Whole-Genome Identification and Salt- and ABA-Induced Expression Trends of the *Nicotiana tabacum* CKX gene family

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Research Article

Keywords: Cytokinin oxidase/dehydrogenase, *Nicotiana tabacum*, stress-induced expression, phylogenetic analysis

Posted Date: July 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-731912/v1>

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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 contain 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 a large number of abiotic stress-responsive cis-acting elements, including DRE, ERE, MBS, MYB, and MYC. Gene expression analysis revealed that each *NtCKX* gene has a different response to salt- and exogenous ABA-stress. 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 have provided basic information about the CKX gene family of *N. tabacum*, and elucidated their gene expression patterns under abiotic stresses, including ABA- and salt-stress. The findings of this work can serve as a foundation for future study of the functions of *N. tabacum* CKX genes.

Introduction

Naturally occurring cytokinins are adenine derivatives with an isopentenyl side chain at the N^6 position. In combination with auxin and abscisic acid (ABA), cytokinins play an important role in plant growth and morphogenesis [1, 2]. 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 [3, 4]. 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^6 position, thus forming *cis*-zeatin (cZ) cytokinins [5]. Adenylate-IPTs may isopentenylate the N^6 position of adenine using dimethylallyl diphosphate as the side chain donor, to form N^6 -(Δ^2 -isopentenyl)adenine (iP) or *trans*-zeatin (tZ) cytokinins [6]. Cytokinin dehydrogenases (CKXs) in particular play an important role in regulating cytokinin levels. CKXs are flavin adenine dinucleotide (FAD) oxidoreductases that cleave the N^6 sidechain of cytokinins to form adenine and the corresponding side chain aldehyde, thus inducing irreversible cytokinin degradation [7, 8].

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 pattern 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 [7]. In *Z. mays*, *ZmCKX1* is strongly expressed in the roots, and both *ZmCKX2* and *ZmCKX12* have been detected in the stem after germination [9]. In *B. rapa*, *BrCKX6* and *BrCKX2-2* are strongly expressed in the roots and gynoecium, respectively [10]. 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 [7]. In *Oryza sativa*, a decrease in *OsCKX2* expression can increase the number of reproductive organs, thus increasing the grain yield [11]. 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 [12]. The silencing of the *HvCKX1* gene decreases CKX enzyme level in *Hordeum vulgare* and leads to higher plant productivity [13].

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* [14]. In the presence of drought-stress, induction of most *Glycine max* CKX genes occurs in the roots [15]. CK-deficient *A. thaliana* plants are known

to exhibit a strong stress-tolerant phenotype [16]. Genetically modified *A. thaliana* plants that overexpress *AtCKX3* have significantly greater root biomass than those of the wildtype (60%), and this expanded root system increases the survival rate of the genetically modified line in drought conditions [17].

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 and exogenous ABA-stress. We have thus conducted a systematic study on the *CKX* genes of *N. tabacum*, which will lay the foundation for future study of their function.

Materials And Methods

The identification and annotation of *N. tabacum* *NtCKX* genes

The genome sequences of *A. thaliana* [18], *O. sativa* [19], and *Solanum lycopersicum* [20] were retrieved from PlantGDB (<http://www.plantgdb.org/>), whereas 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/Nicotiana tabacum/genome](https://solgenomics.net/organism/Nicotiana%20tabacum/genome)), 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 *N. tabacum* *CKX* gene sequences (Table S1). ProtComp 9.0 (<http://linux1.sberry.com/berry.phtml/>

topic = protcomppl&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* gene families of *N. tabacum*, *A. thaliana*, *O. sativa*, *B. rapa*, *P. trichocarpa*, *S. lycopersicum*, and *P. patens* was created using MEGA 6.0 with the neighbor-joining (NJ) method with 1,000 bootstrap replications [21]. DNAMAN was used to investigate the conservativeness of the *CKX* gene family by analyzing the multiple sequence alignment between the *CKX* protein sequences of *N. tabacum*, *A. thaliana*, *O. sativa*, *S. lycopersicum*, and *B. rapa* [22]. The gene structure of the *NtCKX* genes, including the number of introns and exons, and their genomic positions, were visualized using Gene Structure Display Server 2.0 (<http://gsds.gaolab.org/>) [23].

Conserved protein motif analysis, cis-acting element analysis, and protein-protein inter-action network analysis

MEME (<http://alternate.meme-suite.org/tools/meme>) and TBtools [24] were used to analyze the conserved protein motifs of the *CKX* genes. Descriptions of the *cis*-acting elements of the 15 *NtCKX* genes were obtained from PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

The protein–protein interactions of the *CKX* genes 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 with a 16 h:8 h light/dark cycle. After two 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. After collection, all the sample materials were immediately stored in liquid nitrogen and then transferred to a -80°C freezer before measurements of gene expression levels. The stressed K326 plants were cultivated from seeds in soil at 25°C under 16 h: 8 h light/dark cycles. At four-week-old, all of 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 of the samples were immediately stored in liquid nitrogen after collection, and then transferred to a -80°C freezer before being used for stress-induced expression profile analysis.

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

RNA extraction was performed on all *N. tabacum* plant materials according to the Trizol reagent (Invitrogen) protocol. The GoScript™ Reverse Transcription System (Promega) was used to reverse transcribe the extracted RNA to cDNA. Quantitative RT-PCR (qRT-PCR) was performed using a SYBR Premix Ex Taq™ II kit (Takara, Japan) with specific quantitative PCR primers that were de-signed in PrimerQuest Tool (<https://sg.idtdna.com/Primerquest/Home/Index>). *NtEF1a* 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 minutes, 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 hr 6 min. Quantitative verification was performed using the $2^{-\Delta\Delta C_t}$ method [25].

Statistical Analysis

All statistical analysis uses 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 *N. tabacum* CKX 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 *NtCKX* gene family is presented in Table 1. The *NtCKX* gene family has between three–five exons, and the proteins they encode vary between 210–517 amino acids in length and 24,571.53–57,940.98 Da in molecular weight. All the proteins were hydrophilic, and their predicted isoelectric points ranged from 5.58 to 9.21. According to subcellular localization analysis of *NtCKX* gene expression, *NtCKX1* was expressed in the cell membrane; *NtCKX4–7*, *NtCKX10–12*, and *NtCKX14* were expressed in the extracellular matrix; *NtCKX2*, *NtCKX3*, and *NtCKX8* were expressed in the vacuoles; *NtCKX13* was expressed in the cytoplasm; and *NtCKX15* was expressed in the chloroplasts.

Phylogenetic 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 has a total of eight branches, which are denoted as α, β, γ, δ, ε, ζ, η, and θ. The *NtCKX* genes are mainly located on the α, β, δ, ζ, and θ branches. The α branch includes *NtCKX1–3*, *NtCKX5–6* and *NtCKX8*; the β branch includes *NtCKX4* and *NtCKX7*; the δ branch includes *NtCKX10–11*; the ζ branch includes *NtCKX9* and *NtCKX12*; and the θ branch exclusively consists of the *NtCKX13–15* genes. The γ branch consists of eight genes, which are the *OsCKX3* and CKX genes of *P. patens*.

Multiple protein sequence alignment analysis indicated that *N. tabacum* CKX genes exhibit a high degree of homology and conservativeness with respect to the CKX protein sequences of *A. thaliana* and *S. lycopersicum* (Figure S1). We found that genes located on the same branch have similar gene structures (Fig. 1B). For instance, *NtCKX3*, *NtCKX5*, *NtCKX9* and *NtCKX12* have very similar gene structures, and all 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 structure.

Conserved protein motifs of the CKX genes of *N. tabacum*

The MEME tool analysis of the conserved protein motifs of the CKX genes indicated that *NtCKX* genes on the same phylogenetic branch have highly similar motifs and that motifs 6, 9, 13, 17, and 20 are present in all the members of the *NtCKX* gene family (Fig. 2, Table S3). Except for *NtCKX15*, motifs 2, 4, and 15 are also present in all the members of this family. Motif 14 is present in members of the *NtCKX* gene family except for *NtCKX* genes on the α branch. Motifs 10 and 18 are also present in all *NtCKX* genes except for α -branch *NtCKX* genes 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.

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

Figure 3 illustrates the complex network of protein–protein interactions of the *N. tabacum* and *A. thaliana* CKX genes 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; the *NtCKX3*, *NtCKX5*, *NtCKX6*, and *NtCKX8* genes that correspond to *AtCKX1* could have similar functions. The *NtCKX9*, *NtCKX12*, *NtCKX13*, and *NtCKX14* genes that corresponded to *AtCKX3* may also autonomously regulate cytokinin levels and meristem cell viability. *NtCKX4* and *NtCKX7* may function similarly to *AtCKX5*, which plays a role in regulating stem meristem differentiation and limiting the flow of cytokinins from the stem meristem. *NtCKX1* and *NtCKX2* could have the same functions as *AtCKX6*, and play a role in the development of cotyledon, leaf and root vascular systems, and stoma development in *N. tabacum*.

Tissue expression profile of the *NtCKX* genes of *N. tabacum*

To study the functions of the *NtCKX* genes in further detail, RT-PCR was used to measure the expression of *N. tabacum* CKX 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 expressed abundantly in seeds. *NtCKX14* had high expression levels 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, so no data on its expression has been detected.

Changes in the expression of *N. tabacum* *NtCKX* genes because of ABA- and salt-stress

We also investigated the expression of the *NtCKX* gene family in plants that were subjected to ABA- and salt-stress (Fig. 5). In the presence of ABA-stress, the expression of *NtCKX4* was largely unchanged. The expression of *NtCKX5*, *NtCKX7*, *NtCKX12*, *NtCKX13*, and *NtCKX15* fell to undetectable levels, and the changes in the expression levels of the other *NtCKX* genes was variable. For instance, the expression levels of *NtCKX1*, *NtCKX2*, and *NtCKX6* were suppressed at three hour, and

then upregulated, with their expression levels peaking at 24 h. The expression of *NtCKX3* peaked at 6 h and then fell to undetectable levels. The expression levels of *NtCKX8*, *NtCKX9* and *NtCKX14* increased consistently up to 24 h. The expression of *NtCKX10* resembled a parabolic curve; it gradually rose from 0 h to 12 h and then gradually decreased. The expression of *NtCKX11* was strongly upregulated at 3 h and then decreased gradually, returning to normal levels at 24 h.

In salt-treated plants, *NtCKX7*, *NtCKX12*, *NtCKX13* and *NtCKX15* fell to undetectable levels. The expression of *NtCKX4* was continuously down-regulation under salt treatment. The expression of *NtCKX8* did not change significantly, whereas *NtCKX3* expression was suppressed at 3 h but returned to normal levels shortly after. The expression of *NtCKX2* was suppressed starting from 12 h. The expression levels of *NtCKX1*, *NtCKX10*, and *NtCKX11* were parabola-like, as their expression was upregulated from the point of treatment until 6 h, and then downregulated. The relative transcription levels of *NtCKX9* and *NtCKX14* gradually increased over time and peaked at 24 hour.

Cis -acting elements in the promoter region of *N. tabacum* NtCKX genes

The promoter sequences of *N. tabacum* CKX genes were analyzed to study 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, with the exception of *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 a large number of 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 it also had a substantial number of ERE, MBS, MYC and DRE elements.

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.

All flowering plants have undergone at least one whole-genome duplication (WGD) event in their evolutionary history [26]. This phenomenon likely arose as hybrids display enhanced adaptability to environmental changes, termed hybrid vigor [27]. Tobacco is a classic representative of Solanaceae plants. Common tobacco (*N. tabacum*), in particular, is an allotetraploid plant that contains the full genomes of both of its ancestors, diploid *N. sylvestris* and *N. tomentosiformis* [28–30]. *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. We speculate that WGD events account for the comparatively large number of CKX genes in *N. tabacum*. As WGD often leads to gene function redundancy, some of these genes may have been inactivated or differentiated [31, 32]. In this study, we have found 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 have different structures and sequences. *NtCKX13*, *NtCKX14*, and *NtCKX15* are located on the θ branch of the multi-species phylogenetic tree, and all three of these genes have similar gene structures and intact structural domains. In tissue expression and induced expression analysis, it was found that *NtCKX13* and *NtCKX14* are both strongly expressed in seeds and mature leaves and that *NtCKX14* expression changes in response to abiotic stress. However, *NtCKX15* is not expressed in any tissue or organ, with or without stress. We speculate that *NtCKX15* may have been inactivated following WGD because of redundancy.

The tissue expression profile (Fig. 4) indicates that *NtCKX11* is expressed in all tissues, with especially high levels in mature roots. *NtCKX1* is strongly expressed in flowers and mature roots, whereas *NtCKX8* and *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 is indicative of 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 [33]. For instance, the overexpression of *AtCKX1*, *AtCKX2*, and *AtCKX3* increases initial root elongation and lateral root growth rates in *A. thaliana* [7]. Therefore, regulating the expression of *N. tabacum* CKX genes may enhance root system development in these plants.

Cytokinin is one of the five major types of plant hormones. CKX plays an important role in biological adaption to environmental stress through its cytokinin degradation function [14, 15]. Studies have shown that stress induction in *Z. mays* causes *ZmCKX1* to be strongly expressed in the leaves but not in the roots. Our study demonstrated that *NtCKX* genes have various roles in salt, low-temperature, and ABA-induced 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 were also varied. The *cis*-acting element analysis revealed that the *NtCKX* genes contained ABA-responsive, drought-responsive, and salt-responsive *cis*-acting elements. The ABRE element, in particular, is responsive to each of these stressors. Since ABRE is hypersensitive to salt stress and ABA, endogenous ABA synthesis may decrease in response to the exogenous application of ABA. Concurrent regulation of endogenous hormones in the plant body will also lead to the accumulation of cytokinins, resulting in the inhibition of *NtCKX4* expression. CKX overexpressors generally exhibit a strong drought and salt tolerance phenotype, as well as enhanced heat-stress tolerance [34]. *NtCKX8* and *NtCKX9* gene expression was strongly induced by ABA and salt stress, which is consistent with the large number of MYB *cis*-acting elements in their promoter sequences. *NtCKX10* and *NtCKX11* both responded strongly to ABA and drought induction; this behavior may have been caused by the large number of ABA-, drought- and salt-responsive elements in their promoter regions. Some studies have shown that drought induction leads to the accumulation of ABA, which may enhance the tolerance of a plant to drought conditions [17, 35]. It has been demonstrated that this phenomenon arises from the regulation of the ABA-to-cytokinin ratio, which imbues the plant with enhanced drought and salt tolerance [16]. We hope that the findings of this study will serve as a reference for future studies of *N. tabacum* CKX genes and will guide efforts to engineer drought- or salinity-resistant plants via the modification of CKX genes.

Conclusions

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

Abbreviations

CKX Cytokinin oxidase/dehydrogenase

ABA abscisic acid

FAD flavin adenine dinucleotide

IPT Isopentyl transferases

cZ *cis*-zeatin cytokinins

Declarations

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

Funding This study was supported by grants from CTCCC (No. B20202NY1337) and CNTC research program (No.110202001020 (JY-03), No.110202001021 (JY-04), 110202001028 (JY-11) and 110201601034(JY-08)).

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

Compliance with ethical standards

Conflicts of interest The authors declare no competing interests.

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Tables

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

Table 2. Abiotic stress-responsive cis-acting elements in the promoter region of the *NtCKX* genes.

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.

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	1	-
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.

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	41929.17	8.78	-0.017	5	Cell membrane
NtCKX2	Nitab4.5_0008947g0010	352	39461.19	7.27	-0.082	3	Vacuole
NtCKX3	Nitab4.5_0000677g0110	445	49814.47	8.75	-0.083	5	Vacuole
NtCKX4	Nitab4.5_0001861g0120	416	45872.64	6.81	-0.032	4	Extracell matrix
NtCKX5	Nitab4.5_0000364g0020	373	41651.70	6.79	-0.038	5	Extracell matrix
NtCKX6	Nitab4.5_0001618g0190	331	37041.04	6.30	-0.088	3	Extracell matrix
NtCKX7	Nitab4.5_0005779g0060	482	54151.91	7.69	-0.196	5	Extracell matrix
NtCKX8	Nitab4.5_0000826g0120	311	34646.32	6.63	-0.128	3	Vacuole
NtCKX9	Nitab4.5_0008523g0060	493	55517.40	7.65	-0.209	5	Extracell matrix
NtCKX10	Nitab4.5_0000341g0010	517	57940.98	5.75	-0.190	4	Extracell matrix
NtCKX11	Nitab4.5_0000446g0210	517	57838.78	5.58	-0.160	4	Extracell matrix
NtCKX12	Nitab4.5_0000540g0320	451	51146.53	9.21	-0.289	5	Extracell matrix
NtCKX13	Nitab4.5_0010540g0020	474	53372.17	9.09	-0.176	5	Cytoplasm
NtCKX14	Nitab4.5_0010634g0030	379	42987.29	6.83	-0.140	4	Extracell matrix
NtCKX15	Nitab4.5_0007115g0010	210	24571.53	9.21	-0.286	4	Chloroplast

Figures

A

B

Figure 1

Phylogeny and gene structure of the *N. tabacum* CKX gene family. (A) Phylogenetic tree of the CKX gene family in *N. tabacum* and other species, including *A. thaliana* (At), *A. lyrata* (Al), *B. rapa* (Br), *S. lycopersicum* (Sl), *N. tabacum* (Nt), *O. sativa* (Os), and *P. patens* (Pp). The members of the *N. tabacum* CKX gene family are shown in red. (B) Phylogenetic tree and gene structure map (drawn to scale) of the NtCKX gene family.

Figure 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.

Figure 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.

Figure 4

Tissue-specific expression of the NtCKX genes. Pr: young root; Ys: young stem; Yl: young leaves, Fl: flower, Fr: seedlings; Mr: Mature root; Ms: Mature stem; Ml: Mature leaves

Figure 5

NtCKX expression in the presence of ABA and salt-stress. (A) NtCKX expression in the presence of ABA-stress. The indicated time points are 0 h, 3 h, 6 h, 12 h, and 24 h. The significance of the change in gene expression level with respect to the control at 0 h is designated as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. (B) NtCKX expression in the presence of salt-stress. The indicated time points are 0 h, 3 h, 6 h, 12 h and 24 h. The significance of the change in gene expression level with respect to the control at 0 h is designated as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

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