Whole-genome identification and salt- and ABA-induced expression trends of the *Nicotiana tabacum* CKX gene family

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Abstract

Cytokinin hormones are indispensable for plant growth and development. Cytokinin oxidase/dehydrogenase (CKX) helps regulate the dynamic balance of endogenous cytokinin levels. However, little is known about the CKX genes of *Nicotiana tabacum* (*NtCKXs*). In silico analyses were used to isolate, characterize, and elucidate the phylogenetic relationships of 15 *NtCKX* genes. Multi-species phylogenetic tree construction placed *NtCKX1–15* on five of the eight branches of the CKX phylogenetic tree. Protein structure and network analyses revealed that *NtCKX* genes located on the same phylogenetic branch generally contained several conserved motifs and have highly similar structures, with structural domains related to flavin adenine dinucleotide (FAD) and cytokinin-binding found on all of the predicted *NtCKX*-encoded proteins. The upstream promotor region of *NtCKX* genes contained many abiotic stress-responsive cis-acting elements, including DRE, ERE, MBS, MYB, and MYC. Gene expression analysis revealed that each *NtCKX* gene responded differently to salt stress and exogenous abscisic acid (ABA) treatment. Four *NtCKX* genes exhibited ABA-induced expression trends with varying peak times. Under salt stress, *NtCKX* expression was significantly suppressed in two genes and upregulated in five others. In summary, we provided basic information regarding the CKX gene family of *N. tabacum* and elucidated their gene expression patterns under abiotic stresses, including ABA treatment and salt stress. The findings of this work can serve as a foundation for future study of the functions of *NtCKX* genes.

Keywords Cytokinin oxidase/dehydrogenase · *Nicotiana tabacum* · Stress-induced expression · Phylogenetic analysis

Introduction

Naturally occurring cytokinins are adenine derivatives with an isopentenyl side chain at the N6 position. In combination with auxin and abscisic acid (ABA), cytokinins play an important role in plant growth and morphogenesis (Eklöf et al. 1997; Werner et al. 2001). Therefore, the dynamic homeostasis of endogenous cytokinin levels is critical for plants. Isopentyl transferases (IPTs) are responsible for the rate-limiting step in cytokinin biosynthesis, and the two types of IPTs found in plants are tRNA-IPTs and adenylate-IPTs (Kieber and Schaller 2018; Polanska et al. 2007). In
tRNAs that recognize codons beginning with U, the adenine residue immediately 3' to the anticodon may be modified by tRNA-IPT to have an isopentenyl- or cis-hydroxy isopentenyl side chain at the N\textsuperscript{6} position, thus forming cis-zeatin (cZ) cytokinins (Yevdakova and Schwartzenberg 2007). Adenylate-IPTs may isopentenylate the (cZ) cytokinins (Yevdakova and Schwartzenberg 2007). Adenylate-IPTs may isopentenylate the (cZ) cytokinins (Yevdakova and Schwartzenberg 2007).

The number of CKX genes identified in Arabidopsis thaliana, Zea mays, and Brassica rapa are 7, 10, and 12, respectively. The temporal and spatial expression patterns of CKX genes vary between different species. In A. thaliana, AtCKX1 is expressed in the shoots, lateral meristems, and roots. AtCKX5 is related to the development of pollen and axillary buds, whereas AtCKX6 is expressed in the vascular systems of the cotyledons, leaves, and roots (Werner et al. 2003). In Z. mays, ZmCKX1 is strongly expressed in the roots, and both ZmCKX2 and ZmCKX12 have been detected in the stem following germination (Zalabak et al. 2014). In B. rapa, BrCKX6 and BrCKX2-2 are strongly expressed in the roots and gynoecium, respectively (Liu et al. 2013). The functions of the CKX genes have also been extensively studied. The overexpression of AtCKX3 in A. thaliana can increase the growth rate of the primary roots and the length of the lateral and adventitious roots (Werner et al. 2003). In Oryza sativa, a decrease in OsCKX2 expression can increase the number of reproductive organs, thus increasing the grain yield (Ashikari et al. 2005). Overexpression of AtCKX1 in transgenic Nicotiana tabacum causes mature leaves to thicken and harden. Similarly, A. thaliana AtCKX3/AtCKX5 double mutants are known to produce more seedlings (Bartrina et al. 2011). The silencing of the HvCKX1 gene decreases CKX enzyme level in Hordeum vulgare and leads to higher plant productivity (Zalewski et al. 2010).

Cytokinins also play an important role in plant stress responses. Vyroubalova et al. demonstrated that long-term salt stress leads to the persistent and abundant expression of ZmCKX2, ZmCKX3, and ZmCKX6 in Z. mays (Vyroubalova et al. 2009). In the presence of drought stress, most Glycerine max CKX genes are induced in the roots (Le et al. 2012). CK-deficient A. thaliana plants are known to exhibit a strong stress-tolerant phenotype (Nishiyama et al. 2011). Overexpression of AtCKX2 using a root-specific promoter in wheat resulted in significant root enhancement and drought resistance (Chen et al. 2020b). Genetically modified A. thaliana plants that overexpress AtCKX3 have significantly greater root biomass than wildtype plants (60%), and this expanded root system increases the survival rate of the genetically modified line under drought conditions (Werner et al. 2010).

In this study, whole-genome sequencing was performed to identify and sequence the CKX genes of N. tabacum. In addition, we analyzed the expression trends of NtCKX genes under salt stress and exogenous ABA treatment. We conducted a systematic study on the CKX genes of N. tabacum, which will lay the foundation for future studies on their function.

### Materials and methods

#### The identification and annotation of NtCKX genes

The genome sequences of A. thaliana (Takei et al. 2001), O. sativa (Tsai et al. 2012), and Solanum lycopersicum (Matsuo et al. 2012) were retrieved from PlantGDB (http://www.plantgdb.org/). The sequences of these three species were obtained through species search and CKX gene search on the PlantGDB website and were further obtained by blast comparison on the NCBI website. The sequences of Arabidopsis lyrata, B. rapa, Populus trichocarpa, and Physcomitrium patens were obtained from the study conducted by Liu et al. (2013). Basic Local Alignment Search Tool alignment was performed between the protein sequences of A. thaliana AtCKX and the N. tabacum genome (https://solgenomics.net/organism/Nicotianatabacumgenome), with an E-value of 1e^-20. The predicted proteins of the resultant sequences were screened for the presence of FAD- and cytokinin-binding regions to obtain the candidate NtCKX gene sequences (Table S1). ProtComp 9.0 (http://linux1.berry.com/berry.phpml/topic=protcompl&group=programs& subgroup=proloc) was used to predict the subcellular localization of NtCKX proteins, and the ExPaSy ProtParam tool (https://web.expasy.org/protparam/) was used to predict their isoelectric points and affinity coefficients.

### Multi-species phylogenetic analysis, multiple protein sequence alignment, and gene structure mapping

A phylogenetic tree of the CKX families of N. tabacum, A. thaliana, O. sativa, B. rapa, P. trichocarpa, S. lycopersicum, and P. patens was created using MEGA 6.0 using the neighbor-joining (NJ) method with 1000 bootstrap replications (Tamura et al. 2013). DNAMAN was used to investigate the conservativeness of the CKXs family by analyzing the multiple sequence alignment among the CKX protein sequences of N. tabacum, A. thaliana, O. sativa, S. lycopersicum, and B. rapa (Thompson et al. 1997). The structure of the NtCKX genes including the number of introns and exons, and their
was performed using an SYBR Premix Ex Taq™II kit extracted RNA to cDNA. Quantitative RT-PCR (qRT-PCR) using the GoScript™ Reverse Transcriptase System (Promega) was used to reverse-transcribe the RNA according to the manufacturer’s instructions. The protein–protein interactions of the CKX of *A. thaliana* and *N. tabacum* were predicted using STRING (https://string-db.org/).

*N. tabacum* plant materials, the tissue-specific expression of NtCKX genes, and stress-induced expression trends

The *N. tabacum* plant materials were obtained from laboratory-grown *N. tabacum* K326. The seeds were disinfected using 75% alcohol for 10 s, rinsed three times with sterile water, and then subjected to 10% NaOCl for 6–8 min to remove the seed coat. The seeds were washed another five times with sterile water before being cultured onto solid MS media. Cultivation was performed at 25 °C under a 16:8 h light/dark cycle. After 2 months of cultivation, the young leaves, stems, and roots were collected, along with the leaves, roots, stems, flowers, and seeds of mature laboratory-grown *N. tabacum* plants at a vegetative maturation stage of approximately 5–6 months after germination. After collection, all the sample materials were immediately stored in liquid nitrogen and then transferred to a −80 °C until the determination of gene expression levels. The stressed K326 plants were cultivated from seeds in the soil at 25 °C under 16 h:8 h light/dark cycles. At 4 weeks old, all the leaves of each plant were sprayed with a 200 mM NaCl or a 100 µM ABA solution. Leaves from three biological replicates were then collected at 0 h, 3 h, 6 h, 12 h, and 24 h. All samples were immediately stored in liquid nitrogen after collection and then transferred to a −80 °C freezer until they were used for stress-induced expression profile analysis.

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was extracted from all *N. tabacum* plant materials using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The GoScript™ Reverse Transcription System (Promega) was used to reverse-transcribe the extracted RNA to cDNA. Quantitative RT-PCR (qRT-PCR) was performed using an SYBR Premix Ex Taq TMII kit (Takara, Japan) with specific quantitative PCR primers that were designed using PrimerQuest Tool (https://sg.idtdna.com/Primerquest/Home/Index). *NtEF1α* was used as the internal control (Table S2). qTOWER 3.0 real-time PCR system (Analytik Jena AG, Jena, Germany) was used to measure gene expression levels in each tissue sample. Three biological replicates were collected for each treatment at each time point, and three technical replicates were used for each sample. The qRT-PCR regime consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s (denaturation) and 60 °C for 1 min (annealing/elongation). The total duration of this procedure was 1 h 6 min. Quantitative verification was performed using the 2^−ΔΔCt method (Livak and Schmittgen 2001).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 6. ****, ***, **, and * indicate significant differences compared to the control (0 h) at *p* < 0.0001, *p* < 0.001, *p* < 0.01, and *p* < 0.05, respectively.

Results

Identification and annotation of NtCKX genes

Aligning the *A. thaliana* AtCKX sequences with the *N. tabacum* genome revealed 15 *N. tabacum* NtCKX genes with similar structural domains. These genes were named NtCKX1–NtCKX15. The basic information of the NtCKX gene family is presented in Table 1. The NtCKX gene family had three–five exons, and the proteins they encode varied from 5.58 to 9.21. According to the subcellular localization analysis of the NtCKX proteins, NtCKX1 was located in the cell membrane; NtCKX4–7, NtCKX10–12, and NtCKX14 were located in the extracellular matrix; NtCKX2, NtCKX3, and NtCKX8 were located in the vacuoles; NtCKX13 was located in the cytoplasm; and NtCKX15 was located in the chloroplasts.

Phylogenetetic tree, multiple protein sequence alignment, and gene structure analysis

A phylogenetic tree was constructed using MEGA 6.0 for the CKX proteins of *N. tabacum*, *A. thaliana*, *O. sativa*, *B. rapa*, *P. trichocarpa*, *S. lycopersicum*, and *P. patens* to ascertain the evolutionary relationships of the CKX gene family (Fig. 1a). The phylogenetic tree had a total of eight branches, which are denoted as α, β, γ, δ, ε, ζ, η, and θ. The
NtCKX genes were mainly located on the α, β, δ, ζ, and θ branches. The α branch included NtCKX1–3, NtCKX5–6, and NtCKX8; the β branch included NtCKX4 and NtCKX7; the δ branch included NtCKX10–11; the ζ branch included NtCKX9 and NtCKX12; and the θ branch exclusively consisted of the NtCKX13–15 genes. The γ branch consisted of eight genes: the OsCKX3 and CKX genes of P. patens.

Multiple protein sequence alignment analysis indicated that NtCKX proteins exhibit a high degree of homology and conservativeness with the CKX protein sequences of A. thaliana and S. lycopersicum (Fig. S1). We found that the genes on the same branch have similar structures (Fig. 1b). For instance, NtCKX3, NtCKX5, NtCKX9, and NtCKX12 have very similar gene structures, and all of these genes have five exons that are flanked by four introns. NtCKX10 and NtCKX11 have four exons and three introns, whereas NtCKX6 and NtCKX8 have three exons and two introns. NtCKX14 and NtCKX15 contain four exons and three introns. These NtCKX genes have the highest degree of intra-branch similarity in terms of structure and homology and have the same number of exons and introns. In contrast, NtCKX1, NtCKX2, NtCKX4, NtCKX7, and NtCKX13 genes exhibit intra-branch differences in terms of structure.

Conserved motifs of the CKX proteins of N. tabacum

The MEME tool analysis of the conserved motifs of the CKX proteins indicated that NtCKX proteins on the same phylogenetic branch have highly similar motifs and that motifs 6, 7, 9, 13, 17, and 20 were present in all the members of the NtCKX proteins family (Fig. 2, Table S3). Except for NtCKX15, motifs 2, 4, and 15 were also present in all members of this family. Motif 14 was present in all members of the NtCKX proteins family except for the NtCKX proteins on the α branch. Motifs 10 and 18 were also present in all NtCKX members except for α-branch NtCKX members and NtCKX4. Motifs 2 and 16 were annotated as motifs that could be related to the FAD-binding domain and motifs 3, 5, 6, 7, 13, and 17 as possibly related to the cytokinin-binding domain. Motifs 6, 7, 13, and 17 were present in all NtCKX protein family members. NtCKX4 did not have motif 3, and except for NtCKX9 and NtCKX12, other family members had motif 5. NtCKX1, 2, 3, 4, 5, 7, 9, 10, 11, and 12 all had motif 2 and motif 16.

Table 1 Basic information of the cytokinin dehydrogenase/oxidase gene family of Nicotiana tabacum

| Gene name | Gene ID | Protein length (aa) | Molecular weight (Da) | Theoretical PI | Grand average of hydropathicity (GRAVY) | Exon number | Subcellular location |
|-----------|---------|---------------------|-----------------------|----------------|----------------------------------------|-------------|---------------------|
| NtCKX1    | Nitab4.5_0000316g0350 | 375                  | 41,929.17             | 8.78           | −0.017                                 | 5           | Cell membrane       |
| NtCKX2    | Nitab4.5_0008947g0010 | 352                  | 39,461.19             | 7.27           | −0.082                                 | 3           | Vacuole             |
| NtCKX3    | Nitab4.5_0000677g0110 | 445                  | 49,814.47             | 8.75           | −0.083                                 | 5           | Vacuole             |
| NtCKX4    | Nitab4.5_0001861g0120 | 416                  | 45,872.64             | 8.61           | −0.032                                 | 4           | Extracell matrix    |
| NtCKX5    | Nitab4.5_0000364g0020 | 373                  | 41,651.70             | 6.79           | −0.038                                 | 5           | Extracell matrix    |
| NtCKX6    | Nitab4.5_0001618g0190 | 331                  | 37,041.04             | 6.30           | −0.088                                 | 3           | Extracell matrix    |
| NtCKX7    | Nitab4.5_0005779g0060 | 482                  | 54,151.91             | 7.69           | −0.196                                 | 5           | Extracell matrix    |
| NtCKX8    | Nitab4.5_0000826g0120 | 311                  | 34,646.32             | 6.63           | −0.128                                 | 3           | Vacuole             |
| NtCKX9    | Nitab4.5_0008523g0060 | 493                  | 55,517.40             | 7.65           | −0.209                                 | 5           | Extracell matrix    |
| NtCKX10   | Nitab4.5_000341g0010  | 517                  | 57,940.98             | 5.75           | −0.190                                 | 4           | Extracell matrix    |
| NtCKX11   | Nitab4.5_0000446g0210 | 517                  | 57,838.78             | 5.58           | −0.160                                 | 4           | Extracell matrix    |
| NtCKX12   | Nitab4.5_0000540g0320 | 451                  | 51,146.53             | 9.21           | −0.289                                 | 5           | Extracell matrix    |
| NtCKX13   | Nitab4.5_0010540g0020 | 474                  | 53,372.17             | 9.09           | −0.176                                 | 5           | Cytoplasm           |
| NtCKX14   | Nitab4.5_0010634g0030 | 379                  | 42,987.29             | 6.83           | −0.140                                 | 4           | Extracell matrix    |
| NtCKX15   | Nitab4.5_0007115g0010 | 210                  | 24,571.53             | 9.21           | −0.286                                 | 4           | Chloroplast         |

Analysis of the protein–protein interaction network of N. tabacum and A. thaliana CKX proteins

Figure 3 illustrates the complex network of protein–protein interactions of the N. tabacum and A. thaliana CKX proteins predicted using the STRING website. In A. thaliana, AtCKX1 has important functions in the development and morphogenesis of vascular tissues, shoot apical meristems, and axillary meristems (Werner et al. 2010); the NtCKX3, NtCKX5, NtCKX6, and NtCKX8 genes that correspond to AtCKX1 could have similar functions. The NtCKX9, NtCKX12, NtCKX13, and NtCKX14 genes corresponding to AtCKX3 may also autonomously regulate cytokinin levels and meristem cell viability (Bartrina et al. 2011). NtCKX4 and NtCKX7 may function similarly to AtCKX5, which regulates stem meristem differentiation and limits the flow of cytokinins from the stem meristem (Bartrina et al. 2011).
NtCKX1 and NtCKX2 could have the same functions as AtCKX6 and play a role in the development of cotyledon, leaf, stoma, and root vascular systems in N. tabacum (Wer-ner et al. 2010).

**Expression profile of the NtCKX genes in N. tabacum tissues**

To further investigate the functions of the NtCKX genes, RT-PCR was used to measure the expression of NtCKX genes in young roots, leaves, and stems, and mature roots, leaves, stems, flowers, and seeds (Fig. 4). NtCKX11 was expressed in all tested tissues with high expression levels in mature roots. NtCKX4 was strongly expressed in young stems, whereas NtCKX6 was abundantly expressed in seeds. NtCKX14 was highly expressed in seeds and mature leaves. NtCKX1 was abundantly expressed in the flowers and mature roots, whereas NtCKX8 and NtCKX13 were strongly expressed in the seeds; these genes were either expressed at low or undetectable levels in all other tissues. NtCKX9 was abundantly expressed in young leaves, and it was also expressed in young roots and stems, mature stems, and seeds. NtCKX5 was only expressed in mature leaves, whereas NtCKX12 was only expressed in the seeds. NtCKX7 was specifically expressed in flowers and mature stems. The expression of NtCKX2, 3, 10, and 15 was very low in all the sampled tissues and organs; thus, no data on its expression were obtained.
Fig. 2 Conserved protein motif analysis of the NtCKX genes. Each color in the key (upper right corner) represents a different conserved motif, which have been drawn according to their length.

Fig. 3 Protein–protein interaction network analysis of N. tabacum and A. thaliana CKX genes. Interaction map between the proteins coded by NtCKX and AtCKX genes.
Cis-acting elements in the promoter region of NtCKX genes

The promoter sequences of NtCKX genes were analyzed to investigate their response to abiotic stress. The cis-acting elements in the promoter region of each NtCKX gene that were predicted to respond to environmental stress and hormones are shown in Table 2, except for NtCKX14, whose promoter sequences could not be identified. These cis-acting elements include ABA-responsive elements (ABRE), drought-responsive elements (ERE, MBS, and MYB), and salt-responsive elements (DRE). MYC responds to induction by low temperatures, high salinity, and drought. ERE, MBS, and MYB respond to drought conditions and the accumulation of ABA. The promoter regions of NtCKX8 and NtCKX9 contained many MYB elements. ERE was the most abundant element in the promoter region of NtCKX10, which also contained many MYB and MYC elements. The promoter region of NtCKX11 had more MYB elements than any other gene, and a substantial number of ERE, MBS, MYC, and DRE elements.

Changes in the expression of NtCKX genes due to ABA treatment and salt stress

We also investigated the expression of the NtCKX gene family in plants subjected to ABA treatment and salt stress (Fig. 5). Upon treatment with ABA, NtCKX4 expression was largely unchanged. The expression of NtCKX5, NtCKX7, NtCKX12, NtCKX13, and NtCKX15 remained undetectable, and the changes in the expression levels of the other NtCKX genes were variable. For instance, the expression levels of NtCKX1, NtCKX2, and NtCKX6 were suppressed at 3 h, and then were upregulated, with their expression levels peaking at 24 h, reaching 5–10 times that of unstressed conditions. The expression of NtCKX3 peaked at 6 h and then fell...
The expression levels of \( \text{NtCKX8} \), \( \text{NtCKX9} \), and \( \text{NtCKX14} \) increased consistently for up to 24 h and then reached 2–5 times that of unstressed conditions. The expression changes of \( \text{NtCKX10} \) followed a parabolic curve; it gradually rose from 0 to 12 h and then gradually decreased. The expression of \( \text{NtCKX11} \) was strongly upregulated at 3 h and then decreased gradually, returning to normal levels at 24 h.

In salt-treated plants, the expression of \( \text{NtCKX7} \), \( \text{NtCKX12} \), \( \text{NtCKX13} \), and \( \text{NtCKX15} \) remained undetectable. The expression of \( \text{NtCKX4} \) was continuously downregulated under salt treatment, and the difference was evident. At 24 h, after salt treatment, the expression of \( \text{CKX4} \) decreased by about 10 times. The expression of \( \text{NtCKX8} \) did not change significantly, whereas \( \text{NtCKX3} \) expression was suppressed at 3 h but returned to normal levels shortly after. The expression of \( \text{NtCKX2} \) was suppressed starting from 12 h. The expression patterns of \( \text{NtCKX1} \), \( \text{NtCKX10} \), and \( \text{NtCKX11} \) were parabola-like, as their expression was upregulated from the point of treatment until 6 h and then was downregulated. The relative transcription levels of \( \text{NtCKX9} \) and \( \text{NtCKX14} \) gradually increased over time and peaked at 24 h, reaching approximately six times that of unstressed conditions.

### Discussion

Cytokinins are indispensable hormones for plant growth. CKX plays a critical role in the dynamic stabilization of endogenous cytokinin levels. Although the CKX gene family has been identified and studied in many plant species, the CKX genes of Solanaceae plants remain largely unexplored. *A. thaliana*, *A. lyrata*, *P. trichocarpa*, *S. lycopersicum*, and *O. sativa* are all diploids, and their CKX gene families comprise nine, seven, nine, six, and ten members, respectively. The gene sequencing results of this study indicate that common tobacco contains 15 CKX gene family members. In this study, we determined that some genes belonging to the same phylogenetic branch generally have identical or similar sequences and structures, whereas most genes that belong to different branches exhibit different structures and sequences.

The tissue expression profile (Fig. 4) indicates that \( \text{NtCKX11} \) is expressed in all tissues, with exceptionally high levels in mature roots. \( \text{NtCKX1} \) is strongly expressed in flowers and mature roots, whereas \( \text{NtCKX8} \) and \( \text{NtCKX13} \) are strongly expressed in the seeds; these three genes are either unexpressed or expressed at low levels in all other tissues. The tissue- and time-specific expression of these CKX genes indicates functional differentiation, resulting in each gene having a different regulatory role in plant development. Previous studies have shown that CKX genes are critical for root structure development (Reid et al. 2016). For instance, the overexpression of \( \text{AtCKX1} \), \( \text{AtCKX2} \), and \( \text{AtCKX3} \) increased initial root elongation and lateral root growth rates in *A. thaliana* (Werner et al. 2003). Therefore, regulating the expression of \( \text{NtCKX} \) genes may enhance root system development in these plants.

Cytokinins are one of five major plant hormone types. CKX plays an important role in biological adaption to environmental stress through its cytokinin degradation function (Vyroubalova et al. 2009; Le et al. 2012). Studies have

| Gene name | MYB | MYC | MBS | ABRE | ERE | DRE |
|-----------|-----|-----|-----|------|-----|-----|
| NtCKX1    | 7   | 5   | 1   | –    | –   | –   |
| NtCKX2    | 9   | 3   | –   | 1    | 1   | –   |
| NtCKX3    | 8   | 4   | 1   | 2    | –   | –   |
| NtCKX4    | 1   | 4   | –   | 13   | –   | 1   |
| NtCKX5    | 1   | 1   | –   | 1    | 3   | –   |
| NtCKX6    | 8   | 2   | –   | 4    | –   | –   |
| NtCKX7    | 8   | 4   | 2   | 8    | 1   | –   |
| NtCKX8    | 16  | 6   | 3   | 4    | –   | –   |
| NtCKX9    | 10  | 1   | 1   | 4    | –   | –   |
| NtCKX10   | 7   | 5   | 1   | 2    | 4   | –   |
| NtCKX11   | 4   | 8   | 1   | 2    | 2   | 1   |
| NtCKX12   | 4   | 3   | –   | 4    | –   | –   |
| NtCKX13   | 10  | 1   | 1   | –    | 3   | 1   |
| NtCKX15   | 10  | 3   | –   | 6    | –   | 1   |

“–” represented no cis-acting element in the promoter region of the NtCKX gene.

**MYB** drought-responsive element, **MYC** low-temperature, salt, and drought-responsive element, **MBS** drought-responsive element, **ABRE** ABA-responsive element, **ERE** drought-responsive element, **DRE** salt-responsive element.
shown that stress induction in *Z. mays* causes *ZmCKX1* to be strongly expressed in the leaves but not in the roots. CKX overexpressors generally exhibit a strong drought- and salt-tolerance phenotype, and enhanced heat-stress tolerance (Mackova et al. 2013). Our study demonstrated that *NtCKX* genes have various roles in ABA-induced-treatment, salt-, and low-temperature-stress responses of *N. tabacum*. We found that each *CKX* gene had a different response to exogenous ABA, and expression levels in response to salt stress also varied. The cis-acting element analysis revealed that the *NtCKX* genes contained ABA-, drought-, and salt-responsive cis-acting elements. *NtCKX8* and *NtCKX9* gene
expressions were strongly induced by ABA and salt stress, which is consistent with a large number of MYB cis-acting elements in their promoter sequences. \textit{NtCKX10} and \textit{NtCKX11} responded strongly to ABA and drought induction; this behavior may have been caused by many of ABA-, drought- and salt-responsive elements in their promoter regions. Several studies have also demonstrated that stress induction leads to the accumulation of ABA, which in turn inhibits the expression of CKX, which may enhance plant tolerance to stress induction (Werner et al. 2010; Huang et al. 2018). Increased ABA content and decreased CK levels, an increase in the ratio of ABA to CK, are beneficial for plants to adapt to an unfavorable living environment (Nishiyama et al. 2011). In contrast, the dynamic changes of \textit{NtCKX} expression were related to the antagonism of ABA and CK. The increase of ABA content will reduce the level of CK, but when the CK level drops to the critical threshold level, it may inhibit the CK signal transduction pathway, which has a negative effect on stress and/or ABA-responsive gene expression. The regulatory functions are alleviated, resulting in enhanced drought and salt tolerance of the plant (Liu et al. 2013; Nishiyama et al. 2011). We hope that the findings of this study will serve as a reference for future studies of \textit{NtCKX} genes and will guide efforts to engineer drought- or salinity-resistant plants via the modification of \textit{CKX} genes.

Conclusions

A total of 15 \textit{NtCKX} genes were identified in this study. The proteins were located on five of the eight phylogenetic branches of the CKX family. According to gene structure maps, conserved motif analysis, and multiple sequence alignment, CKX proteins in the same phylogenetic branch usually have identical or similar gene structures, conserved motifs, and a high degree of homology. Most of the \textit{NtCKX} genes contain many environmental stress-responsive elements in their upstream regulatory region, namely ABA-, drought-, and salt-responsive elements. The expression of each \textit{NtCKX} gene varies spatially and temporally, as well as in their response to stress induction. In summary, this work has provided basic information regarding the \textit{CKX} genes of \textit{N. tabacum}, and it has established a foundation for future research into the functions of these genes.

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Author contributions WWC performed the experiments, analyzed the data, prepared figures and tables, and authored drafts of the paper. GHW conceived and designed the experiments. MQY, TKG, and CMS analyzed the data and contributed reagents/materials/analysis tools. QG analyzed the data, and contributed reagents/materials/analysis tools. QYX and HYX authored or reviewed the manuscript. All the authors have read and agreed to the published version of the manuscript.

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Data availability The data sets supporting the results of this article are included within the article and its additional file.

Declarations

Conflict of interest The authors declare no competing interests.

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