Identification and Expression Analysis of CKX Gene Family in *Brassica juncea* var. *tumida* and Their Functional Analysis in Stem Development

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Abstract: Cytokinin oxidase/dehydrogenase (CKX) is a specific enzyme affecting plant growth, development, and yield by mediating the metabolism of endogenous cytokinins in a dual catalytic mode. This study aims to reveal the distribution and associated properties of CKX gene family members in the whole genome of *Brassica juncea*, lay a theoretical basis for further exploration of the biological function of *BjuCKX* genes, and provide genetic resources to improve the breeding quality of *B. juncea*. We screened *BjuCKX* genes with typical FAD-binding and CK binding domains and identified them using bioinformatics methods. In addition, we analyzed physicochemical properties, phylogenetic relationship, gene structure, conservative motif, cis-acting element of promoter, and expression pattern of *BjuCKX* gene family members. Endogenous hormone levels (GA3, ZR, IAA, ABA, BR and MeJA) were also determined in different developmental periods using an indirect enzyme-linked immunosorbent assay (ELISA). A total of 23 *BjuCKX* genes were identified, and they were renamed *BjuCKX01* ~ *BjuCKX23*. Further analysis revealed that the amino acid number of 23 *BjuCKX* proteins ranged from 333 to 1337 aa, the molecular weight ranged 36.58 to 148.49 kDa, whereas the theoretical isoelectric point ranged from 4.94 to 9.10. The phylogenetic tree clustering analysis can group family members into four subgroups. Collinearity analysis revealed that genes were not evenly distributed on the chromosome, with a pair of tandem repeats. Meanwhile, *BjuCKX* genes located on each chromosome revealed cross collinearity caused by fragment replication. The genes were more conserved in structure. In the upstream region of promoter, there were several cis-acting elements, including plant growth and development, hormone response, and biological and abiotic stress. Combined with transcriptome data, *BjuCKX* gene expression has been demonstrated to be different at varying developmental stages of the stem. RT-qPCR further confirmed that *BjuCKX* genes were involved in stem development and affects growth by regulating endogenous hormone levels.

Keywords: CKX gene family; phylogenetic analysis; collinearity analysis; expression analysis; stem development; endogenous hormone; *Brassica juncea*

1. Introduction

Cytokinins play a key role in plant source-sink coordination, stress response, and nitrogen metabolism, in addition to being involved in a series of plant growth and development processes, including root and stem growth, flower bud differentiation, fruit development, and leaf senescence [1–4]. Endogenous cytokinin metabolism in plants is a delicate and complex network system, in which catabolic is primarily accomplished through reversible
glycosylation, conversion to cytokinin nucleotides by adenine phosphoribosyltransferase genes, and irreversible breakdown of cytokinin-by-cytokinin oxidase/dehydrogenases [5]. Cytokinin oxidase/dehydrogenase can degrade active cytokinins using quinone type electron receptors, hence regulating cytokinin homeostasis in plant cells, tissues, and organs [6]. In general, mature cytokinin oxidase/dehydrogenase proteins contain FAD conserved domains at N-terminal and CK conserved domains at C-terminal, which can cut the unsaturated isoprenoid side chain at N6 end and degrade cytokinin in a dual catalytic mode [7]. Currently, studies have demonstrated that regulating cytokinin levels by inhibiting cytokinin oxidase/dehydrogenase can positively affect plant growth, development, and yield [8]. Additionally, a series of novel cytokinin oxidase/dehydrogenase inhibitors have become targets for improving plant yield and stress resistance and are widely employed in modern plant biotechnology and agriculture [8,9].

CKX genes primarily encode cytokinin oxidase/dehydrogenase. CKX gene family is a small family containing several subtypes with different substrate specificities, spatiotemporal expression patterns, and subcellular localization, and the number of genes contained in different plant genomes varies [10–12]. Identification and characterization of CKX gene families in various plant species revealed 7 members in Arabidopsis, 11 in rice, 13 in corn, 17 in soybean, 12 in Chinese cabbage, and 23 in Brassica napus [12–14]. CKX genes have been proved to have various functions, such as yield regulation and stress response. In B. napus, mutations in specific CKX genes can regulate cytokinin status to attain dicotyledonous crop yield, with four BnCKX3 and two BnCKX5 mutants demonstrating more flowers and main stem pod numbers [15]. Studies in rice have found that OsCKX11 knockout mutants exhibit moderate leaf senescence and higher photosynthetic performance and increase tillering and grain count [4]. OsCKX2-2 with 3-UTR mutation enhanced the accumulation of endogenous cytokinin by affecting the activity of cytokinin oxidase/dehydrogenases, thus regulating inflorescence development and grain size [16]. In Lotus japonicus, LjCKX3 maintains nodule development efficiently by regulating cytokinin accumulation, and ckx3 mutants reduce nodule formation, rhizobia infection, and root growth [17]. Root-specific expression of chickpea CKX6 leads to enhanced root growth, drought tolerance, and yield without compromising nodulation [18]. Ectopic expression of CKX1 gene in Arabidopsis enhances drought and heat resistance in tobacco plants [19].

Allotetraploid Brassica juncea (AABB, 2n = 36) is formed by cross-doubling between Brassica rapa (AA, 2n = 20) and Brassica nigra (BB, 2n = 16) that underwent a genome-wide tripling event [20]. As a stem variety of mustard, tumorous stem mustard (Brassica juncea var. tumida) uses fleshy tumorous stems for fresh food and mustard processing; nonetheless, the formation and expansion of tumorous stems is a complex process involving structural changes, substance accumulation, and gene regulation [21]. Based on the contribution of cytokinin oxidase/dehydrogenase in plant growth and development, a comprehensive investigation of CKX gene family in tumorous stem mustard was performed. We demonstrate the member characteristics, evolutionary expansion, and transcriptional expression of BjuCKX gene family and have a deeper insight into the mechanism of CKX gene involved in enlargement of stem tumors. Additionally, it provides a reference for further studying the function of CKX proteins.

2. Materials and Methods

2.1. Identification of CKX Gene Family Members in B. juncea

The genome sequence information of B. juncea were downloaded in the Brassicaceae Database (BRAD, http://brassicadb.cn/; accessed on 1 May 2021). All protein sequences of AtCKXs were downloaded in Arabidopsis thaliana genome database (https://www.arabidopsis.org/; accessed on 10 May 2021) and used as query sequences. The blastp comparison was performed in B. juncea genome database. The screening threshold was set to 1e−10 to obtain candidate genes. The protein sequences of candidate genes were further submitted to Pfam database (http://pfam.xfam.
org/; accessed on 10 May 2021) for FAD-binding domain (PF01565.23) and CK binding domain (PF09265.10) validation. Protein sequences containing both domains were selected.

2.2. Sequence Analysis and Phylogenetic Tree Construction

The online ProtParam tool (http://web.expasy.org/protparam/; accessed on 5 July 2021) was used to predict molecular weight, isoelectric point and amino acid size [22]. Subcellular localization was predicted in WOLF PSORT (https://www.genscript.com/wolf-psort.html; accessed on 5 July 2021). Multiple sequence alignments were made using Clustal W, and MEGA X software was performed to construct the phylogenetic tree using the neighbor-joining method with 1000 bootstrap replicates [23].

2.3. Chromosomal Localization and Collinearity Analysis

Chromosomal localization and intraspecific and interspecific collinearity plot were carried out by TBtools software [24]. The chromosomal localization of CKX genes was performed using the GFF3 annotation file of B. juncea. Genomic sequences and GFF3 annotation files of B. juncea, Arabidopsis thaliana, B. rapa and B. nigra were used for alignment and analysis using MCscan X tool, and the alignment results were mapped and visualized [25].

2.4. Analysis of Gene Structure, Conserved Motifs and Cis-Acting Elements in Promoter Region

The online MEME tool (http://meme-suite.org/; accessed on 20 July 2021) was used to predict the conservative motif of BjuCKX gene family members, and the number of motif searches was set to 15 [26]. TBtools was used to draw the gene structure, conservative motif and conserved binding domain map of BjuCKX gene family [24]. The 2000 bp sequence upstream of the BjuCKX genes was defined as the promoter and further submit to the online website PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; accessed on 22 July 2021) for cis-acting element prediction [27].

2.5. Expression Pattern Analysis of CKX Genes in B. juncea var. tumida

The transcriptome sequencing data of four developmental stages (stem diameter: 2, 4, 6 and 8 cm) of the stem nodulation mustard cultivar ‘Fuza No.2’ were downloaded from the NCBI website under the accession number SRP151320. FPKM values were used to reflect the expression abundance of genes based on the transcriptome data, and Heatmap program under TBtools tool was used for heatmap plotting.

2.6. Plant Materials, RNA Isolation and cDNA Synthesis, and Real-Time Quantitative PCR (RT-qPCR) Validation

Based on the transcriptome data, CKX genes of subgroup IV and V with high transcriptional abundance were selected. Through their mRNA sequences, RT-qPCR primers were designed by online IDT (https://sg.idtdna.com/pages; accessed on 20 September 2021) and handed over to Beijing Qingke Biotechnology Co., Ltd. (Beijing, China) for primer synthesis (Table S1).

In this study, tumorous stem mustard cultivar ‘Fuza No.2’ was planted and used for gene expression pattern analysis. This variety is a hybrid variety, which is an early-maturing and high-yielding variety bred by Agricultural Science in Fuling District, and has a wide range of adaptability. The seeds were grown in the modern agricultural research and development base of Sichuan Agricultural University (Chengdu, China), when the expanded stems were on 2 cm, 4 cm, 6 cm, and 8 cm in diameter, stem were collected and frozen under liquid nitrogen. Total RNA was extracted using the Total RNA kit and cDNA was synthesized using the Goldenstar™ RT6 cDNA Synthesis Mix kit for subsequent real-time fluorescent quantitative PCR. RT-qPCR was performed using TaKaRA SYBR Premix Ex TaqTM (Perfect Real Time) fluorescence quantitative kit method. TUB was used as an internal reference gene [28]. The experiments were repeated three bio-replicates and tech-replicates, and the relative expression levels of genes were calculated using $2^{-\Delta\Delta CT}$ algorithm [29]. Statistical analysis was performed using multivariate logistic regression
with the IBM SPSS statistics program (SPSS Version 27), and Origin software was used to
draw expression profile.

2.7. Hormone Extraction and Purification

The extraction and purification of endogenous levels of GA3, ZR, IAA, ABA, BR and
MeJA, were measured by an indirect ELISA technique that was a modification of that from
Wang et al. [30]. The samples (0.5 g) were homogenized in liquid nitrogen and extracted
in cold 80% (v/v) methanol containing 1 mm butylated hydroxytoluene as an antioxidant.
The extract was incubated at 4 °C for 4 h and centrifuged at 4000 × g for 15 min at the
same temperature. Supernatants were then filtered through Chromoseq C18 columns (C18
Sep-Pak Cartridge, WatersCorp., Millford, MA, USA). The resulting elution was dried
under N2 and dissolved in 2 mL phosphate-buffered saline (PBS) containing 0.1% (v/v)
Tween 20 and 0.1% (w/v) gelatin (pH 7.5) for ELISA analysis.

3. Results

3.1. Identification and Analysis of Physicochemical Properties of CKX Gene Family Members in
B. juncea

By searching and comparing the whole genome database of B. juncea and using
Pfam database to verify the typical domains, 23 CKX genes were identified, named
BjuCKX01~BjuCKX23, respectively (Table 1). Further analysis of the physicochemical
properties of proteins of CKX gene family members of B. juncea demonstrated that the
number of amino acids encoded by 23 BjuCKX genes ranged from 333 (BjuCKX17) to
1337 (BjuCKX09), and the relative molecular weight of proteins ranged from 36.58 to
148.49 kDa, showing significant differences. The theoretical isoelectric points of BjuCKX
proteins were located at 4.94~9.10, among which the theoretical isoelectric points for sub-
groups I, IV, and V (4.94~6.81) were located in the acidic range, rich in acidic amino acids.
Theoretical isoelectric points of subgroup VI were primarily located in the basic range,
and the primary amino acids accounted for a large proportion. The results of predicting
hydrophobicity of BjuCKX protein revealed that hydrophobicity of BjuCKX protein was
mostly negative, indicating that most of this family were hydrophilic proteins. Following
subcellular localization prediction, 15 BjuCKX proteins were located in chloroplast, endo-
plasmic reticulum, vacuole, and other organelles, and BjuCKX11~BjuCKX14 belonging to
subgroup IV were located in the cytoplasm. The remaining BjuCKX03, BjuCKX04, and
BjuCKX09 proteins were located in the extracellular matrix, plasma membrane, and nucleus,
respectively.

Table 1. Basic information of CKX gene family members of B. juncea.

| Gene Name | Accession Number | Amino Acids | Molecular Weight/Da | Isoelectric Point | Protein Hydrophobicity | Subgroup | Subcellular Localization |
|-----------|------------------|-------------|---------------------|------------------|------------------------|----------|-------------------------|
| BjuCKX01  | BjuB029649       | 505         | 56,097.41           | 5.75             | -0.019                 | I        | chloro/-                |
| BjuCKX02  | BjuA046407       | 450         | 49,748.58           | 5.17             | -0.055                 | I        | chloro/-                |
| BjuCKX03  | BjuA017172       | 502         | 55,439.79           | 5.63             | 0.019                  | I        | chloro/-                |
| BjuCKX04  | BjuB028610       | 504         | 55,806.39           | 6.23             | 0.024                  | I        | plas/-                  |
| BjuCKX05  | BjuB046928       | 524         | 58,189.70           | 5.78             | 0.016                  | I        | chloro/-                |
| BjuCKX06  | BjuA013849       | 524         | 57,978.45           | 5.54             | 0.030                  | I        | chloro/-                |
| BjuCKX07  | BjuO004995       | 515         | 58,506.51           | 5.62             | -0.220                 | I        | vacu/-                  |
| BjuCKX08  | BjuO002898       | 519         | 59,054.18           | 5.88             | -0.215                 | I        | vacu/-                  |
| BjuCKX09  | BjuA006752       | 1337        | 148,490.79          | 6.00             | -0.214                 | I        | chloro/-                |
| BjuCKX10  | BjuA016599       | 411         | 46,156.67           | 6.81             | -0.201                 | I        | chloro/-                |
| BjuCKX11  | BjuA006478       | 470         | 51,673.56           | 5.44             | -0.183                 | IV       | chloro/-                |
Table 1. Cont.

| Gene Name | Accession Number | Amino Acids | Molecular Weight/Da | Isoelectric Point | Protein Hydrophobicity | Subgroup | Subcellular Localization |
|-----------|------------------|-------------|---------------------|------------------|------------------------|----------|-------------------------|
| BjuCKX12  | BjuO011542       | 534         | 59,220.25           | 5.21             | −0.126                 | IV       | cyto/-                  |
| BjuCKX13  | BjuA038850       | 523         | 57,967.61           | 4.94             | −0.136                 | IV       | cyto/-                  |
| BjuCKX14  | BjuO008975       | 525         | 57,914.52           | 4.97             | −0.127                 | IV       | cyto/-                  |
| BjuCKX15  | BjuB029847       | 533         | 59,905.92           | 5.69             | −0.249                 | V        | vacu/-                  |
| BjuCKX16  | BjuA029462       | 535         | 60,082.13           | 5.56             | −0.241                 | V        | vacu/-                  |
| BjuCKX17  | BjuA029463       | 333         | 36,584.35           | 5.15             | −0.074                 | V        | E.R./-                  |
| BjuCKX18  | BjuO00929        | 507         | 57,217.29           | 6.69             | −0.257                 | VI       | vacu/-                  |
| BjuCKX19  | BjuA017827       | 535         | 60,758.18           | 7.14             | −0.260                 | VI       | vacu = golo/-           |
| BjuCKX20  | BjuB027127       | 549         | 62,108.95           | 8.80             | −0.259                 | VI       | mito/-                  |
| BjuCKX21  | BjuA017334       | 548         | 61,990.78           | 9.10             | −0.247                 | VI       | mito/-                  |
| BjuCKX22  | BjuB017009       | 526         | 59,226.41           | 7.02             | −0.237                 | VI       | chlo/-                  |
| BjuCKX23  | BjuA010583       | 545         | 61,374.01           | 8.72             | −0.191                 | VI       | mito/-                  |

Note: chlo: chloroplast; extr: extracellular matrix; Plas: plasma membrane; cyto: cytoplasm; vacu: vacuole; nucl: nucleus; E.R.: endoplasmic reticulum; golo: golgi apparatus; mito: mitochondria; -: Any other parts.

3.2. Phylogenetic Analysis of CKX Gene Family

To investigate the phylogenetic correlation between BjuCKX proteins as well as CKX proteins of other species, multiple sequences were compared with protein sequences of CKX gene family members of Arabidopsis (Arabidopsis thaliana), rice (Oryza sativa), and maize (Zea mays), and the neighbor-joining method (NJ) was used to construct a phylogenetic tree (Figure 1). Phylogenetic tree analysis revealed that 54 CKX genes were grouped into six subgroups, and BjuCKX genes were categorized into four subgroups: I, IV, V, and VI. Among them, subgroup I had a significant number of BjuCKX family members for ten. The first subgroup included only dicotyledonous plant genes and subgroups II and III included only monocotyledonous plant genes. Meanwhile, IV, V, and VI included monocotyledons and dicotyledons, indicating that amplification of these genes was earlier than the differentiation of monocotyledons and dicotyledons. Compared with CKX genes of rice and maize, CKX genes of B. juncea are phylogenetically closer related to Arabidopsis, suggesting that there were functional similarities between BjuCKX and AtCKX.

3.3. Analysis of Chromosome Localization and Gene Replication

The chromosome distribution of 23 BjuCKXs was mapped using TBtools software (Figure 2). The result demonstrated that 18 genes were unevenly distributed on 11 chromosomes, and five were located in unanchored contigs and scaffolds. Segment replication and tandem replication are common and essential in gene family expansion and differentiation, leading to genome and genetic system evolution. Identification of CKX gene replication events indicated that BjuCKX16 and BjuCKX17 located on chromosome A07 were a pair of tandem repeat genes. Meanwhile, CKX genes located on each chromosome presented cross-collinearity caused by fragment replication (Figure 2). Therefore, it has been speculated that tandem and fragment replication may be fundamental to homologous gene generation and gene diversity in BjuCKX gene family.

3.4. Collinearity Analysis

To investigate in depth the genetic correlation CKX gene family members between B. juncea and A. thaliana, collinearity analysis was performed. As revealed in Figure 3 and Table S2, 27 pairs of collinear CKX genes in the genomes of B. juncea and Arabidopsis, among which 17 BjuCKX genes were collinear, denoting that fragment duplication was common between BjuCKX and AtCKX, and most CKX genes were homologous in the two species. Meanwhile, except for AtCKX6, AtCKX genes located on different chromosomes of Arabidopsis had a collinear relationship with more than two BjuCKX genes, respectively. For instance, AtCKX4 and AtCKX2 on chromosomes 4 and 2 of Arabidopsis had collinearity with 7 BjuCKX genes, respectively. These findings indicated that BjuCKX gene family
expanded and evolved mainly through fragment duplication. The deletion of AtCKX6 homologous gene in B. juncea showed that some genes were lost in B. juncea.

Figure 1. Phylogenetic tree of CKX gene families in B. juncea, Arabidopsis, Oryza sativa, and Zea mays. The tree was generated based on protein sequences when aligned with Clustal W, following the neighbor-joining method. CKX proteins were divided into six subgroups (I, II, III, IV, V, VI), and each color group represented a subgroup.

B. juncea (AABB, 2n = 36) is formed by hybridization and doubling of B. rapa (AA, 2n = 20,) and B. nigra (BB, 2n = 16). To clarify the homologous relationship of the three species, collinearity analysis was conducted on B. juncea, B. rapa, and B. nigra (Figure 4 and Table S2). A total of 37 orthologous CKX gene pairs were identified in B. rapa and B. juncea. A total of 35 pairs were detected in B. juncea and B. nigra, and 17 BjuCKX genes were involved. The results manifested that BjuCKX genes were derived from large fragment duplication of B. rapa and B. nigra. Meanwhile, the orthologous gene pairs were evenly distributed on each chromosome of B. juncea excepting for chromosome A03.

3.5. Analysis of Gene Structure and Conserved Motifs

Analysis of CKX gene structure in B. juncea demonstrated that BjuCKX09 contained 9 introns and 10 exons, and the range of the number of exons of the remaining BjuCKX genes varied slightly, ranging from 3 to 5 (Figure 5). Among them, 16 BjuCKX genes contained 5 exons, 5 BjuCKX genes had 4 exons, and 1 BjuCKX gene (BjuCKX17) only contained 3 exons and the length of exons was highly similar.

In particular, 70% of genes in subgroup I (BjuCKX1-BjuCKX10) had the same number of highly similar exons, and the only difference was in the distribution of exons. Further analysis of the conserved structural domains of genes demonstrated that highly similar exons were FAD-binding-4 and cytokine-binding conserved binding domains of CKX genes, which fully demonstrated the conserved evolution of BjuCKX genes.
Figure 2. Collinearity analysis of CKX gene family in *B. juncea*. Eighteen chromosomes, four contigs, and one scaffold are arranged into a single-colored circle. The colored line connecting the chromosomes represents the different collinear region of *BjuCKX* genes. Blue, pink, red, cyan line represents the I, IV, V, VI subgroups, respectively.

Figure 3. Collinearity analysis of CKX gene family between *B. juncea* and *A. thaliana*. The colored round rectangles represent the chromosomes of two plants. The gray curves connect protein-coding genes in the synteny blocks, and the orange curves represent gene pairs that are collinear with UBC genes in *B. juncea*/*A. thaliana*.

The software MEME was used to search the motifs conserving on *BjuCKX* proteins, and the results revealed that the motifs distributed on each *BjuCKX* were highly similar in number and order. It was found that motif 2–5, 7, 10, and 15 belonged to cytokine-binding domain, and motifs 1, 6, 8, 9, and 11 were the components of FAD-binding-4 conserved binding domain.
3.6. Analysis of Promoter Cis-Acting Elements

Cis-acting elements as binding sites for transcription factors can regulate plant growth and development and stress resistance mechanism by driving downstream expression of genes. To study the regulatory of BjuCKX genes, the Plant-CARE database was used to analyze the cis-acting promoter elements located in the upstream 2000 bp sequence of genes (Figure 6 and Table S3). The results revealed that BjuCKX genes had multiple cis-acting elements involved in plant growth and development, hormone response, biological and abiotic stress. The types and number of light-response items were the highest, up to 22 element types in 221 times, mainly Box 4, GT1-Motif, and G-box. Six hormone response elements were found, including an auxin response element (AuxRR-core and TGA element), gibberellin response element (GARE-motif, P-box, and TATC-box), abscisic acid response element (ABRE), methyl jasmonate response element (CGTCA-motif and TGACG-motif), ethylene response element (ERE), and salicylic acid response element (TCA-element). Abscisic acid response element (ABRE) was the most abundant one, with a frequency of 71 times, especially in subgroup V, with the frequency of 13, 13, and 14 times in BjuCKX15–BjuCKX17, respectively. The response element has been speculated to play a significant role in promoting abscisic acid metabolism.
Studies have indicated that abiotic stress such as drought and low temperature can affect the expression level of CKX genes. This study identified 63 anaerobic response elements (ARE), 18 drought-induced elements, and 15 low-temperature response elements. Meanwhile, stress response element (STRE), defense and stress response element (TC-rich repeats), pathogen response element (W box), and trauma response element (WUN-Motif) were also found in BjuCKX genes. These stress-related cis-acting elements may significantly contribute to the resistance mechanism of B. juncea.

3.7. Transcriptional Expression Analysis of CKX Gene during Tumorous Stem Development

To investigate the potential functions of different members of BjuCKX gene family in mustard stem enlargement, the expression pattern of CKX gene family was analyzed based on transcriptomic data at different stages of mustard tumor stem enlargement (Figure 7). The results revealed that the related expression levels of the remaining 19 genes were detected except for BjuCKX05, BjuCKX08, BjuCKX10, and BjuCKX17. In combination with family clustering, the expression patterns of BjuCKX genes in different subgroups varied. BjuCKX01 in subgroup I was insignificantly expressed in the first two stages of stem development, and the expression level in the last two stages was significantly increased. This gene may be involved in the late development of mustard stem; nevertheless, other genes were insignificantly expressed. The expression levels of subgroup IV genes (BjuCKX11–BjuCKX14) was generally higher, indicating that members of subgroup IV might play an essential role in stem development. Meanwhile, compared with the expression levels of the first two stages of stem development, the expression level of BjuCKX12 was significantly increased in the last two stages. On the contrary, the expression level of
BjuCKX13 suggested that these two genes played different roles in stem development. In the VI subgroup, except for BjuCKX19, the expression level was highest in the second stage. Meanwhile, the expression of BjuCKX18 and BjuCKX22 was the highest in the first two stages and significantly decreased in the last two stages. These results suggested that the early development stage of mustard stem may be the main expression stage of these genes. In brief, it can be concluded that CKX gene family regulates the normal development of stem product organs through specific roles played by different members at varying stages of stem development in mustard.

Figure 7. Transcriptional expression analysis of CKX gene family in B. juncea var. tumida. The color scale bar on the right side of heatmap corresponds to the value converted by log2FPKM; the more red, the more the transcripts were abundant.

3.8. RT-qPCR Validation of CKX Gene in B. juncea var. tumida during Stem Formation

To further verify the expression pattern of CKX genes in tumorous stem mustard, six BjuCKXs with high transcriptional abundance in subgroups IV and V were selected from the expression profile for RT-qPCR analysis (Figure 8). The results indicated that the changing trend of gene expression was basically consistent with the result of transcriptional expression analysis. The four genes in subgroup IV exhibited different expression patterns. The expression levels of BjuCKX11 and BjuCKX13 were significantly up-regulated in S2 period and were the highest in the four periods, while the highest expression levels of BjuCKX12 and BjuCKX14 appeared in S4 period. Expression of the two genes in V subgroup demonstrated the same trend, increasing first, then decreasing, and then increasing in the four periods of S1-S4, reaching the peak in S2. Further studies revealed that BjuCKX15 and BjuCKX16 of subgroup V were a pair of paralogous genes produced by fragment duplication, illustrating similarities in the functional stages of the two genes.
3.9. Dynamic Changes in Hormone Levels during Stem Development

To examine the changes in endogenous hormones during stem development, the contents of GA3, ZR, IAA, ABA, BR, and MeJA were measured. As showed in Table 2, the hormone levels showed dynamic changes in accordance with the developmental stages. GA3, ZR, IAA, BR, and MeJA decrease over time, whereas ABA reach their highest value during the S3 period and then decrease rapidly. Correlation analysis showed that GA3, ZR, IAA, ABA, BR, and MeJA changes were positively correlated, implying that these hormones may crucial for stem development and that they cooperate to regulate stem development (Figure 9). The expression of most CKX genes was significantly positively correlated with the changes of GA3, ZR, IAA and BR. Overall, the trend of gene expression was the same as that of the accumulation of GA3, ZR, IAA, ABA, BR, and MeJA, indicating that these genes could promote the growth of mustard. In contrast, BjuCKX13 was negatively correlated with GA3, ZR, IAA and BR. Overall, the trend of gene expression was the same as that of the accumulation of GA3, ZR, IAA, ABA, BR, and MeJA, suggesting that CKX gene could promote plant growth by regulating endogenous hormone levels in the four stages of stem development in mustard.

Table 2. Hormone levels during the four periods of stem development in B. juncea var. tumida.

| Period | GA3 (ng/g FW) | ZR (ng/g FW) | IAA (ng/g FW) | ABA (ng/g FW) | BR (ng/g FW) | MeJA (ng/g FW) |
|--------|---------------|--------------|---------------|---------------|--------------|---------------|
| S1     | 6.601 ± 0.816a | 12.191 ± 1.197a | 62.815 ± 5.003a | 55.976 ± 3.521b | 8.654 ± 0.656a | 16.922 ± 1.084a |
| S2     | 4.718 ± 0.264b | 4.870 ± 0.728b | 58.301 ± 7.164a | 53.812 ± 2.329b | 7.364 ± 0.396b | 12.235 ± 0.901b |
| S3     | 4.471 ± 0.229b | 2.915 ± 0.329c | 47.196 ± 5.811b | 84.153 ± 7.329a | 6.904 ± 0.310b | 13.975 ± 1.131b |
| S4     | 4.053 ± 0.309b | 1.817 ± 0.219c | 38.476 ± 2.237b | 25.348 ± 2.466b | 5.272 ± 0.464c | 13.871 ± 1.026b |

Note: Different lowercase letters between different periods of the same hormone indicated a significant difference at the 0.05 level.
4. Discussion

Cytokinins are positive regulators of stem growth and can regulate stem development [31]. Downregulation of endogenous cytokinin levels in plants mainly depends on a class of essential enzymes, cytokinin oxidase/dehydrogenase. The enzyme is mainly encoded by CKX genes. Through identification and functional analysis of CKX genes in various plants, CKX genes played a significant role in nutrition, embryo growth, seed development, stem and root meristem formation, as well as flower organ development [32].

Based on whole-genome data of B. juncea, 23 CKX genes were screened by identifying typical domains of cytokinin oxidase/dehydrogenase, which was distinctively more than that of Arabidopsis (7 members) and B. rapa (12 members). It has been shown that the genome of B. rapa has undergone triplication since it diverged from A. thaliana [33]. However, the CKX gene numbers in B. rapa are considerably fewer than the simple triplication of CKX genes in Arabidopsis genome, suggesting that massive gene losses and chromosome rearrangements have occurred in diploid Brassica species during the triploidization [34]. In our study, collinearity analysis disclosed that AtCKX6 has no corresponding homologous gene, which be caused by asymmetry or loss in doubling replication events during Brassica evolution. As an important member of 'U-triangle', B. junce is derived from the hybridization of B. rapa and B. nigra [20]. The ratio between the number of CKX genes in B. juncea and B. rapa is about 2:1, which roughly conforms to 'U-triangle' theory [35,36]. Collinearity analysis of B. rapa, B. nigra, and B. juncea revealed that BjuCKX genes of B. juncea A subgenome had direct homology with B. rapa. In conclusion, the expansion of CKX gene family in B. juncea is involved in genome-wide doubling and tandem duplication events.

Based on the results and the classification of CKX gene family in other plants, BjuCKX family members were divided into four subgroups I, IV, V, and VI. However, the phylogenetic tree showed that CKXs from monocotyledons and dicotyledons could be divided into six groups. Among them, group I specificity exists in dicotyledonous plants (B. juncea and Arabidopsis), while groups II and III exist in monocotyledonous plants (O. sativa and Z. mays), suggesting that gene members in subgroups I, II and III were emerged before the divergence of monocot and dicot plants, whereas subgroups IV, V, and VI appeared relatively later. Similarly, a study by Schmülling et al., also believed that several CKX genes occurred before the separation of monocot and dicot plants [12].

Although there are differences in the number of genes in different subgroups, the structure and conservative sequence of all BjuCKX genes are highly similar, especially the members in the same subfamily, whose motif number and order are almost the same, revealing that the CKX family is highly conserved in evolution. The subcellular localization

Figure 9. (A) Correlation analysis between BjuCKX genes and six hormones. (B) Principal Component Analysis (PCA) plot of mustard in four developmental periods. Note: * means significant differences at 0.05 level; ** mean significant differences at 0.01 level.
prediction revealed that subcellular localization sites of different BjuCKX proteins varied. BjuCKX11–BjuCKX14 belonging to subgroup IV were mainly present in the cytoplasm, while the rest were located in organelles, plasma membrane, and nuclei, such as chloroplast, endoplasmic reticulum, and vacuole. In B. napus, BnCKX proteins were also present in different cells, including chloroplast, cytoplasm, mitochondria, vacuole, and nucleus [15]. Similar results were found in A. thaliana, multiple AtCKX proteins had different localization at the subcellular level, among which four AtCKX proteins were located in the exoplasm, two in vacuoles, and one in the cytoplasm [37]. However, in monocot maize, except for ZmCKX1 localized to the exoplasma and ZmCKX10 to the cytoplasm, the remaining CKX proteins were mainly localized in the vacuol [38].

Prediction of cis-acting elements revealed the presence of multiple response factors that regulate plant growth and development and biotic and abiotic stress responses, such as light, meristem expression, circadian rhythm, low temperature, and drought. The analysis of BjuCKX genes promoter sequences demonstrated that CKX gene family members may differ in their functions due to presence of various cis-elements in their promoter regions. For example, BjuCKXs belong to subgroup V are rich in cis-elements related to hormone response, BjuCKXs belong to subgroup IV is rich in cis-elements related to plant growth and development. In addition, various hormone response elements have been widely detected in BjuCKX gene family, demonstrating that BjuCKX genes can also respond to hormones to varying degrees. ZmCKX gene can respond to hormones such as auxin, abscisic acid, and gibberellin [39]. In Fragaria vesca, there is at least one hormone response element in each FeCKX, indicating the FeCKX genes is involved in the hormone response [40]. Stress response factors such as drought and low temperature may be linked to drought and cold resistance of B. juncea. Studies have demonstrated that plants with CKX overexpression exhibit stronger drought resistance and salt tolerance [41]. The expression pattern of BjuCKX genes was analyzed based on transcriptome analysis of tumorous stem mustard. BjuCKX genes of different evolutionary branches were found to have differences in stem development stage. Consequently, it can be speculated that genes may have differentiated into various functions during evolution. Plant hormones are crucial for physiological processes such as growth, development, and adaptation to environmental changes [42]. Endogenous hormones including GA3, ZR, IAA, ABA, BR, and MeJA showed dynamic changes during the process of stem swelling, implying that these hormones may crucial for stem development. Of some concern, there were high correlation between expression levels of BjuCKX genes and hormone levels. These results provide a basis for further elucidating the functions of CKX genes at plant development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8080705/s1, Table S1: RT-qPCR primer sequence of BjuCKX genes. Table S2: Sequence identify of homologous CKX genes in Arabidopsis thaliana, B. rapa, B. juncea, and B. nigra. Table S3: Analysis of cis-regulatory elements in the promoter regions of BjuCKX genes.

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