Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2024.051207

ARTICLE

Genome-Wide Identification of Tomato (*Solanum lycopersicum* L.) *CKX* Gene Family and Expression Analysis in the Callus Tissue under Zeatin Treatment

Zhengfeng Lai, Dongmei Lian, Shaoping Zhang, Yudong Ju, Bizhen Lin, Yunfa Yao, Songhai Wu, Jianji Hong and Zhou Li*

Subtropical Agriculture Research Institute, Fujian Academy of Agricultural Sciences, Zhangzhou, 363005, China

*Corresponding Author: Zhou Li. Email: chqhlj@163.com

Received: 29 February 2024 Accepted: 18 April 2024 Published: 27 June 2024

ABSTRACT

The cytokinin oxidase/dehydrogenase (CKX) enzyme is essential for controlling the fluctuating levels of endogenous cytokinin (CK) and has a significant impact on different aspects of plant growth and development. Nonetheless, there is limited knowledge about *CKX* genes in tomato (*Solanum lycopersicum* L.). Here we performed genome-wide identification and analysis of nine *SlCKX* family members in tomatoes using bioinformatics tools. The results revealed that nine *SlCKX* genes were unevenly distributed on five chromosomes (Chr.1, Chr.4, Chr.8, Chr.10, and Chr.12). The amino acid length, isoelectric points, and molecular weight of the nine SlCKX proteins ranged from 453 to 553, 5.77 to 8.59, and 51.661 to 62.494 kD, respectively. Subcellular localization analysis indicated that SlCKX2 proteins were located in both the vacuole and cytoplasmic matrix; SlCKX3 and SlCKX5 proteins were located in the vacuole; and SlCKX1, 4, 6, 7, 8, and 9 proteins were located in the cytoplasmic matrix. Furthermore, we observed differences in the gene structures and phylogenetic relationships of SlCKX proteins among different members. *SlCKX1-9* were positioned on two out of the three branches of the *CKX* phylogenetic tree in the multispecies phylogenetic tree construction, revealing their strong conservation within phylogenetic subgroups. Unique patterns of expression of *CKX* genes were noticed in callus cultures exposed to varying concentrations of exogenous ZT, suggesting their roles in specific developmental and physiological functions in the regeneration system. These results may facilitate subsequent functional analysis of *SlCKX* genes and provide valuable insights for establishing an efficient regeneration system for tomatoes.

KEYWORDS

Tomato; *SlCKX* gene family; phylogenetic relationships; trans-zeatin; expression pattern

1 Introduction

Cytokinins (CKs), a type of phytohormone, play a pivotal role in the regulation and modulation of plant growth and development [1]. They induce cytoplasmic division and have diverse applications in plant tissue culture because of their ability to stimulate plant regeneration [2]. The biosynthesis and degradation of CKs control their internal levels in plant cells [3]. Trans-zeatin (ZT), a plant growth factor extracted from maize whiskers, exists in two forms: trans and cis. ZT regulates growth and exhibits anti-aging effects. Two biosynthetic pathways for CKs in plants are the transfer RNA (tRNA) pathway and the AMP (ATP/ADP)

pathway. In the tRNA pathway, an isopentenyl adenosine residue can be modified by tRNA isopentenyl transferase (tRNA IPT, EC 2.5.1.8), leading to the subsequent conversion of cis-ZT to active trans-ZT catalyzed by cis-trans isomerase [4–6]. The degradation of CKs is primarily catalyzed by CK oxidase/dehydrogenase (CKX), which is the only known enzyme associated with the irreversible degradation of CKs and is present in all plant tissues [7]. The CKX enzyme is vital for controlling processes that depend on CK [8,9]. The *CKX* gene families have been discovered in millet [10], maize [11], soybean [12], oilseed rape [13], and wheat [14], with the reported numbers of gene family members being 11, 13, 17, 23 and 11, respectively.

Although CKX isoforms catalyze similar reactions, they exhibit distinct patterns of subcellular localization and tissue specificity in different plant species. In *Arabidopsis*, *AtCKX1* and *AtCKX3* are localized in the vacuole and *AtCKX2* is localized in the endoplasmic reticulum [15]. Likewise, in maize, *ZmCKX1* is found in the apoplast, while *ZmCKX10* is situated in the cytosol [16]. Expression analyses in *Arabidopsis* revealed that *AtCKX1* and *AtCKX2* are predominantly expressed in the shoot apex; *AtCKX4* in stomatal precursor cells, trichomes, and root cap; and *AtCKX5* in the root meristem and stamen. In addition, *AtCKX6* expression was associated with the gynoecium at various development [4,17]. Likewise, in maize, the genes *ZmCKX2*, *ZmCKX3*, *ZmCKX4*, and *ZmCKX5* exhibited high levels of expression in mature tassels, aged leaves, young tassels, and developing ears, respectively [18].

The impact of external phytohormones on the expression levels of *CKX* in various plant tissues remains poorly understood. For instance, 6-benzylaminopurine (6-BA) could counteract poor growth and decrease chlorophyll content in wheat under low temperatures by modulating endogenous hormone levels [19], In addition, 6-BA could reduce CKX activity, improve antioxidant enzyme activity, promote grain filling, and increase wheat yield [20]. Treating trifoliate orange plants with exogenous 6-BA resulted in increased expression of *PsCKX1*, *PsCKX2*, and *PsCKX5* in both the leaves and roots. Furthermore, this treatment slightly increased the expression of *PsCKX7* in the leaves but not in the roots [21]. Under abscisic acid (ABA) stress, the expression of *CKX* was increased in *Medicago sativa* [22], and the heightened expression of *MdCKX5.2* caused an increased sensitivity to external ZT which affected the primary root elongation [23]. These findings suggest that different CKXs typically exhibit varying subcellular localization, tissue specificity, and spatial and temporal expression patterns, and the physiological functions of most species remain unexplored.

Tomato (*Solanum lycopersicum* L.) is a vital horticultural crop in China [24], It has various varieties with broad adaptability to diverse environments, and its production and consumption continue to rise with the discovery of its high nutritional values and medicinal applications [25]. However, the tomato industry faces challenges such as a long growth cycle and susceptibility to pests and diseases during the seedling stage [26]. Cellular engineering offers a promising solution to enhance tomato production efficiency by reducing the effects of season, pest, and disease, and reducing cultivation time. High-quality callus tissue is crucial for cell culture in plant asexual propagation [27]. Thus, investigating tomato CKX family genes and their expression during callus development is crucial for developing efficient tomato *in vitro* regeneration systems.

This study aimed to identify nine *CKX* genes in tomato plants, examine their structural features, and evaluate the expression of *CKX* in callus tissues under ZT treatments through qRT-PCR. The findings of this research establish a foundation for further investigations into the biological roles of *CKX* genes in tomatoes and offer insights for improving callus induction techniques.

2 Materials and Methods

2.1 Identification of CKX Family in Tomato

To identify the *CKX* gene family members in tomatoes, we conducted a comparison of gene sequences with the tomato genome database found on the Sol Genomics Network website (https://solgenomics.net/), which is derived from the Ensembl Plants database (https://plants.ensembl.org/index.html). This analysis allowed us to establish the total count of tomato *CKX* gene family members, which were then assigned the names *SlCKX1-9*.

2.2 Analysis of Physicochemical Properties of SlCKXs Gene

The Expasy website (https://web.expasy.org/protparam/) was utilized to analyze the physicochemical characteristics of members of the *SlCKXs* family. This analysis encompassed a range of properties such as molecular weights, theoretical isoelectric points, protein instability indexes, and fat indexes (which represent the relative values of aliphatic amino acid content) [28]. Subcellular localizations were predicted using Cell-Ploc 2.0 (www.csbio.sjtu.edu.cn).

2.3 Analysis of SlCKXs Protein Structure

We used the Expasy website (https://swissmodel.expasy.org/interactive) to analyze the secondary structure of the SlCKX proteins and predict their tertiary structures. Additionally, we constructed protein tertiary structure models.

2.4 Analysis of Chromosome Localization, Gene Structure, Conserved Motifs, and Conserved Domains

The MapChart tools (v 2.3.2) were used to visually show the localization of *SlCKX* genes on the chromosome [29]. By utilizing the MEME online software (https://meme-suite.org/meme/tools/meme), conserved motifs (motif) in the SlCKX protein sequences were identified and then visualized with TBtools.

2.5 Comparison of SlCKX Gene Family Members with CKX Gene Members of Other Species and Collinearity Analysis

We used MEGA 7.0 (www.megasoftware.net) to determine the evolutionary relationships of the SlCKX tree [30], and the results were displayed using evolutionary trees from the online website iTOL (https://itol.embl.de/itol.cgi) [31].

We utilized the MapChart tool (v. 2.3.2) [29] for mapping the *SlCKX* genes on chromosomes. Furthermore, we employed MCScanX software [32] to study the evolutionary relationship of homologous *CKX* genes among tomato and Arabidopsis, eggplant, and cabbage. Subsequently, KaKs_Calculator 2.0 was applied to determine the nonsynonymous (Ka) and synonymous (Ks) substitutions for each iteration of the *SlCKX* gene [33]. The divergence time was calculated using the formula T = Ks/2R, with R representing 1.5×10^8 synonymous substitutions per year.

2.6 Analysis of Cis-Acting Elements

To investigate the cis-acting elements found in the promoter regions of *SlCKX* family members, 2000-base pair DNA sequences upstream of the start codon of *SlCKX* family members were obtained through TBtools software. Subsequently, PlantCARE, an online tool available at http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ [34], was employed for analyzing cis-elements.

2.7 Sterile Seedling Culture

To establish a sterile seedling culture, seeds of 'Micro Tom' were immersed in sterilized water at 55°C for 30 min, and the water temperature was lowered to 30°C for germination by immersion. Under aseptic conditions, the seeds were soaked in 75% alcohol for 30 s, rinsed three times with sterile water, dried on filter paper, soaked in 4%–5% NaClO for 10 min, rinsed four to five times with sterile water, and blotted

on filter paper to dry the seed surface. The sterile seeds were placed on MS solid medium (0.7% agar) amended with 3% sucrose and allowed to germinate and grow for 10 days in a constant incubator (25°C, 4,000 lux light intensity, and 12/12 h day/night rhythm).

2.8 Exogenous ZT Treatment

Young leaves of 10-day-old tomato seedlings were cut into 0.5 cm × 0.5 cm squares on an ultra-clean bench, then inoculated onto the MS co-culture medium (composition: MS nutrition powder 4.42 $g \cdot L^{-1}$, IAA 1.0 $mg \cdot L^{-1}$, agar 7 $g \cdot L^{-1}$, sucrose 30 $g \cdot L^{-1}$; pH 5.8) supplemented with five different concentrations of ZT (0.1, 0.5, 1.0, 1.5 and 2.0 $mg \cdot L^{-1}$). 20 explants were cultured in one vial, each replicated three times in a completely randomized design. Whole explants were kept at 25°C, 12 h photoperiod, and the light intensity was adjusted to 3,000 lux. According to the induction of the callus, the growth of the callus was counted regularly.

2.9 qRT-PCR Analysis

Callus explants with different ZT concentrations were selected to extract RNA from tomato calli by following the method of the Vazyme Plant Total RNA Extraction Kit (RC401), and the reverse transcription kit (KR118) of the Tiangen Company was then used to synthesize cDNA using RNA as a template. Genstar Biological 2 × RealStar Green Power Mixture (A311-01) was used for the quantitative procedures, and the website Sangon.com was employed to design specific primers for qRT-PCR analysis, with the gene sequence provided in Supplementary S1. A LightCycler 96 fluorescence quantitative PCR system from Roche was utilized for the PCR, with 20 μL of *SlActin* serving as the reference gene. The gene-specific primers can be found in Table 1. The PCR protocol comprised denaturation at 95°C for 15 s, annealing/extension at 60°C for 15 s, repeated for 40 cycles, followed by the generation of a melting curve. The relative gene expressions were calculated using the $2^{-\Delta\Delta Ct}$ method [35]. Each treatment was conducted with three biological replicates.

Table 1: Primers used for qRT-PCR

Target genes	Upstream primer	Downstream primer
SlActin	GTCCTCTTCCAGCCATCCAT	ACCACTGAGCACAATGTTACCG
SlCKX1	CTTCGGAGTTCACAACGGACCTTC	TGGATGTGGCACTTCCCATAAACC
SlCKX2	TTACGCGGATGTTGGAGGTGAAC	TGTCCATGAAACAGGTGCTAAGCC
SlCKX3	GTTGCTGCTAGAGGACATGGTCAC	GCTGGTGCTCGAAGCGATTCC
SlCKX4	TGGGCTTGCACCTAAATCTTGGAC	TGATCTGCGGTCCGTGCTTAAAAG
SlCKX5	TGGCATCAGTGGACAGGCATTTC	ACCAAGTCCTCCAAGAACAGCATG
SlCKX6	CGCAAGGGGTAACGGTCATTCAG	AATGCTCCACCACCAACATCGAC
SlCKX7	GGCGAGAGGCAACGGTCATTC	ACTCCATAATGCTCCACCACCAAC
SlCKX8	GCACCCTGGAATATGGCTTAGC	CTGTCCCAAACCTCCTAAAACTCC
SlCKX9	TTACGCGGATGTTGGAGGTGAAC	TGTCCACGAAACAGGTGCTAAGC

2.10 Data Analysis

Statistical analysis was performed on the collected data using a one-way analysis of variance (ANOVA), followed by Tukey's test with SPSS (IBMSPSS Statistical Version 24). Statistical significance was determined by a p-value of <0.05. The graphs were created using GraphPad Prism 8 (San Diego, CA, USA).

3 Results

3.1 Discovery of the CKX Gene Family in Tomato across the Entire Genome

To identify the *CKX* family proteins in tomatoes, we conducted a query of the tomato genome (v4.0) (https://solgenomics.net/ftp/tomato_genome/assembly/build_4.00/). This search yielded nine *SlCKX* sequences: *SlCKX1* (Solyc04g080820.2.1), *SlCKX2* (Solyc12g008900.2.1), *SlCKX3* (Solyc04g016430.3.1), *SlCKX4* (Solyc10g079870.3.1), *SlCKX5* (Solyc01g088160.4.1), *SlCKX6* (Solyc08g061920.3.1), *SlCKX7* (Solyc08g061930.4.1), *SlCKX8* (Solyc10g017990.2.1), and *SlCKX9* (Solyc12g008920.3.1). All these sequences contain the typical flavin adenine dinucleotide (FAD) binding and CK-binding domains, which are unique features of CKX family members. Table 2 presents basic information on *SlCKXs* including loci, chromosome positioning, and open reading frame (ORF) length, the nine members of the *SlCKXs* gene family exhibit various physicochemical properties, The analysis revealed that these proteins consist of amino acid sequences ranging from 453 to 553 residues, with corresponding relative molecular weights falling within the range of 51.661 to 62.494 kD, and isoelectric points varying from 5.77 to 8.59. Among them, SlCKX1, 4, 5, 6, 7, and 9 are acidic proteins, while SlCKX2, 3, and 8 are basic proteins. The stability coefficient of SlCKX1, 4, 5, 6, 7, and 8 is less than 40, indicating that they are all stable proteins. However, SlCKX2, 3, and 9 have coefficients greater than 40, suggesting that they are unstable proteins.

3.2 Analysis of the Protein Structure of CKX in Tomatoes

To explore the structural characteristics of SlCKX proteins, we used the online tool SOPMA for secondary structure prediction. As shown in Table 3, we determined that all SlCKX proteins β-sheets, extended chains, and random coils. The proportion of α-helices, β-sheets, extended chains, and random coils ranged from 32.26% to 36.98%, from 5.47% to 6.98%, from 17.54% to 20.87%, and from 38.11% to 42.94%, respectively. These structural elements were found to differ among the nine SlCKX proteins. Following the assessment of the secondary structure, we analyzed the tertiary structure (Fig. 1). Protein modeling indicated that the global model quality estimation (GMQE) scores for the SlCKX1-9 protein sequences were all close to 1, indicating a high degree of consistency within the protein family and greater reliability of the related sequences.

3.3 Chromosome Distribution, Gene Structure, Conserved Motifs, and Conserved Domains Analysis

Based on tomato genome annotation, we identified nine members that belonged to the *SlCKXs* gene family and were distributed across five tomato chromosomes (Fig. 2). Eight of these genes were evenly located on four chromosomes, with *SlCKX5* being the only gene located on chromosome 1. Furthermore, *SlCKX1* and *SlCKX3* were located on chromosome 4, *SlCKX6* and *SlCKX7* on chromosome 8, and *SlCKX4* and *SlCKX8* on chromosome 10. Additionally, *SlCKX2* and *SlCKX9* were found on chromosome 12. Notably, the proximity of *SlCKX6* and *SlCKX7* on chromosome 8, as well as *SlCKX2* and *SlCKX9* on chromosome 12, suggests that these genes may be orthologs resulting from segmental duplication events.

We identified 15 conserved motifs of SlCKX proteins by using MEME software. As depicted in Fig. 3, we observed some similarity in the number and arrangement of conserved motifs among the nine protein sequences of SlCKX. Each protein within the same subfamily exhibited a unique motif pattern, where SlCKX8 lacked motif9, SlCKX9 lacked motif4 and motif7, and SlCKX4 lacked motif2. All nine members of the SlCKX family possessed six highly conserved motifs, including motif1, motif3, motif5, motif6, motif8, and motif10. Furthermore, motif10 was located closer to the 5′-end, whereas motif5 and motif7 were located closer to the 3′-end, suggesting the high conservation of these six motifs during evolution.

Table 2: Physicochemical properties of tomato *CKX* family members

Locus name	Protein name	Number of amino acids	Molecular weight (kD)	Isoelectric point (pI)	Instability index	Aliphatic index	Grand average of hydropathicity	Subcellular localization
Solyc04g080820.2.1	SlCKX1	524	58.964	6.04	37.78	90.59	−0.221	Cytoplasmic matrix.
Solyc12g008900.2.1	SlCKX2	527	60.123	7.99	43.54	91.33	−0.196	Cytoplasmic matrix; Vacuole.
Solyc04g016430.3.1	SlCKX3	543	61.362	8.59	40.11	89.74	−0.157	Vacuole.
Solyc10g079870.3.1	SlCKX4	496	56.742	6.81	34.60	93.95	−0.186	Cytoplasmic matrix.
Solyc01g088160.4.1	SlCKX5	553	62.494	6.97	35.83	97.23	−0.091	Vacuole.
Solyc08g061920.3.1	SlCKX6	530	58.968	6.40	29.97	95.21	−0.060	Cytoplasmic matrix.
Solyc08g061930.4.1	SlCKX7	516	58.188	6.10	35.27	90.79	−0.173	Cytoplasmic matrix.
Solyc10g017990.2.1	SlCKX8	471	53.569	8.52	39.97	93.72	−0.195	Cytoplasmic matrix.
Solyc12g008920.3.1	SlCKX9	453	51.661	5.77	42.72	91.17	−0.279	Cytoplasmic matrix.

Table 3: Secondary structure of SlCKXs protein

Name	Length (aa)	α-helix (%)	*β*-turn (%)	Random coil (%)	Extended strand (%)
SlCKX1	524	32.82	6.30	42.94	17.94
SlCKX2	527	33.21	6.45	39.47	20.87
SlCKX3	543	35.73	6.26	39.78	18.23
SlCKX4	496	32.26	6.85	42.94	17.94
SlCKX5	553	35.62	5.61	41.23	17.54
SlCKX6	530	36.98	5.47	38.11	19.43
SlCKX7	516	32.75	6.98	41.67	18.60
SlCKX8	471	36.31	5.52	39.28	18.90
SlCKX9	453	33.55	6.40	42.38	17.66

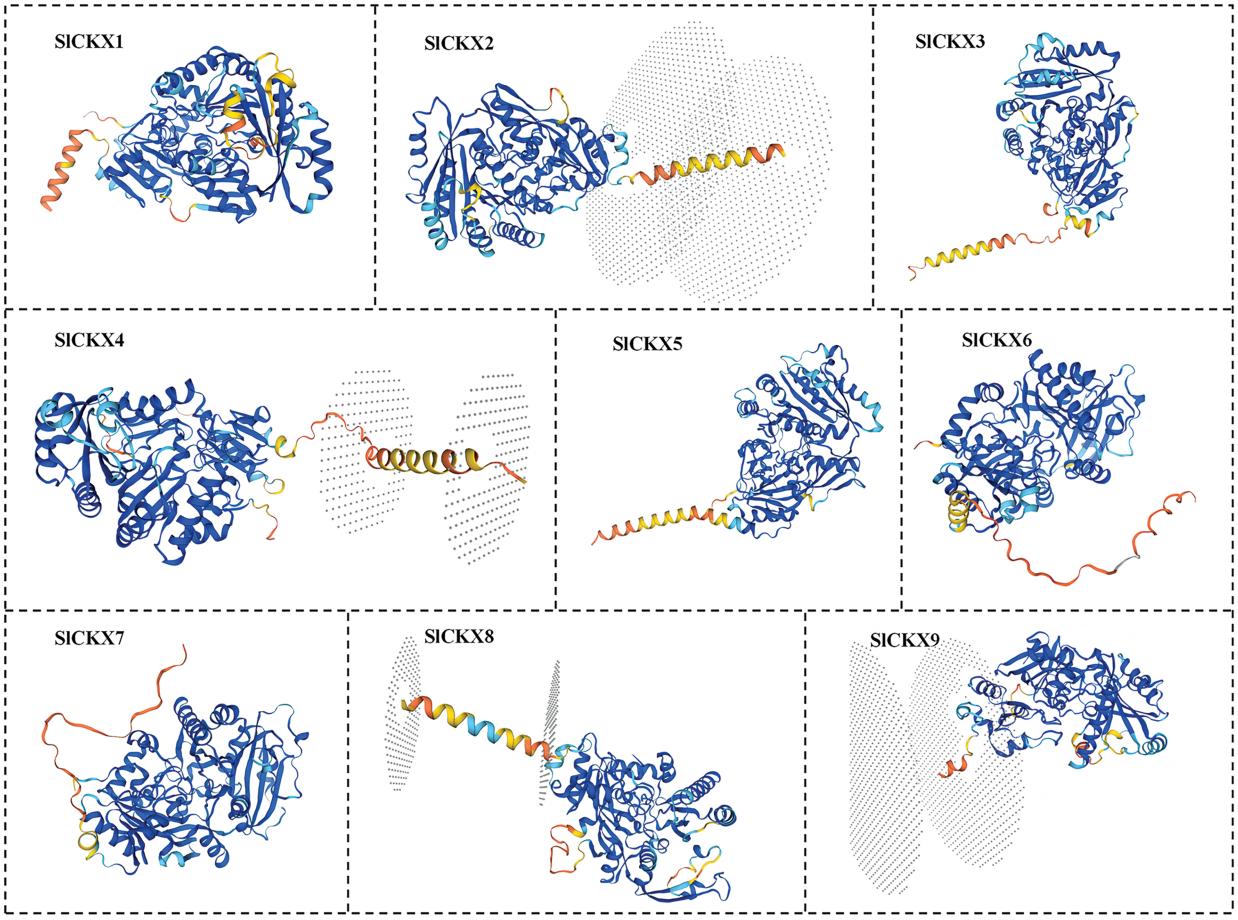

Figure 1: Tertiary structure of SlCKXs protein

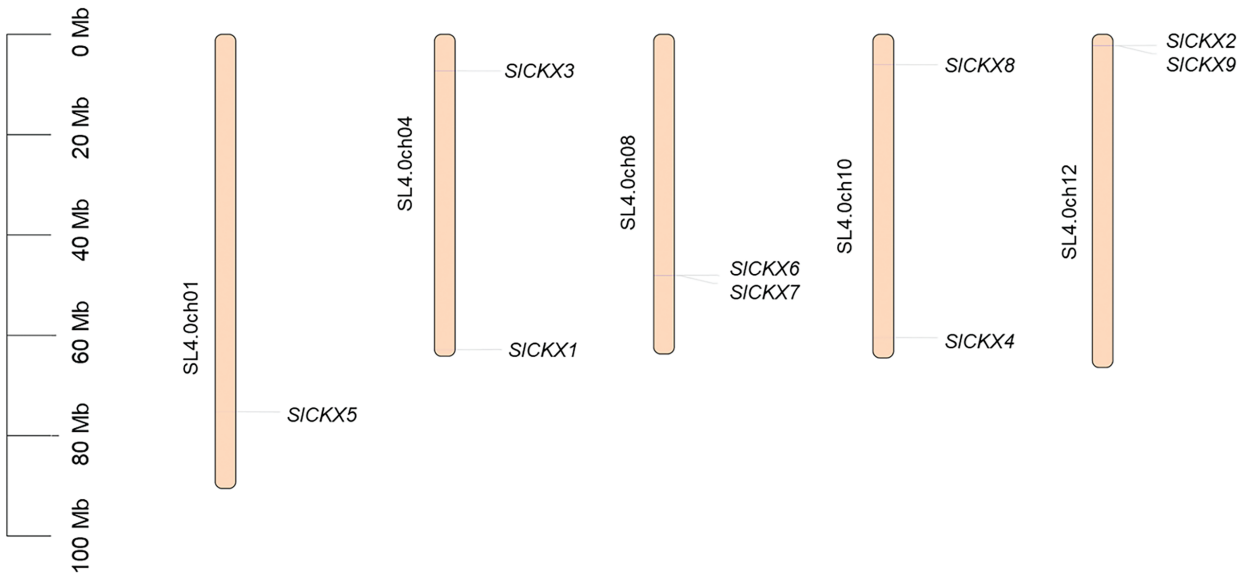

Figure 2: Localization of *CKX* in chromosomes of tomato

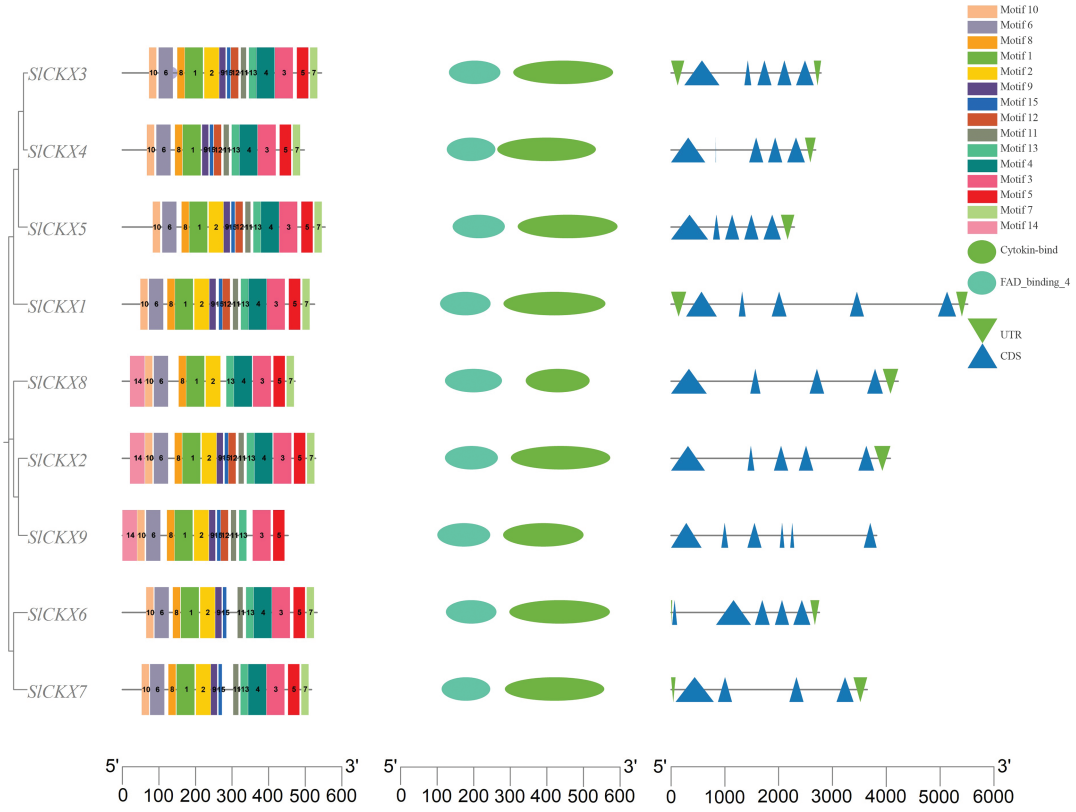

Figure 3: Conserved motif, conserved domain, and gene structure of *SlCKXs* gene family

All the nine members of the *SlCKXs* family possess conserved domains for FAD-binding and CK-binding 2, respectively. The FAD-binding domains are located at the 5′-end, whereas the CK-binding

domains are situated at the 3′-end. Members within the *SlCKXs* family exhibit variations in untranslated regions and coding sequences (CDS), with a number of these CDS ranging between 4 and 6. This diversity may arise from evolutionary changes in *CKX* genes.

3.4 Phylogeny and Synteny Analyses of the CKX Gene Family

To better understand the phylogenetic relationships of *CKX* genes in tomato, *Arabidopsis*, eggplant, and nonbulb cabbage, we constructed a phylogenetic tree. This tree was based on conserved domains of the *CKX* genes, utilizing the neighbor-joining (NJ) method and multiple sequence alignments. Analysis of the results revealed that the 34 *CKX* genes can be classified into four distinct subgroups: I, II, III, and IV (Fig. 4). Among these groups, groups I and II were the largest, each comprising 12 members. Subgroup I consisted of three tomatoes, two *Arabidopsis*, four nonbulbous cabbage, and three eggplant species, whereas subgroup II consisted of three tomatoes, three *Arabidopsis*, five nonbulbous cabbage, and one eggplant species. The phylogeny diagram indicated a high homology between tomato and eggplant likely due to their shared membership in the Solanaceae family. Overall, members in the same subfamily are likely to share similar functions, allowing for further exploration into the potential biological roles of the *SlCKX* family.

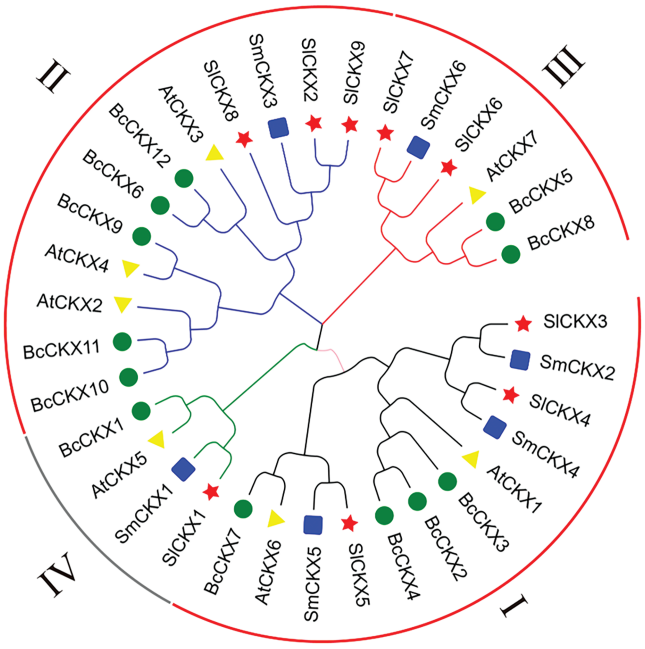

Figure 4: Rootless phylogenetic trees from the *CKX* family of *Arabidopsis thaliana* (triangle), *Solanum melongena* (square), Tomato (pentagram), and Brassica (round) with different colored lines in the outer ring indicating different subgroup

To delve deeper into the evolutionary relationships of the tomato *CKX* family, a comparative collinear analysis was conducted between the *CKX* family members of *Arabidopsis* and tomato. This analysis identified strong collinear relationships between the nine *SlCKX* family genes and other species and identified the presence of a strong collinear region between tomato and *Arabidopsis* (Fig. 5). Moreover, The collinearity analysis of *AtCKXs* and *SlCKX* revealed that three tomato chromosomes were collinear with five *Arabidopsis* chromosomes. Moreover, SL4.0ch12 corresponded to three *Arabidopsis*

chromosomes, demonstrating high homology and providing valuable insights into the genetic relationships and gene functions of these species.

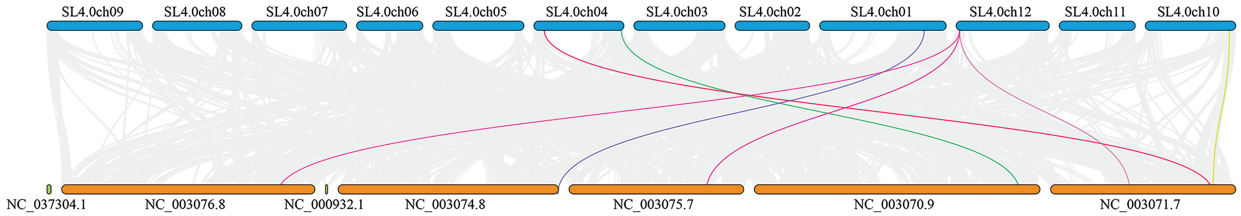

Figure 5: Between-species collinear relationship between tomato and *Arabidopsis*. Tomato and *Arabidopsis* chromosomes are marked with different colors, the tomato chromosome in blue and the *Arabidopsis* chromosome in orange. Chromosome numbers are marked above the chromosome. Different colored lines connect collinear relationships between members of the *CKX* gene family of different species

3.5 Analysis of Promoter Cis-Acting Elements of the SlCKX Gene Family

We analyzed cis-acting elements located in the promoter region 2000 bp upstream of the *SlCKX* gene. We found that the *SlCKX* gene family primarily comprises four categories of cis-acting elements (Fig. 6A): light-response elements, hormone-response elements, stress-response elements, and growth-response elements. The details are provided in the Supplementary S2. The number of hormone-response elements in the *SlCKX* gene family was five (Fig. 6B), distributed across all eight members except for *SlCKX6*. The most widely distributed element was the ABA-response element, with the highest number found in *SlCKX1* and *SlCKX9*. Auxin-response elements were found in *SlCKX1*, *2*, and *8*; methyl jasmonate-response elements were detected in *SlCKX1*, *3*, 8 and *9*; ABA-response elements in *SlCKX1*, *2*, *3*, *4*, *5*, *7*, *8*, and *9*; salicylic acid-response elements in *SlCKX2*, *5*, *7*, and *9*; and gibberellin-response elements in *SlCKX5*, *8*, and *9*, these cis-elements indicated the functional diversity of *SlCKX* genes, particularly in hormone response, suggesting the involvement of *SlCKX* in plant growth regulation.

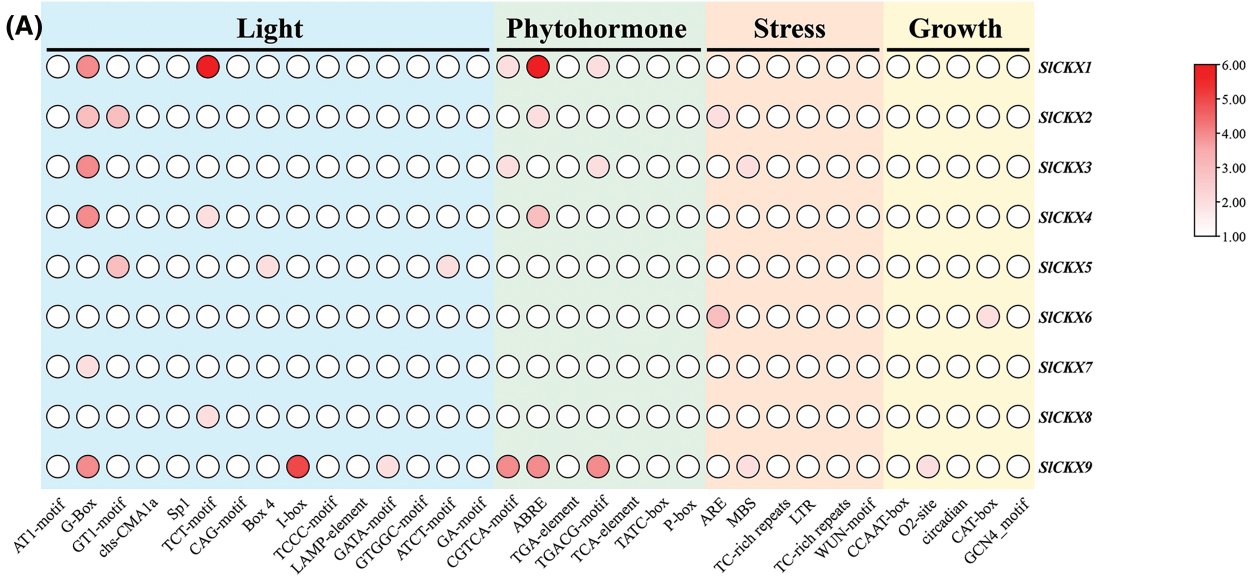

Figure 6: (Continued)

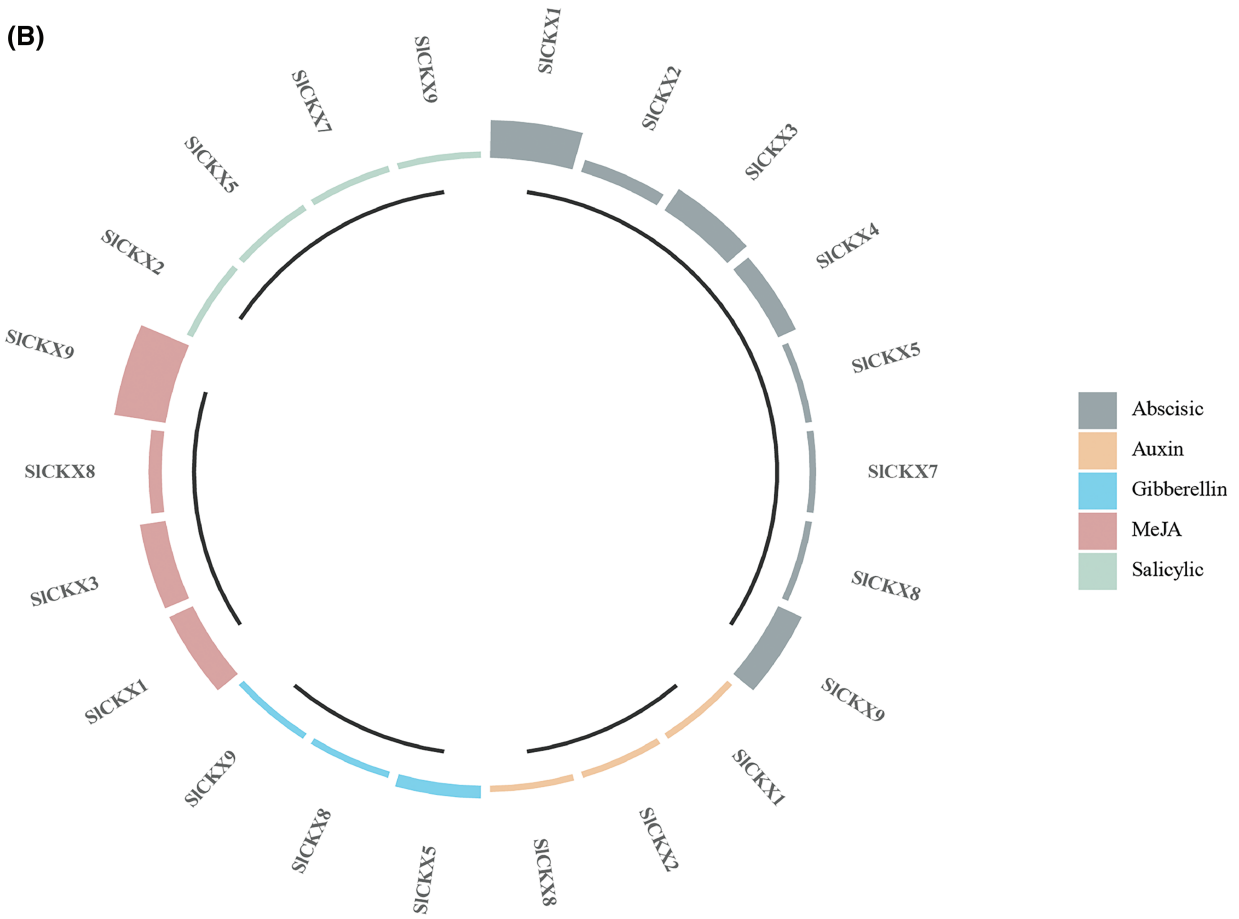

Figure 6: Distribution of cis-acting elements of the *SlCKX* gene family (Fig. 6A) and hor-mone response elements (Fig. 6B)

3.6 Validation of Expression of Tomato CKX Gene Family in Callus

To further examine the expression patterns of the *SlCKX* gene family in tomato (Fig. 7), We isolated RNA from the calli exposed to varying concentrations of trans-ZT and subsequently reverse-transcribed into cDNA for quantitative real-time PCR. The findings revealed that the transcriptional activity of *SlCKX5* and *SlCKX9* genes initially increased and then decreased, whereas the transcriptional activity of *SlCKX2*, *3*, *4*, *6*, *7* and *8* genes initially increased and then decreased at a concentration of 2.0 $mg{\cdot}L^{-1}$. The most pronounced difference was observed in the medium supplemented with 1.0 $mg{\cdot}L^{-1}$ IAA and 1.0 $mg{\cdot}L^{-1}$ ZT, suggesting that a hormonal concentration of 1.0 $mg{\cdot}L^{-1}$ is optimal for the growth of tomato calli.

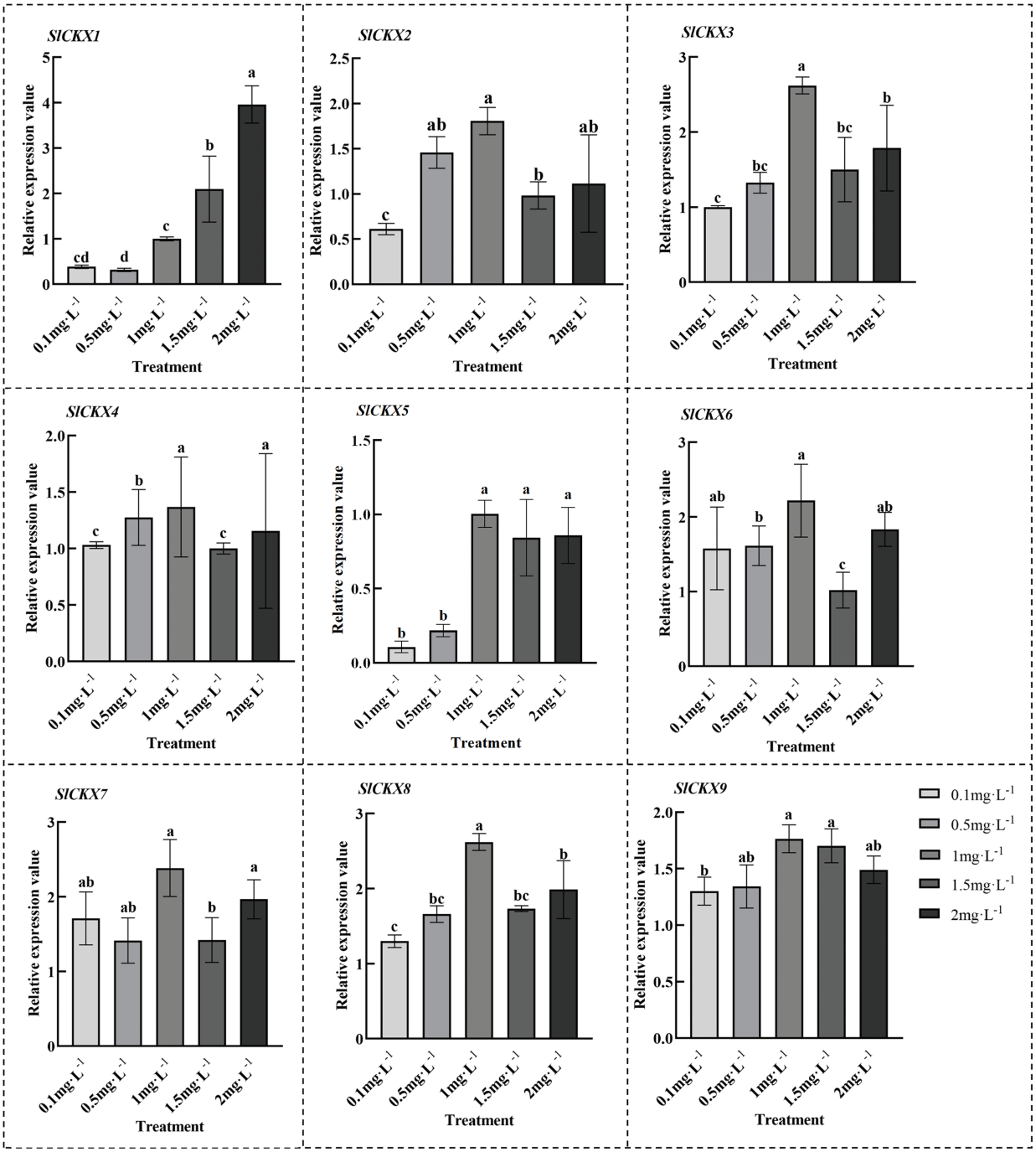

Figure 7: Levels of gene expression for the *SlCKX* family members in callus were examined under varying zeatin concentrations. The results are displayed as the average ±SD, with a sample size of n = 3. Distinct lowercase letters above the bars indicate statistically significant differences ($p < 0.05$)

4 Discussion

Endogenous hormones play a crucial role in regulating plant growth and development [36], affecting various processes such as seed germination [37], cell differentiation [38], root elongation [39], and fruit

ripening and abscission [40]. In plant tissue culture, exogenous hormone application is a standard technique to promote explant regeneration. These exogenous hormones can affect the synthesis and distribution of endogenous hormones, thereby influencing plant growth and development. CK dehydrogenase, encoded by the *CKX* gene, plays a crucial role in controlling CK homeostasis by degrading CKs. The degradation process is crucial for controlling plant growth, development, stress tolerance, and productivity [41]. They are ensuring an appropriate conducive to maintaining cell division capacity in plant cells.

The *SlCKX* gene family in tomatoes was found to be larger than in *Arabidopsis* but notably smaller than in cabbage. This finding indicates that the events of gene expansion and reduction have occurred in the *CKX* family during plant evolution. The finding is consistent with those reported by Guo et al. [42]. The lengths of the CKX proteins in tomatoes vary from 453 to 553 amino acids, corresponding to molecular weights ranging from 51.66 to 52.49 kDa. The theoretical isoelectric points of these proteins range from 5.77 to 8.59, suggesting their hydrophilic nature. Furthermore, each of these comprises a corresponding *CKX* gene in eggplant, indicating their common ancestry within the Solanaceae family. Moreover, we observed that motifs1, 3, 5, 6, 8, and 10 are conserved among all the nine SlCKX proteins. This finding indicates that these motifs are essential for the biological functions of CKX proteins in tomatoes.

All nine *CKX* genes in tomato exhibit conserved FAD-binding and cytokinin-binding 2 domains situated at the 5′ and 3′ termini, respectively. This organization aligns with findings in wheat as reported by Feng et al. [43]. The functional diversity of *SlCKX* genes is highlighted by the presence of different cis-elements, especially in hormone-response pathways, suggesting their involvement in growth regulation in plants. Further investigation of the expression of tomato genes revealed the effect of CK on callus growth and development. The expression of *SlCKX1* increased with trans-ZT concentration. However, the transcriptional activity of *SlCKX5* and *SlCKX9* genes increased initially and then decreased, whereas the transcriptional activity of *SlCKX2*, *3*, *4*, *6*, *7*, *8* genes initially increased and then decreased at a concentration of 2.0 $mg{\cdot}L^{-1}$. The most pronounced difference was observed in the medium supplemented with 1.0 $mg{\cdot}L^{-1}$ IAA and 1.0 $mg{\cdot}L^{-1}$ ZT, implying that a hormonal concentration of 1.0 $mg{\cdot}L^{-1}$ is optimal for the growth of tomato calli.

5 Conclusion

In this study, we identified nine *SlCKX* genes in the tomato genome. We constructed a phylogenetic tree of SlCKX proteins from tomato and other species and examined their evolutionary relationships. We investigated the gene structure and conserved motifs of all nine SlCKX proteins, as well as the stress response of functional cis-elements in the promoter region. Furthermore, we analyzed the expression of the *SlCKXs* gene under different hormonal concentrations with the most significant expression observed in the medium supplemented with 1.0 $mg{\cdot}L^{-1}$ IAA and 1.0 $mg{\cdot}L^{-1}$ ZT, indicating that 1.0 $mg{\cdot}L^{-1}$ is the optimal hormone concentration for tomato callus growth.

Acknowledgement: Not applicable.

Funding Statement: This work was funded by the Special Project for Science and Technology Innovation Platform of Fujian Academy of Agricultural Sciences, China (CXPT2023003), the Freely Explore Scientific and Technology Innovation Program of Fujian Academy of Agricultural Sciences (ZYTS202207), and the Program for Innovative Research Team of Fujian Academy of Agricultural Sciences, China (CXTD2021006-3).

Author Contributions: Z. L. was responsible for designing the experiments, analyzing the results, performing the experiments, and wrote the manuscript. D. L., Y. J. and S. W. performed the experiments. S. Z. and Y. Y. performed the experiments and took part in data analysis. B. L. collected the plant samples. J. H. conceptualized and designed the research. Z. L. interpreted the results and revised the paper.

Availability of Data and Materials: All the data supporting the findings of this study are included in this article.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declared that they have no conflicts of interest to report regarding the present study.

Supplementary Materials: The supplementary material is available online at https://doi.org/10.32604/phyton.2024.051207.

References

1. Mishra DC, Arora D, Budhlakoti N, Solanke AU, Mithra S, Kumar A, et al. Identification of potential cytokinin responsive key genes in rice treated with trans-zeatin through systems biology approach. Front Genet. 2021;12:780599.
2. Grzegorczyk-Karolak I, Hnatuszko-Konka K, Krzeminska M, Olszewska MA, Owczarek A. Cytokinin-based tissue cultures for stable medicinal plant production: regeneration and phytochemical profiling of *Salvia bulleyana* shoots. Biomolecules. 2021;11(10):1513. doi:10.3390/biom11101513.
3. Uniyal S, Bhandari M, Singh P, Singh RK, Tiwari SP. Cytokinin biosynthesis in cyanobacteria: insights for crop improvement. Front Genet. 2022;13:933226. doi:10.3389/fgene.2022.933226.
4. Kakimoto T. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyl transferases. Plant Cell Physiol. 2001;42:677–85. doi:10.1093/pcp/pce112.
5. Mok DWS, Mok Machteld C. Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol. 2001;52:89–118. doi:10.1146/arplant.2001.52.issue-1.
6. Hluska T, Sebela M, Lenobel R, Frebort I, Galuszka P. Purification of maize nucleotide pyrophosphatase/phosphodiesterase casts doubt on the existence of Zeatin cis-trans isomerase in plants. Front Plant Sci. 2017;8:1473. doi:10.3389/fpls.2017.01473.
7. Gouda G, Gupta MK, Donde R, Kumar J, Vadde R, Mohapatra T, et al. Computational approach towards understanding structural and functional role of cytokinin oxidase/dehydrogenase 2 (*CKX2*) in enhancing grain yield in rice plant. J Biomol Struct Dyn. 2020;38(4):1158–67. doi:10.1080/07391102.2019.1597771.
8. Gu R, Fu J, Guo S, Duan F, Wang Z, Mi G, et al. Comparative expression and phylogenetic analysis of maize cytokinin dehydrogenase/oxidase (*CKX*) gene family. J Plant Growth Regul. 2010;29(4):428–40. doi:10.1007/s00344-010-9155-y.
9. Galuszka P, Popelková H, Werner T, Frébortová J, Pospíšilová H, Mik V, et al. Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. J Plant Growth Regul. 2007;26(3):255–67. doi:10.1007/s00344-007-9008-5.
10. Wang Y, Liu H, Xin Q. Genome-wide analysis and identification of cytokinin oxidase/dehydrogenase (*CKX*) gene family in foxtail millet (*Setaria italica*). Crop J. 2014;2(4):244–54. doi:10.1016/j.cj.2014.05.001.
11. Zalabak D, Galuszka P, Mrizova K, Podlesakova K, Gu R, Frebortova J. Biochemical characterization of the maize cytokinin dehydrogenase family and cytokinin profiling in developing maize plantlets in relation to the expression of cytokinin dehydrogenase genes. Plant Physiol Biochem. 2014;74:283–93. doi:10.1016/j.plaphy.2013.11.020.
12. Le DT, Nishiyama R, Watanabe Y, Vankova R, Tanaka M, Seki M, et al. Identification and expression analysis of cytokinin metabolic genes in soybean under normal and drought conditions in relation to cytokinin levels. PLoS One. 2012;7(8):e42411. doi:10.1371/journal.pone.0042411.
13. Liu P, Zhang C, Ma JQ, Zhang LY, Yang B, Tang XY, et al. Genome-wide identification and expression profiling of cytokinin oxidase/dehydrogenase (*CKX*) genes reveal likely roles in pod development and stress responses in oilseed rape (*Brassica napus* L.). Genes. 2018;9(3):168. doi:10.3390/genes9030168.
14. Jain P, Singh A, Iquebal MA, Jaiswal S, Kumar S, Kumar D, et al. Genome-wide analysis and evolutionary perspective of the cytokinin dehydrogenase gene family in wheat (*Triticum aestivum* L.). Front Genet. 2022;13:931659. doi:10.3389/fgene.2022.931659.

15. Werner T, Motyka V, Laucou V, Smets R, van Onckelen H, Schmulling T. Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell. 2003;15(11):2532–50. doi:10.1105/tpc.014928.
16. Smehilova M, Galuszka P, Bilyeu KD, Jaworek P, Kowalska M, Sebela M, et al. Subcellular localization and biochemical comparison of cytosolic and secreted cytokinin dehydrogenase enzymes from maize. J Exp Bot. 2009;60(9):2701–12. doi:10.1093/jxb/erp126.
17. Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T. New insights into the biology of cytokinin degradation. Plant Biol. 2006;8(3):371–81. doi:10.1055/s-2006-923928.
18. Massonneau A, Houba-Herin N, Pethe C, Madzak C, Falque M, Mercy M, et al. Maize cytokinin oxidase genes: differential expression and cloning of two new cDNAs. J Exp Bot. 2004;55(408):2549–57. doi:10.1093/jxb/erh274.
19. Panda BB, Sekhar S, Dash SK, Behera L, Shaw BP. Biochemical and molecular characterisation of exogenous cytokinin application on grain filling in rice. BMC Plant Biol. 2018;18(1):89. doi:10.1186/s12870-018-1279-4.
20. Zhang W, Xia L, Peng F, Song C, Manzoor MA, Cai Y, et al. Transcriptomics and metabolomics changes triggered by exogenous 6-benzylaminopurine in relieving epicotyl dormancy of *Polygonatum cyrtonema* Hua seeds. Front Plant Sci. 2022;13:961899. doi:10.3389/fpls.2022.961899.
21. Ma YY, Zheng L, Xie R, He SL, Deng L. Genome-wide identification and analysis of *CKX* genes in *Poncirus trifoliata*. J Hortic Sci Biotech. 2016;91(6):592–602. doi:10.1080/14620316.2016.1194171.
22. Li S, An Y, Hailati S, Zhang J, Cao Y, Liu Y, et al. Overexpression of the cytokinin oxidase/dehydrogenase (CKX) from *Medicago sativa* enhanced salt stress tolerance of Arabidopsis. J Plant Biol. 2019;62(5):374–86. doi:10.1007/s12374-019-0141-z.
23. Liu Y, Wang X, Wang X, Gao W, You C. Identification and functional characterization of apple *MdCKX5.2* in root development and abiotic stress tolerance. Horticulturae. 2022;8(1):62. doi:10.3390/horticulturae8010062.
24. Qi S, Zhang S, Islam MM, El-Sappah AH, Zhang F, Liang Y. Natural resources resistance to tomato spotted wilt virus (TSWV) in tomato (*Solanum lycopersicum*). Int J Mol Sci. 2021;22:10978. doi:10.3390/ijms222010978.
25. Gerszberg A, Hnatuszko-Konka K, Kowalczyk T, Kononowicz AK. Tomato (*Solanum lycopersicum* L.) in the service of biotechnology. Plant Cell Tiss Org (PCTOC). 2014;120(3):881–902.
26. Gatahi DM. Challenges and opportunities in tomato production chain and sustainable standards. Int J Hortic Sci Technol. 2020;7(3):235–62.
27. Hnatuszko-Konka K, Gerszberg A, Weremczuk-Jezyna I, Grzegorczyk-Karolak I. Cytokinin signaling and de novo shoot organogenesis. Genes. 2021;12(2):265. doi:10.3390/genes12020265.
28. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23(21):2947–8. doi:10.1093/bioinformatics/btm404.
29. Voorrips RE. MapChart: software for the graphical presentation of linkage maps and QTLs. J Heredity. 2002;93:77–8. doi:10.1093/jhered/93.1.77.
30. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–4. doi:10.1093/molbev/msw054.
31. Letunic I, Bork P. Interactive tree of life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 2019;47(W1):W256–9. doi:10.1093/nar/gkz239.
32. Wang Y, Tang H, Debarry JD, Tan X, Li J, Wang X, et al. MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. Nucleic Acids Res. 2012;40(7):e49. doi:10.1093/nar/gkr1293.
33. Wang D, Zhang Y, Zhang Z, Zhu J, Yu J. KaKs_Calculator 2.0: a toolkit incorporating gamma-series methods and sliding window strategies. Genom Proteom Bioinf. 2010;8(1):77–80. doi:10.1016/S1672-0229(10)60008-3.
34. Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Peer YVD, et al. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 2002;30(1):325–7. doi:10.1093/nar/30.1.325.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 2001;25(4):402–8. doi:10.1006/meth.2001.1262.

36. Shimotohno A, Aki SS, Takahashi N, Umeda M. Regulation of the plant cell cycle in response to hormones and the environment. Annu Rev Plant Biol. 2021;72:273–96. doi:10.1146/arplant.2021.72.issue-1.
37. Ozden E, Light ME, Demir I. Alternating temperatures increase germination and emergence in relation to endogenous hormones and enzyme activities in aubergine seeds. S Afr J Bot. 2021;139:130–9. doi:10.1016/j.sajb.2021.02.015.
38. Long TA, Benfey PN. Transcription factors and hormones: new insights into plant cell differentiation. Curr Opin Cell Biol. 2006;18(6):710–4. doi:10.1016/j.ceb.2006.09.004.
39. Chen H, Lei Y, Sun J, Ma M, Deng P, Quan JE, et al. Effects of different growth hormones on rooting and endogenous hormone content of two *Morus alba* L. Cuttings Hortic. 2023;9(5):552. doi:10.3390/horticulturae9050552.
40. Arnao M, Hernández-Ruiz J. Melatonin in flowering, fruit set and fruit ripening. Plant Reprod. 2020;33(2):77–87. doi:10.1007/s00497-020-00388-8.
41. Rodriguez RE, Ercoli MF, Debernardi JM, Breakfield NW, Mecchia MA, Sabatini M, et al. MicroRNA miR396 regulates the switch between stem cells and transit-amplifying cells in Arabidopsis roots. Plant Cell. 2015;27(12):3354–66. doi:10.1105/tpc.15.00452.
42. Guo B, Yang H, Dai L, Zhao X, Wang LF. Genome-wide identification and response stress expression analysis of the *BES1* family in rubber tree (*Hevea brasiliensis* Muell. Arg.). PeerJ. 2022;10:e13189. doi:10.7717/peerj.13189.
43. Feng X, Yu Q, Zeng J, He X, Liu W. Genome-wide identification and characterization of GATA family genes in wheat. BMC Plant Biol. 2022;22(1):372. doi:10.1186/s12870-022-03733-3.