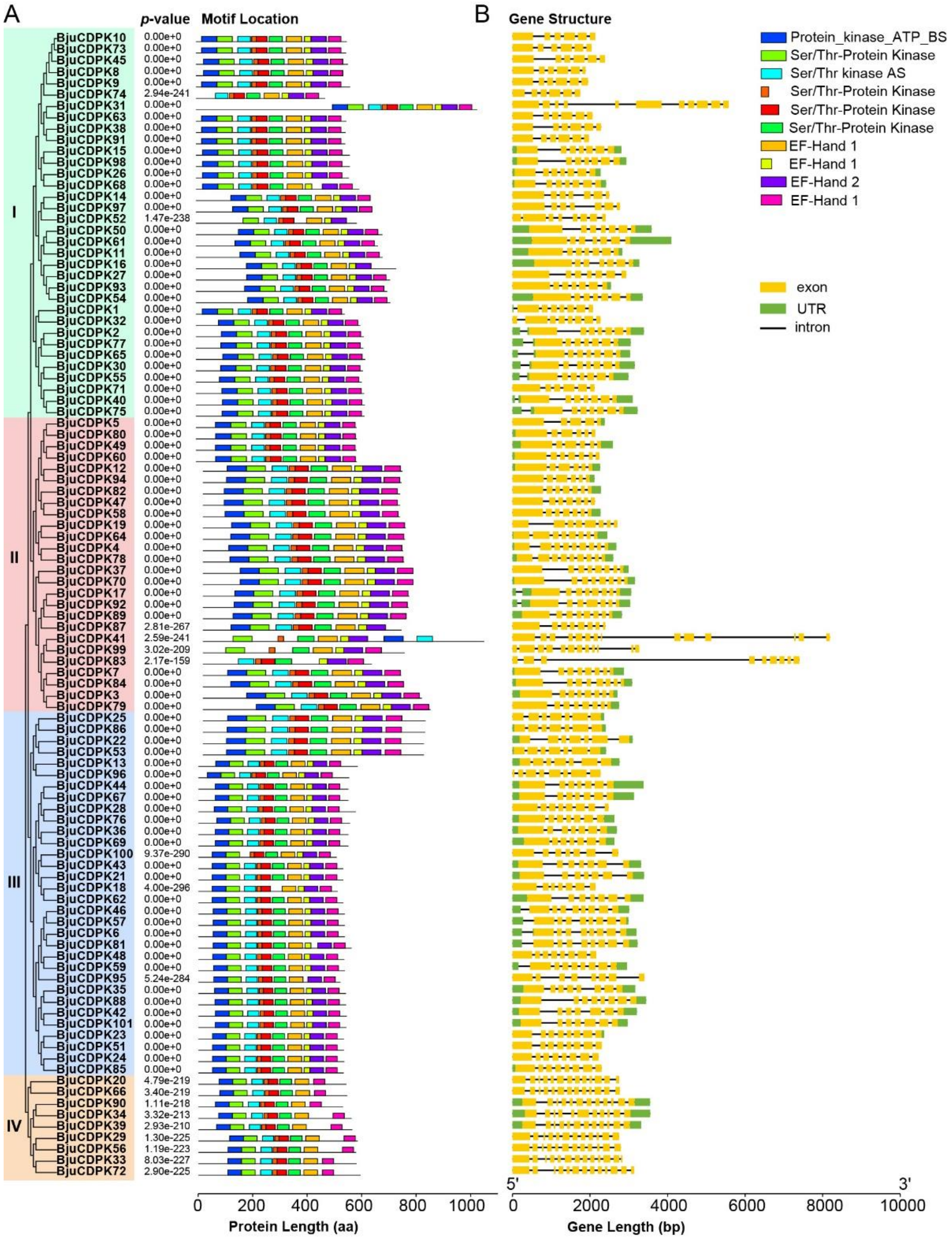

Figure 3. Phylogenetic analysis, conserved motifs, and structure of encoding genes of BjuCDPKs. (**A**) Motif composition and distribution of 101 BjuCDPK proteins based on the phylogenetic relationship analysis. (**B**)Ten motifs are indicated by different colors. The lengths of the boxes and lines are proportional to protein lengths. (**C**) Structure analysis of *BjuCDPKs* genes. UTRs and exons are indicted by green and yellow boxes, respectively, and introns are shown by black lines. The lengths of the boxes and lines are proportional to gene lengths.

3.4. Gene duplication of BjuCDPKs

The obviously larger number of *BjuCDPK* genes indicated that the *BjuCDPK* gene family may undergone significant expansion compared with the family of other plants. To investigate the underlying mechanism, we analyzed the gene duplication patterns of *BjuCDPK* genes, including tandem and segmental duplications. As shown in Figure 4, a total of 188 segmental duplication events were observed, but no tandem duplication event was detected.

To further understand the selective pressure and evolutionary fates of the *CDPK* gene pairs resulting from duplication, the nonsynonymous/synonymous substitution ratio (Ka/Ks) was calculated for the full-length gene regions. Generally, a Ka/Ks value greater than 1 indicates positive selection, and a value of 1 indicates neutral selection, while a value lower than 1 indicates purifying or negative selection [55]. In this study, the Ka/Ks values of all 188 *BjuCDPK* gene pairs resulting from segmental duplication were lower than 0.6 (ranging from 0.028 to 0.476, with an average of 0.123) (Table S3). These results suggested that the *BjuCDPK* genes have undergone strong purifying selection and slow evolution at the protein level.

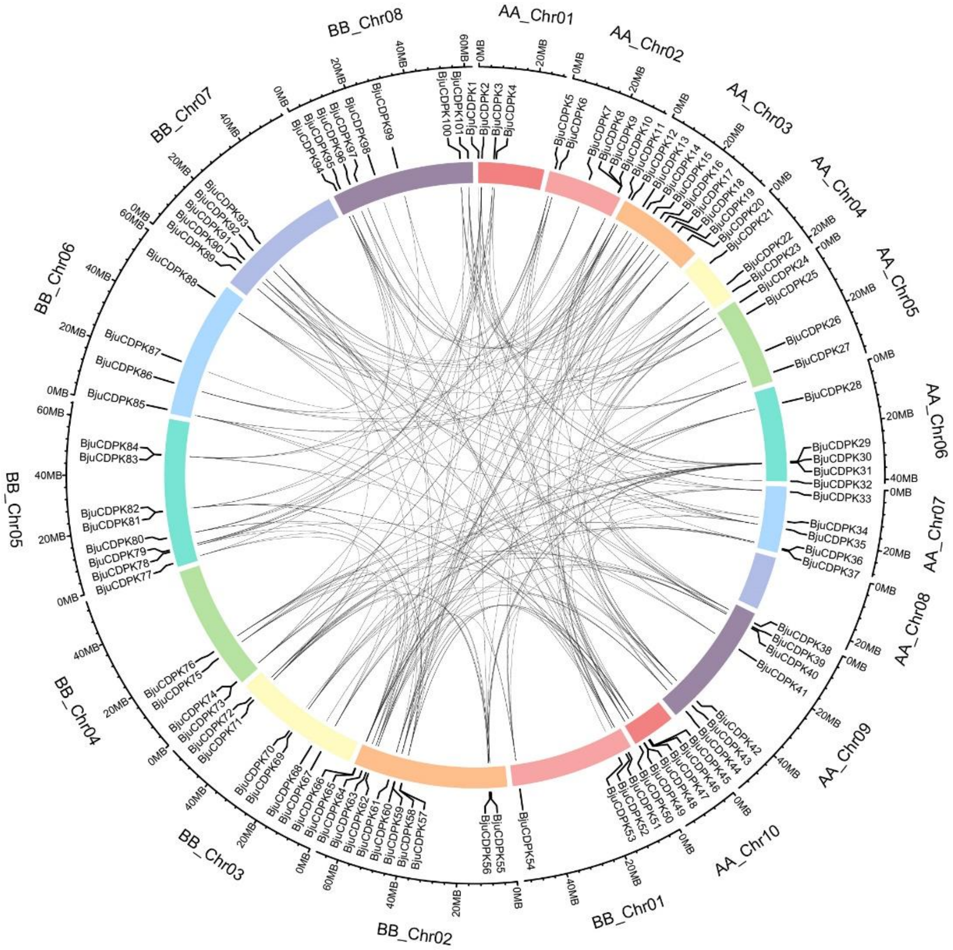

Figure 4. Synteny analysis of interchromosomal relationships of *BjuCDPK* genes. *BjuCDPK* gene pairs in the *B. juncea* genome are indicated by black lines.

3.5. Expression analysis of BjuCDPK genes in various organs of mustard

To reveal the potential biological functions of *BjuCDPKs* during the growth and development of mustard, the RNA-seq data from different mustard organs, including the leaf, stem, root, flower, pod, pod shell, and seed, were obtained (Figure 5). A total of 83 *BjuCDPK* genes showed TPM values higher than 1 in at least one of the tested organs. The *BjuCDPK* genes from different groups exhibited inconsistent expression patterns in different organs, but those closely related *BjuCDPKs* showed similar expression patterns. The majority of *BjuCDPKs* exhibited organ-specific expression. The *BjuCDPK* genes were predominantly expressed in the root and flower bud, suggesting that these genes possibly have important roles in the development of these two organs. In vegetative organs, 33.66% (34/101) of *BjuCDPKs* showed a root-specific expression pattern, while 12 and five *BjuCDPKs* were preferentially expressed in the stem and leaf, respectively. In reproductive organs, 16 *BjuCDPKs* were only expressed in the flower bud, among which six belonged to Group II, and eight and two were clustered in Group III and IV, respectively. At different developmental stages of the pod, seven

BjuCDPKs were preferentially expressed at 7 days after pollination (DAP); only two *BjuCDPKs* were preferentially expressed at 15 DAP; while eight *BjuCDPKs* showed preferential expression in the pod shell of 20 DAP. No *BjuCDPKs* showed preferential expression in the seed. These results suggested that the *BjuCDPK* family genes may be involved in the development of multiple mustard organs.

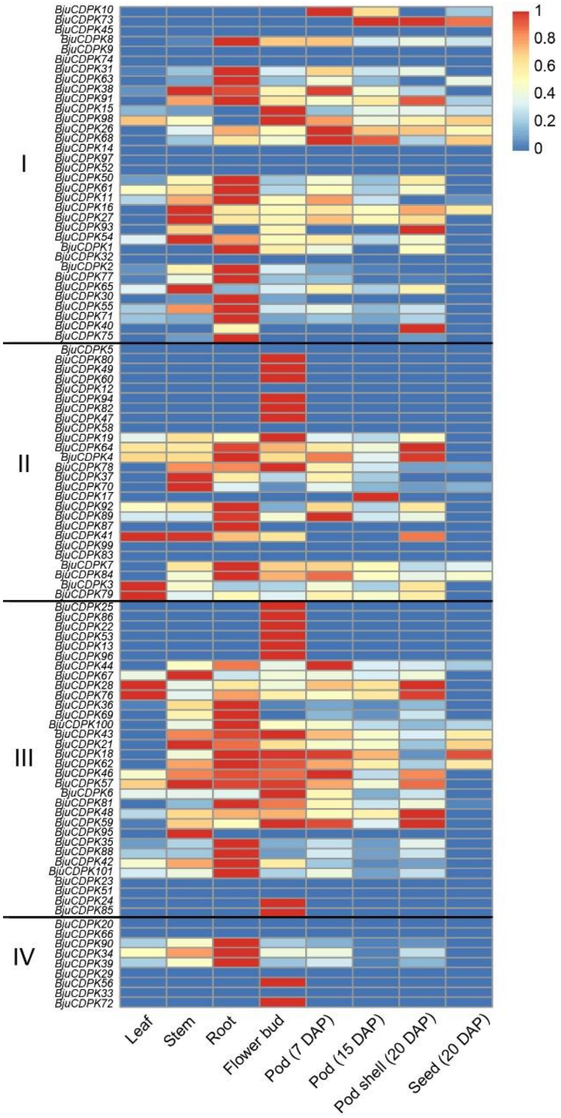

Figure 5. Expression patterns of 101 *BjuCDPK* genes in the leaf, stem, root, flower bud, and pod at different developmental stages. The relative expression levels were determined by RNA-seq data and normalized to 0–1.

3.6. Expression analysis of BjuCDPK genes under cold stress

To assess the potential function of *BjuCDPK* genes in response to cold stress, their transcript accumulation was assessed using RNA-seq data from mustard leaves at 0, 1, 3, 6, 10, and 12 h after exposure to cold stress at 4 °C (Table S4). As a result, 62 *BjuCDPKs* averagely presented TPM values higher than 1 in at least one sample. These *BjuCDPKs* were analyzed and clustered into three groups, with 23 *BjuCDPKs* in Group a, 20 *BjuCDPKs* in Group b, and 19 *BjuCDPKs* in Group c (Figure 6A). In Group a, most *BjuCDPKs* showed up-regulated expression at 6 h after cold treatment, while a few exhibited down-regulated expression. In contrast, most members in Group b exhibited the highest expression levels before cold treatment, while only six members showed increases in expression levels at 6 h after cold treatment. In addition, *BjuCDPK* genes from both Group a and Group b displayed lower expression levels at 1, 3, and 10 h after cold treatment. In Group c, over half of the members were up-regulated at 10 h, while the remaining members were highly expressed at 0, 1, 3, and 6 h after cold treatment (Figure 6A).

qRT-PCR was performed to validate the expression changes of ten randomly selected *BjuCDPK* genes under cold stress treatment (Figure 6B). *BjuCDPK39*, *BjuCDPK68*, *BjuCDPK75*, and *BjuCDPK90* exhibited similar trends with the highest expression at 6 h, while the expression of *BjuCDPK10* and *BjuCDPK67* peaked at 10 h. *BjuCDPK13*, *BjuCDPK71*, *BjuCDPK76*, and *BjuCDPK96* showed the highest expression level at 24 h.

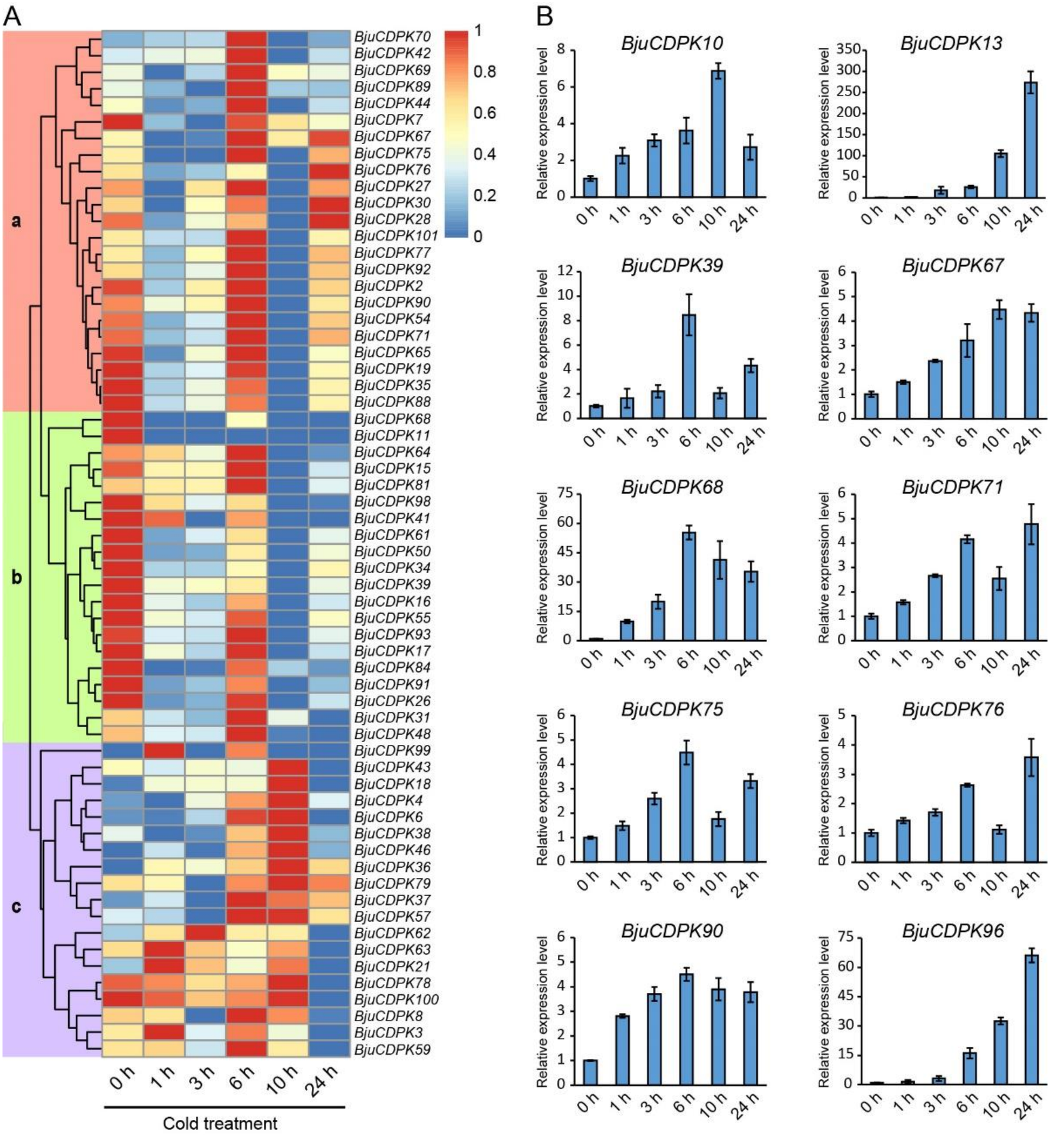

Figure 6. Expression patterns of *BjuCDPK* genes under cold stress. (**A**). Heatmap of expression profiles of *BjuCDPK* genes in seedlings under cold (4 °C) treatment, and the TPM value for each gene was normalized to 0–1. (**B**). qRT-PCR validation of ten selected *BjuCDPK* genes under cold stress.

3.7. Analysis of cis-acting elements of CDPK family genes in mustard

To illustrate how the promoter structure influences the gene expression levels, we extracted and analyzed 2000 bp upstream sequences of 101 *BjuCDPKs*. A total of 31 types of *cis*-elements were identified, including 12 hormone-responsive elements and 19 stress-responsive elements (Figure 7). Notably, all *BjuCDPK* genes contained at least one type of hormone-responsive and stress-responsive elements. For instance, the ABRE element is one of the ABA-responsive elements with a highly conserved ACGTG motif, and 84 *BjuCDPK* genes contained at least one ABRE element, indicating that they might play crucial roles in ABA signaling response. Two MeJA-responsive elements, CGTCA and TGACG motifs, were present in the promoter regions of 68.32% (69/101) of the *BjuCDPK* genes. All *BjuCDPK* genes had the MYB element and almost all *BjuCDPKs* (except for *BjuCDPK84*) had the MYC element, suggesting that the *BjuCDPK* genes may be regulated by MYB and MYC transcription factors. Furthermore, 94 *BjuCDPK* genes had the anaerobic induction element ARE, and 42 *BjuCDPK* genes contained at least one low-temperature responsive element LTR. These results indicated that *BjuCDPK* genes might play vital roles in the growth, development, and environmental stress response of mustard.

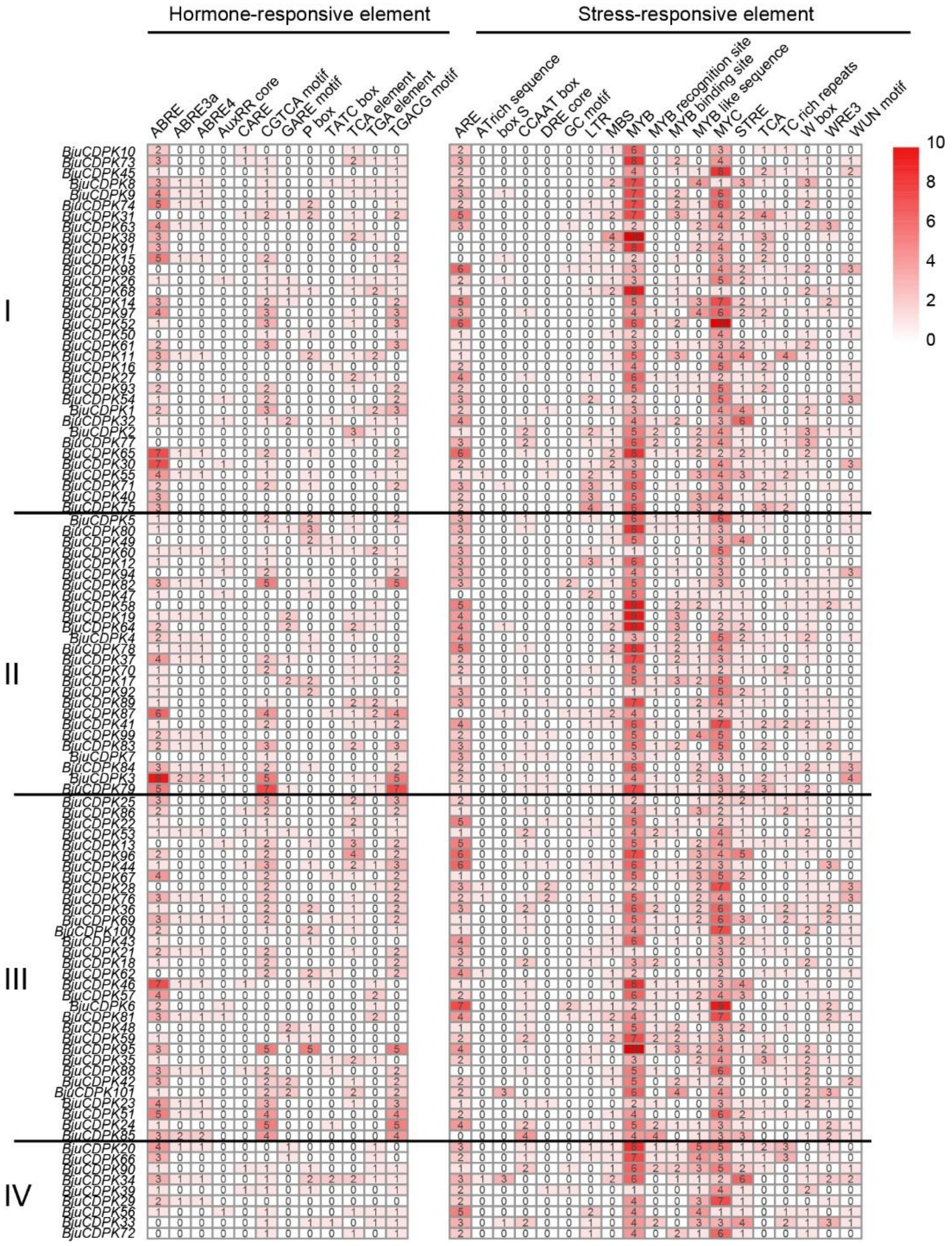

Figure 7. Numbers of 12 types of hormone-responsive elements and 19 types of stress-responsive elements in *BjuCDPK* gene promoter regions.

3.8. Protein-protein interaction network analysis of BjuCDPKs

To explore the potential interaction and regulatory network between BjuCDPKs and other proteins, we predicted the interaction partners of key BjuCDPK proteins using the STRING software based on the orthologs in *Arabidopsis*. Overall, three pairs of protein-protein interactions were predicted within BjuCDPKs. BjuCDPK34, BjuCDPK68, and BjuCDPK75 might interact with

BjuCDPK101, BjuCDPK96, and BjuCDPK76, respectively. In addition, 53 pairs of protein-protein interactions were predicted between BjuCDPKs and other proteins, including SLAH3 (slow anion channel 1 homolog 3, 12 pairs), ABF4 (ABA-responsive element binding factor 4, 12 pairs), ABF1 (ABA-responsive element binding factor 1, 11 pairs), OZS1 (ozone-sensitive, 11 pairs), and DI19 (dehydration-induced 19, seven pairs) (Figure 8 and Table S5).

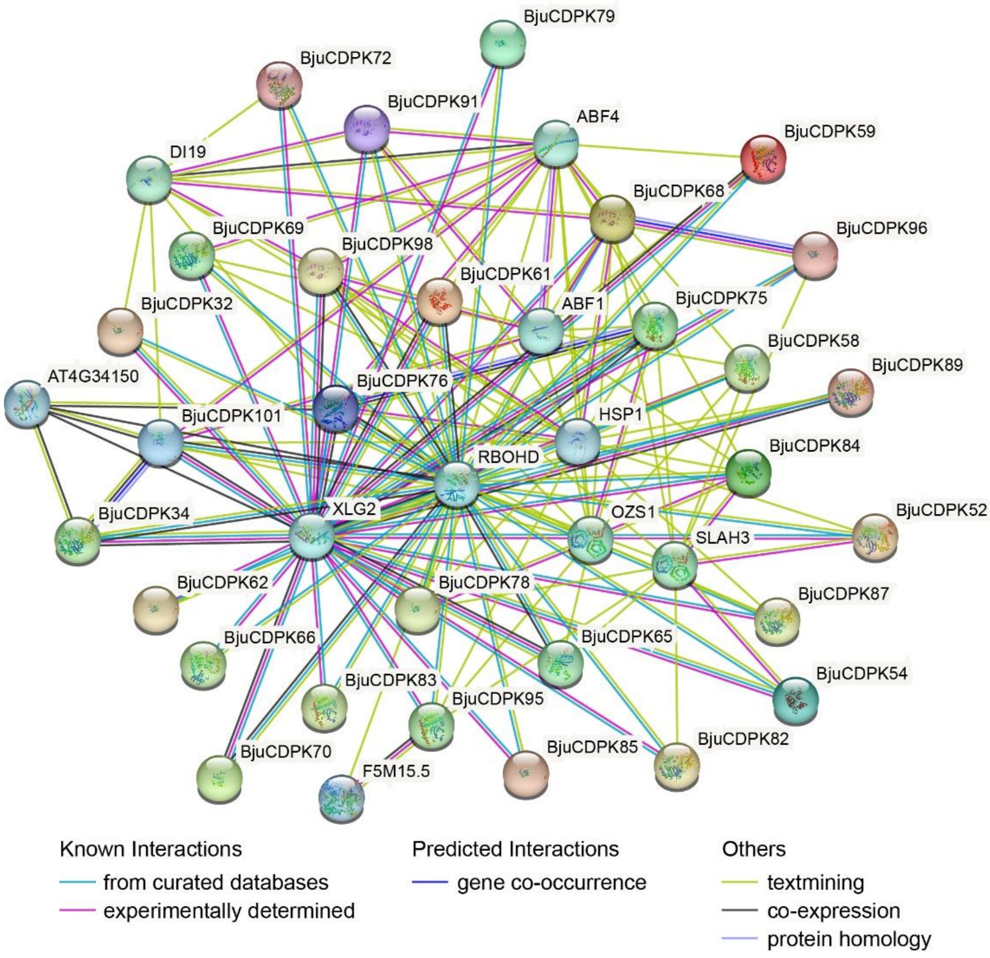

Figure 8. Protein-protein interaction network of BjuCDPKs.

4. Discussion

CDPKs are serine/threonine protein kinases playing crucial regulatory roles in the development and stress response of plants. Various numbers of *CDPK* genes have been identified in different species through bioinformatics analysis of their genomes, such as maize (40 *CDPKs*), *Arabidopsis* (34 *CDPKs*), rice (29 *CDPKs*), peach (17 *CDPKs*), and strawberry (11 *CDPKs*) [27,28,37,38]. In this study, 101 *CDPK* genes were identified in mustard genome, which were unevenly distributed in different chromosomes, indicating possible genetic variation and species evolution. Gene duplication analysis revealed that 99 out of the 101 *BjuCDPK* genes (98.02%) are associated with segmental duplication, suggesting that large fragment duplication is the main force driving the expansion of *CDPK* gene family in mustard. Gene structure analysis revealed that great variations were present in the number of introns and exons among the 101 *BjuCDPK* genes, indicating that the *CDPK* family genes might have differential biological functions in mustard.

Previous studies showed that plant *CDPK* genes have distinct structural features. Based on the evolutionary distance, the 101 BjuCDPKs were clustered into four groups (Group I to IV), all of which were more closely related to AtCPKs than to OsCPKs. The N-terminal of 101 BjuCDPKs varied in length and sequence, and they were predicted to be located at the cell membrane (84 BjuCDPKs), cytoplasm (16 BjuCDPKs), and nucleus (one BjuCDPK). The cytoplasm-located BjuCDPKs consisted of 41 members in Group I and two members in Group II, which had no myristoylation site or S-palmitoylation site in their N-terminus. The only protein located in the nucleus was BjuCDPK31, which had the highest protein length, particularly the length of the N-terminus, and it was lack of myristoylation sites. Typically, the N-terminal domains of CDPKs are characterized by potential N-myristoylation and N-palmitoylation sites, which determine the membrane targeting of plant CDPKs [56]. For example, AtCPK5 is myristoylated and membrane-targeted in *Arabidopsis*, while the mutation in the myristoylation (G2A) site resulted in a defect in its membrane localization [57]. The catalytic kinase region, including the typical Ser/Thr protein kinase, and the regulatory region containing 1-4 EF-hand domains, were present in all BjuCDPK proteins, which is consistent with the previous speculation that the *CDPK* genes may have evolved from the fusion of an ancestral CaM kinase gene with a calmodulin gene [58,59].

CDPKs have been reported to coordinate developmental processes and potentially interact with the phytohormone network. In *Arabidopsis*, AtCPK28 regulates the transition from vegetative growth to reproductive growth and stem elongation during plant development by altering the expression of NAC transcriptional and gibberellic acid homeostasis regulators [60]. OsCPK13 is a callus- and leaf sheath-specific expressed protein with important functions in rice leaf sheath elongation, and its transcription levels and protein accumulation are induced by gibberellins (GA) but suppressed by abscisic acid (ABA) and brassinolide (BL) [61]. In this study, 12 hormone-responsive elements were found in the promoter regions of *BjuCDPK* genes. The differential gene expression patterns of *CDPKs* in different organs may suggest their differential roles in plant growth and development. In eight different organs of mustard (such as leaf, stem, root, flower bud, pod at 7 DAP, pod at 15 DAP, pod shell at 20 DAP, and seed at 20 DAP), 83 *BjuCDPK* genes were expressed in at least one organ as indicated by RNA-seq data analysis and the TPM values were more than 1. Notably, most *BjuCDPK* genes were preferentially expressed in the roots and flower buds of mustard (Figure 5), indicating that these genes may play important roles in regulating the development of these two organs of mustard.

Cis-element analysis revealed the presence of various hormone- and stress-responsive elements in the promoter regions of *BjuCDPK* genes, suggesting that these genes may play important roles in response to stress, which is consistent with previous findings in other species [62–65]. In particular, 42 out of 101 *BjuCDPK* genes contained at least one LTR element (responding to cold stress) in their promoter regions (Figure 7), and a large number of *BjuCDPK* genes displayed altered expression under cold stress via RNA-seq data (Figure 6A), indicating their possible roles in response to cold stress. In *Solanum habrochaites*, 11 *ShCDPK* family members were found to have LTR element, and the expression of a set of *ShCDPK* genes changed to a certain extent under cold stress [66]. Similar results were also found in other plants, such as *Gossypium barbadense* [67], *Medicago truncatula* [68]. Moreover, the expression of ten seleted *BjuCDPK* genes were observably induced by cold stress based on qRT-PCR analysis (Figure 6B), further supported the RNA-seq data. And the expression profiles of *CDPK* orthologous genes in different plants were found to regulated by cold stress, which indicated that the role of *CDPK* genes in cold stress response is conserved across different plants. As a key Ca^{2+} sensor group of protein kinase, CDPK proteins play important roles in Ca^{2+}-mediated signaling pathways under cold stress, and the studies of CDPK roles in cold tolerance were also reported in other species, including *VaCPK20* in *Vitis amurensis* [69], *MdCPK1a* in *Malus domestica* [70], *SpCPK33* in *Solanum pennellii* [71], as well as in other reports [21–23,72,73].

The interaction network prediction by STRING revealed three pairs of protein interactions between BjuCDPK proteins, including BjuCDPK34 and BjuCDPK101, BjuCDPK68 and BjuCDPK96, and BjuCDPK75 and BjuCDPK76. Furthermore, BjuCDPKs were predicted to interact with several other proteins, including SLAH3, ABF4, ABF1, OZS1, and DI19. In the pollen tube tip, phosphorylation by AtCPK2 and AtCPK20 activates the S-type anion channel SLAH3, which regulates pollen tube growth through Ca^{2+} and cytosolic anion gradients [74]. AtCPKs have been reported to participate in response to stress by interacting with ABF1, ABF4, and DI19 in *Arabidopsis*. *AtCPK32* can be induced by high-salt stress, and its overexpression lines are sensitive to ABA and NaCl during seed germination. *AtCPK32* regulates gene expression in response to ABA by phosphorylating ABF4 [75]. Under salt stress, AtCPK4 and AtCPK11 participate in ABA signal transduction by phosphorylating the ABA-responsive transcription factors ABF1 and ABF4 [76]. AtDi19, a stress-induced protein, can also be phosphorylated by AtCPK11 to regulate plant stress response via ABA signal transduction pathway [77]. In this study, five BjuCDPKs (BjuCDPK68, BjuCDPK76, BjuCDPK91, BjuCDPK98, and BjuCDPK101) were predicted to interact with ABF1, ABF4 and/or DI19, while six other BjuCDPKs (BjuCDPK58, BjuCDPK59, BjuCDPK69, BjuCDPK75, BjuCDPK78, and BjuCDPK84) might interact with ABF1 and/or ABF4. Based on the qRT-PCR result that *BjuCDPK68*, *BjuCDPK75*, and *BjuCDPK76* were up-regulated after cold treatment, it can be speculated that these proteins play crucial roles in response to cold stress as interaction partners of ABF1, ABF4 and/or DI19.

5. Conclusions

In this study, 101 *BjuCDPK* genes were systematically identified and characterized in mustard. According to the diversity of conserved domains, BjuCDPK proteins could be divided into four groups, in which proteins with close phylogenetic relationships exhibited strong conservation in gene structure and motif organization. Additionally, the *BjuCDPK* genes harbor many *cis*-elements responsive to various hormones and stresses. RNA-seq analysis revealed that most *BjuCDPK* genes were upregulated upon cold stress treatment. Moreover, proteins predicted to interact with BjuCDPKs are associated with abiotic stress responses in plants. Overall, this study provides vital information for further biological functional characterization of *BjuCDPK* genes and a theoretical basis for improvement of cold stress tolerance of mustard.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Motif analysis of BjuCDPKs using MEME; Table S1: Information of the primers used in this study; Table S2: Identification and characterization of *CDPK* family genes in *Brassica juncea*; Table S3: Ka/Ks values of duplicated *BjuCDPK* gene pairs; Table S4: TPM values of 101 *BjuCDPK* genes under cold treatment; Table S5: Protein-protein interaction network prediction of BjuCDPKs.

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