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Antioxidant system was triggered to alleviate salinity stress by cytokinin oxidase/dehydrogenase gene *GhCKX6b-Dt* in cotton

Mengyue Liu[1,2†], Yupeng Cui[1†], Fanjia Peng[3†], Shuai Wang[1†], Ruifeng Cui[1†], Xiaoyu Liu[1], Yuexin Zhang[1], Hui Huang[1], Yapeng Fan[1], Tiantian Jiang[1], Xixian Feng[1], Yuqian Lei[1], Kesong Ni[1], Mingge Han[1], Wenhua Chen[1], Yuan Meng[1], Junjuan Wang[1], Xiugui Chen[1], Xuke Lu[1], Delong Wang[1], Lixue Guo[1], Lanjie Zhao[1], Jing Jiang[2*] and Wuwei Ye[1,2*]

Abstract

Cytokinin oxidase/dehydrogenase (CKX) is a key regulatory enzyme for the irreversible degradation of the plant hormone cytokinin (CK), which is important in growth and development and response to abiotic stresses in cotton. In this study, 27, 28, 14 and 14 *CKXs* were screened by FAD structural domain and cytokinin binding structural domain in *Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium arboreum* and *Gossypium raimondii*, respectively. Their phylogenetic relationships and expression patterns were analyzed, and most *GhCKXs* were found to be tissue-specific and responsive to various abiotic stresses such as cold, heat, salt and PEG. *GhCKX6b-Dt* was selected for gene silencing in evolutionary branch II for salt stress, because its expression increased after salt stress in cotton plants. An increase in PRO and MDA content and a decrease in SOD activity due to this gene were found after inducing salt stress, contributing to oxidative damage and decreased salt tolerance. In this study, *CKXs* were analyzed to reveal the possible role of *GhCKXs* against abiotic stresses in cotton, which provides a basis for further understanding of the biological functions of CK in plants such as growth and development and stress resistance.

Keywords Cytokinin oxidase/dehydrogenase, CKX, Salinity stress, *GhCKX6b-Dt*, Cytokinin, Antioxidant

Introduction

As the plants are sessile, they must respond quickly to damage from complex environments, including biotic (e.g., microbial and insect pathogens) and abiotic (e.g., drought, salt, and heavy metals) stresses. Among them, salt stress is a primary global environmental factor that limits plant growth and crop productivity [1], more than 6% of the world's land area is saline (about 800 million hectares of land worldwide) [2]. Poor irrigation practices, inappropriate application of fertilizers and industrial pollution have increased salinity in the soil [3]. When the concentration of soluble salts in the soil exceeds a threshold value, saline soils were formed [4, 5]. Salt stress may adversely affect cells by causing osmotic stress [6, 7], ionic stress [8] and oxidative stress [9]. Na has been

†Mengyue Liu, Yupeng Cui, Fanjia Peng, Shuai Wang and Ruifeng Cui contributed equally to this work.

*Correspondence:
Jing Jiang
jiangjing@henu.edu.cn
Wuwei Ye
yew158@163.com
[1] Institute of Cotton Research of Chinese Academy of Agricultural Sciences/Research Base, Anyang Institute of Technology, National Key Laboratory of Cotton Bio-Breeding and Integrated Utilization, Anyang 455000, Henan, China
[2] National Key Laboratory of Cotton Bio-Breeding and Integrated Utilization, Henan University, Kaifeng 475004, Henan, China
[3] Hunan Institute of Cotton Science, Changde 415101, Hunan, China

shown to be an essential nutrient required by animals [10], and excess Sodium is detrimental to animals and plants [11]. The accumulation of Na^+ in plants can result in disturbed ionic dynamic balance, imbalance of potassium ion (K^+)/Na^+ ratio and Na^+ toxicity, which could lead to secondary stresses including oxidative stress. Oxidative stress will lead to cell membrane damage, ion leakage or direct damage to proteins and other macromolecules, which in turn will result in membrane dysfunction and even cell death. Both ion stress and oxidative stress can expedite leaf senescence by degrading chlorophyll, inhibiting photosynthesis and reducing yield [12, 13].

Natural cytokinin (CK) is a plant hormone that is derived from adenine and possesses either an isoprenoid or aromatic side chain at the N^6 position of the adenine ring. It can be obtained through isolation or synthesis from maize or other plant sources [14]. Therefore, CK can be categorized into isoprenoid and aromatic CK, with the former being more prevalent in plants and more abundant than the latter [15]. CK is essential for regulating plant growth, development and adaptation to environmental stresses. It is primarily synthesized in plant roots and functions as a group of compounds that stimulate cytoplasmic division. In growing seedlings, CK regulates lateral root organogenesis, root meristem size, and hypocotyl elongation [16]. Moreover, CK exerts a protective effect against plant senescence by inhibiting the breakdown of nucleic acids, proteins and other substances in the plant, while concurrently redistributing essential amino acids, hormones, inorganic salts and other compounds to other parts of the plant [17]. Since CK is a negative regulator of plant root growth and branching, CK can make plants exhibit long-term drought resistance by promoting the degradation of CK in the root system to expand the root system and increase the root to crown ratio and thus the water absorption area of the root system [12]. An experimental result showed that heat stress reduced the content of CK, and the heat resistance of plants was enhanced by exogenous treatment with CK [18].

Cytokinin oxidase/dehydrogenase (CKX) catalyzes the degradation of CK in plant tissues and is a critical negative regulator of endogenous CK content in the plant kingdom [19–21]. CKX was detected for the first time in crude extracts of tobacco tissues [22]. It catalyzes the cleavage of unsaturated N^6 side chains of CK, such as zeatin, isopentenyl adenine or their ribosyl derivatives, resulting in the release of free adenine or free adenine nucleoside, leading to complete inactivation of CK [18]. Several studies have shown that CKX is involved in various physiological processes in a variety of plants, including CK catabolism metabolism, root structure and resistance to abiotic stresses. *AtCKX* overexpression in *Arabidopsis* induces CK deficiency which enhances salt tolerance and drought tolerance [23]. Moderate increase in CK levels by down-regulating *GhCKXs* expression resulted in higher fiber and seed yield in cotton [24, 25]. A *CKX* gene was isolated from Medicago sativa, *MsCKX*, and its expression was found to increase under salt stress and abscisic acid (ABA) treatment. Overexpression of the *MsCKX* gene increased the activity of CKX, which resulted in root expansion in transgenic *Arabidopsis*. Meanwhile, overexpression of *MsCKX* enhanced salt tolerance in transgenic plants by maintaining a high K^+/Na^+ ratio, enhancing the ROS scavenging activity of antioxidant enzymes and improving the expression levels of stress-related genes (ion transport proteins and H^+ pumps) [26]. In rice, *OsCKX11* had a role in delaying leaf senescence, increasing seed number and coordinating the regulation of the source pool, thus suggesting that CK plays an overwhelming role in leaf senescence and determining seed number [27], and disruption of *OsCKX3* enhances CK content in the articular layer and also negatively regulates leaf angle [28].

Cotton (*Gossypium* spp.) is an extremely valuable fiber crop and oilseed crop, accounting for 35% of global fiber usage. Additionally, it is also a moderately salt tolerant crop [29]. While the impact of *CKX* genes on CK homeostasis and regulating growth and development in plants such as *Arabidopsis thaliana* and rice has been extensively studied, further research is necessary to elucidate the biological function of the CKX family in cotton. In this study, a total of 83 *CKX*s in four major cotton species were identified, and a systematic analysis of the *CKX*s was performed, including phylogenetic relationships, chromosomal localization and protein interaction networks, and their expression patterns under abiotic stresses and different tissues in cotton were also investigated. The function of *GhCKX6b-Dt* in salt tolerance was investigated using the virus induced gene silencing (VIGS) technique. The results of this study will contribute to future research by providing insight into the function into the phytohormone CK in cotton.

Materials and methods

Identification of CKX family members

CDS sequences and protein sequences of *Gossypium hirsutum* (*G. hirsutum*) (NAU), *Gossypium barbadense* (*G. barbadense*) (ZJU), *Gossypium arboreum* (*G. arboreum*) (CRI), and *Gossypium raimondii* (*G. raimondii*) (JGI) were obtained from the cotton database Gossypium Resource and Network Database (http://grand.cricaas.com.cn) and Cotton Functional Genomic Database (CottonFGD) (https://cottonfgd.net/) in this study. CKX protein conserved structural domains were identified by Pfam database (https://pfam.xfam.org/): FAD binding domain (PF01565) and Cytokin-bind domain (PF09265)

[30]. These two Hidden Markov Models (HMM) were utilized as query files for protein screening in the HMMER (version 3.3.1) [31] to obtain candidate genes. The genes common to both screens were set as the final CKX members. CKXs were renamed according to their homology with *Arabidopsis thaliana* [25].

Phylogenetic analysis and sequences alignments

CKX protein sequences were downloaded from CottonFGD (https://cottonfgd.net/) for *G. hirsutum* (NAU), *G. barbadense* (ZJU), *G. arboreum* (CRI), and *G. raimondii* (JGI) and from the online database Phytozome v13 (https://phytozome-next.jgi.doe.gov/) for *Arabidopsis thaliana*. The software MEGA5 was used for sequence alignment, the results were analyzed to construct intraspecific (Neighbor Joining (NJ) method) [25].

Chromosomal locations of *CKXs* from four *Gossypium* species

The genome annotation files of the four *Gossypium* species were downloaded from the CottonFGD (https://cottonfgd.net/about/download/annotation). The software TBtools was employed to visualize the chromosome locations of *CKXs* of four *Gossypium* species [32].

Analysis of *GhCKXs* promoter regions and different expressions

The upstream sequence (2000 bp) preceding the start codon (ATG) of *GhCKXs* was obtained from the CottonFGD database (https://cottonfgd.net) and submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) for the identification of *cis*-acting elements in the promoter of the *GhCKXs*. The expression levels of *GhCKXs* under different abiotic stresses (cold, heat, salt, and PEG) and different tissues (root, stem, leaf, torus, petal, stamen, pistil, and calycle) were shown as FPKM values from RNA-Seq data (PRJNA490626) [33]. Images containing evolutionary trees, *cis*-acting elements, and heat maps of expression levels were drawn for visual observation using TBtools software.

Gene ontology (GO) annotation analysis of *GhCKXs*

To explore the function of *GhCKXs*, GO annotation analysis was performed by CottonFGD (https://cottonfgd.net).

Materials, plant growth and treatments

The *G. hirsutum* cultivar Zhong 9807 was used as experimental material and seeds were sown on a 1:1.5 substrate of sand to vermiculite and grown in an incubator at 25 °C/23 °C with 16 h of light/8 h of darkness. The cotton seedlings were treated with 100 mmol/L NaCl solution, and samples were taken after 0, 6, 12 and 24 h, snap-frozen in liquid nitrogen and stored at − 80 °C.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Tissue (leaf) grinding and extraction of total RNA according to EasySpin plus plant RNA rapid isolation Kit (aidlab Co., Ltd, Beijing, China). RNA was reverse transcribed to cDNA using the HiScript Ill RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co., LTD, Nanjing, China). qRT-PCR experiments were performed using an Applied Biosystems@7500 Fast instrument and a quantification kit (*PerfectStart*® Green qPCR SuperMix) (TransGene Biotech Co., LTD, Beijing, China), with the internal reference gene (*GhUBQ7*) as a control. $2^{-\Delta\Delta CT}$ method to calculate the relative expression of *GhCKXs* [34]. Primers were designed using the website (https://www.genscript.com/) and the specificity of the primers was checked at the NCBI (National Center for Biotechnology Information) (https://www.ncbi.nlm.nih.gov/) website. Gene-specific primers for qRT-PCR are listed in Additional file 1: Table S1.

Virus induced gene silencing (VIGS) experiment

GhCKX6b-Dt was significantly up-regulated at 6 h, 12 h and 24 h after treatment with 100 mmol/L concentration of NaCl solution, so this gene was selected for silencing, and then the *GhCKX* family function was explored. The upland cotton cultivar 9807 was cultivated in nutrient soil and subsequently placed in an incubator with a light–dark cycle of 16/8 h, ambient humidity of 50%, and temperature of 25/20 °C (light/dark). *Agrobacterium tumefaciens* was injected into the cotton plant when the two cotyledons were unfurled and the treatment occurred during the two leaf one heart stage of cotton. A silencing fragment of around 300 bp was designed by SGN-VIGS (https://vigs.solgenomics). The vector construction is outlined in Additional file 2: Materials and methods for a more comprehensive operating procedure for reference. The gene-specific primers for qRT-PCR are listed in Additional file 1: Table S1. The fragment was ligated into the pYL156 vector and after transformation into *Agrobacterium*, and pYL156: *GhCKX6b-Dt*, pYL156: PDS and pYL192 was cultured to OD600 = 1.2–1.5. Each mixture was injected into the lower side of cotyledons of *G. hirsutum* material Zhong 9807. After injection, seedlings were placed in the dark for 24 h, followed by a 16 h light/8 h dark cycle at 25/20 °C (light/dark). The cotton seedlings were treated with 100 mmol/L NaCl solution for 24 h which occurred during the two leaf one heart stage of cotton. Subsequently, the leaves were taken

as samples, which were quick-frozen with liquid nitrogen and stored in a −80 ℃ refrigerator for subsequent experiments.

Determination of PRO, MDA content, SOD enzyme activity and DAB staining

0.1 g of fresh leaves were taken to determine the content or enzyme activity of each substance in the plants using Proline (PRO) Content Assay Kit (Nanjing Jiancheng Institute of Biological Engineering, A107-1-1), Malondialdehyde (MDA) Assay Kit (Nanjing Jiancheng Institute of Biological Engineering, A003-3-1) and Superoxide Dismutase (SOD) Activity Assay Kit (Beijing Solarbio Science & Technology Co., Ltd., BC0170), respectively. Three biological replicates were available for each sample. The DAB staining method called diaminobenzidine method was used to detect the active site of peroxidase in cells [35]. Three leaves each of pYL156 and pYL156: *GhCKX6b-Dt* were taken after NaCl stress and placed in DAB solution, darkened for 12 h, and observed after decolorization with 95% ethanol. The dark brown polymerization products represent the reaction of DAB with hydrogen peroxide.

Gene interaction network of the GhCKX6b-Dt protein

GhCKX6b-Dt protein interaction network was analyzed by STRING database (https://string-db.org/) [36]. The *Arabidopsis thaliana* homolog of GhCKX6b-Dt was used to predict the interactions of GhCKX6b-Dt with other genes in cotton.

Results

Identification of CKX proteins

To investigate the precise information and potential functions of cytokinin dehydrogenase (CKX) in cotton, the putative CKXs in the cotton genome was subjected to systematic genome-wide characterization. For this purpose, Hidden Markov model (HMM) profiles were generated based on the reported protein sequences of *Brassica napus* [37], *Vitis vinifera* [30], and *Medicago truncatula* CKX proteins [38], respectively. Two Hidden Markov Model (HMM) profiles, FAD-binding domain (PF01565) and Cytokinin-binding domain (PF09265), were used for protein screening in the HMMER, and further analysis using the Pfam database identified 27, 28, 14, and 14 CKX members in *G. hirsutum*, *G. barbadense*, *G. arboreum*, and *G. raimondii*, respectively. Then renamed the genes according to their *Arabidopsis* homologs (Additional file 1: Table S2) [25]. A phylogenetic tree of four *Gossypium* species and *Arabidopsis* was constructed based on the full-length amino acid sequences of 83 CKXs proteins in cotton and 7 AtCKXs proteins using the NJ method (Fig. 1A). As shown in Fig. 1B, the CKX family in cotton was clearly divided into 5 subgroups (labeled as groups I, II, III, IV and V) based on the branch in

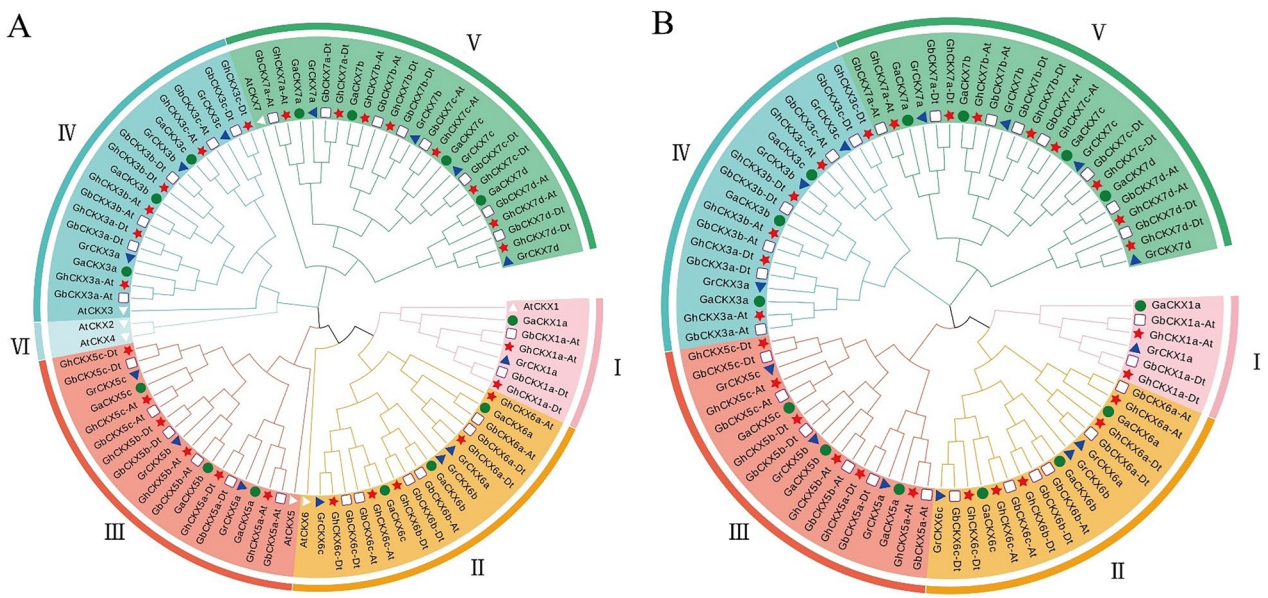

Fig. 1 Phylogenetic analysis of CKX proteins. **A** Phylogenetic relationship of the 83 identified CKXs from four *Gossypium* species and 7 AtCKXs in *Arabidopsis* using MEGA 5.0 by the Neighbor-Joining (NJ) method, the tree showed 6 major phylogenetic subgroups, designated as I to VI. **B** Phylogenetic relationship of the 83 identified CKXs from four *Gossypium* species using MEGA 5.0 by the Neighbor-Joining (NJ) method, the tree showed 5 major phylogenetic subgroups, designated as I to V. The suffix "At" and "Dt" indicates the origin of genes from the cotton A subgenome or D subgenome, respectively

which *Arabidopsis thaliana* was located. The CKX proteins of the four *Gossypium* species were spread across subgroups, containing proteins from both diploid and allotetraploid *Gossypium* species in each branch, and almost twice as many CKX proteins in tetraploid cotton as in diploid cotton.

Chromosomal locations of *CKXs* from four *Gossypium* species

To investigate the chromosomal distribution of *CKXs*, based on the Cotton Genome Database, the predicted *CKXs* were located to the physical locations of genes on the cotton chromosome. To study the chromosome distribution of *CKX* members, the predicted *CKXs* were mapped into the physical locations of genes on chromosomes according to the Cotton Genome Database. As shown in Fig. 2, 81 of the 83 *CKXs* were unequally distributed on their respective specific chromosomes, but 2 *CKXs*, *GhCKX5a-At* and *GhCKX3c-Dt*, were not annotated and not located on any of the chromosomes. In terms of chromosome distribution, there were 1–3 genes on each chromosome. In *G. hirsutum*, there were 13 genes in subgenome A and 14 genes in subgenome D. Chromosomes A02, A03, A4, A11, A12, D02, D03, D07, D11, and D12 did not have *GhCKXs*. In *G. barbadense*, there were 14 genes each in subgenome A and subgenome D. Chromosomes A02, A03, A4, A11, A12, D02, D03, D11, and D12 did not contain *GbCKXs*. At different from *G. hirsutum*, *GbCKX6b-At* was located on the lower part of chromosome D05 in *G. barbadense*; *GbCKX5a-At* was located on chromosome GbA06 instead of the scaffold; and *GbCKX3c-Dt* appeared on chromosome GbD07 instead of the scaffold. In *G. arboreum*, there were 14 genes irregularly arranged on their respective chromosomes, and chromosomes Chr02, Chr03, Chr11 and Chr12 had no *GaCKXs* distribution. In *G. raimondii*, there were 14 genes unevenly distributed on their respective chromosomes and no *GrCKXs* distribution on chromosomes Chr03, Chr05, Chr07 and Chr08. In the four *Gossypium* species, slight differences in the number and distribution of chromosomes were noted, so it was hypothesized that this could be due to the duplication or loss of *CKX* members during evolution.

Analysis of *GhCKXs* promoter and expression pattern

An increasing body of evidence demonstrates that *cis*-acting elements in gene promoters can impact gene expression and function [39]. To further analyze the transcriptional regulation and potential functions of *GhCKXs*, their evolutionary tree, promoter analysis and expression heat map were correlated, and potential stress-responsive *cis*-regulatory elements were identified using *GhCKXs* protein sequences, promoters in 2kb sequences upstream of the transcription start site and *GhCKXs* expression in the RNA-Seq database under different stresses (cold, heat, salt and PEG). As shown in Fig. 3, several light-responsive components, hormone-responsive components, and components related to abiotic stresses were identified, such as light responsiveness, auxin responsiveness, gibberellin-responsiveness, salicylic acid responsiveness, MeJA-responsiveness, abscisic acid responsiveness, wound-responsiveness, defense and stress responsiveness, meristem expression, drought-inducibility and low-temperature responsiveness and so on. The most light responsive elements were detected, with each *GhCKX* member containing multiple light responsive elements; secondly 18 *GhCKXs* contained abscisic acid responsive elements; 17 *GhCKXs* contained gibberellin-responsive element; 16 *GhCKXs* contained MeJA-responsive element; 14 *GhCKXs* contained auxin responsive element; 12 *GhCKXs* contained salicylic acid responsive element; 11 *GhCKXs* contained defense and stress responsive element and meristem expression element; 9 *GhCKXs* contained drought-inducibility element; 7 *GhCKXs* contained low-temperature responsive element; and *GhCKXs* contained wound-responsive element; 7 *GhCKXs* contained low-temperature responsive element; and only 2 *GhCKXs* contained wound-responsive element. This leads to the conclusion that *GhCKXs* were strongly associated with regulatory hormones and abiotic stresses.

Meanwhile, the response of *GhCKXs* to various abiotic stresses was thus verified by analyzing the heat map of *GhCKXs* expression under different stresses (cold, heat, salt and PEG) in the RNA-Seq database. In addition, the expression levels of eight cotton tissues (root, stem, leaf, torus, petal, stamen, pistil and calycle) were analyzed using RNA-Seq data (Fig. 4). The results showed that *GhCKXs* were expressed in various tissues

(See figure on next page.)

Fig. 2 Chromosome localization of *CKXs* from four *Gossypium* species. **A** Chromosomal location of *CKXs* on chromosomes in *G. hirsutum* A subgenome (GhAt). **B** Chromosomal location of *CKXs* on chromosomes in *G. hirsutum* D subgenome (GhDt). **C** Chromosomal location of *CKXs* on chromosomes in *G. barbadense* A subgenome (GbAt). **D** Chromosomal location of *CKXs* on chromosomes in *G. barbadense* D subgenome (GbDt). **E** Chromosomal location of *CKXs* on chromosomes in *G. arboreum* (Ga). **F** Chromosomal location of *CKXs* on chromosomes in *G. raimondii* (Gr). The scale of the genome size was given on the left

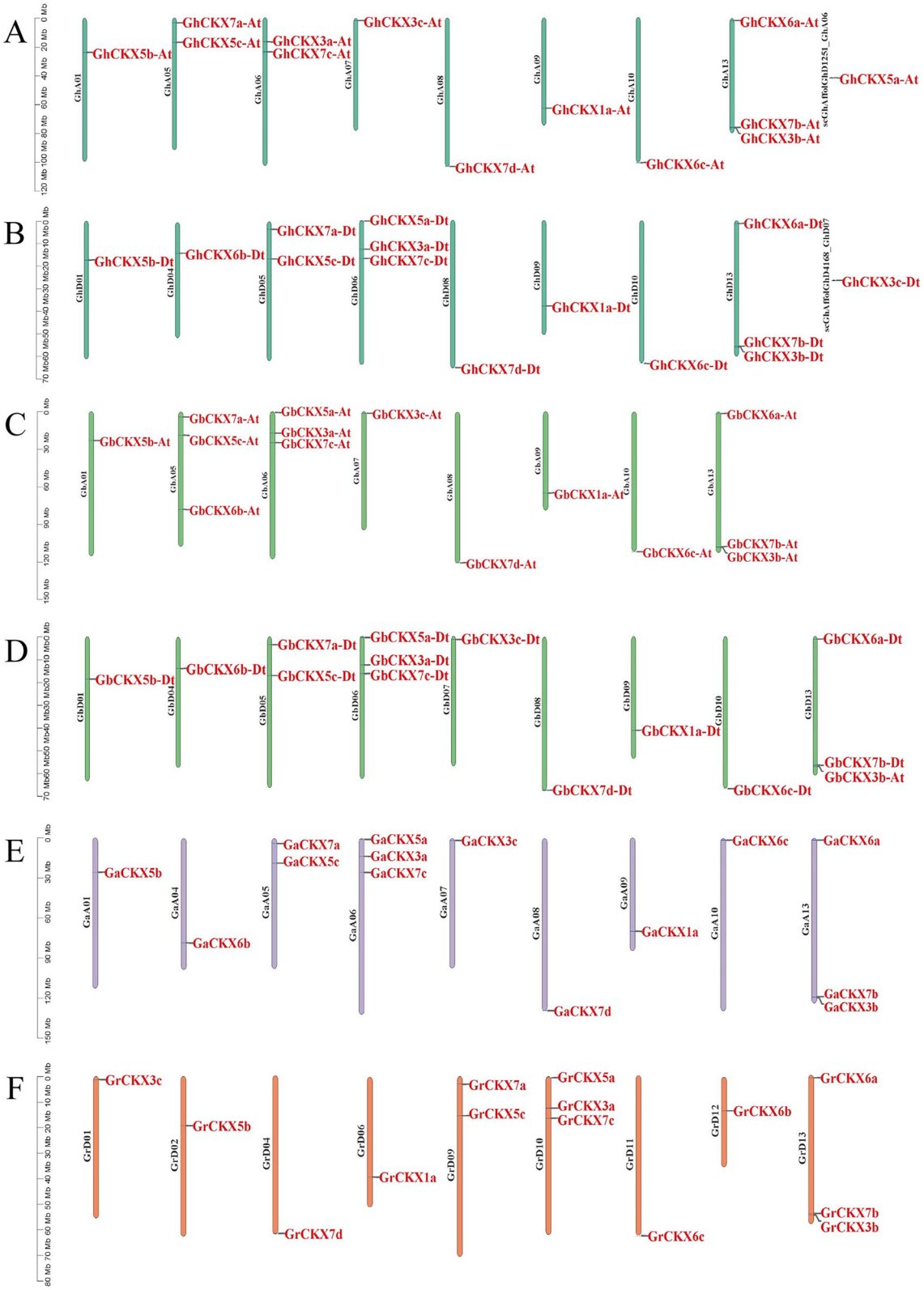

Fig. 2 (See legend on previous page.)

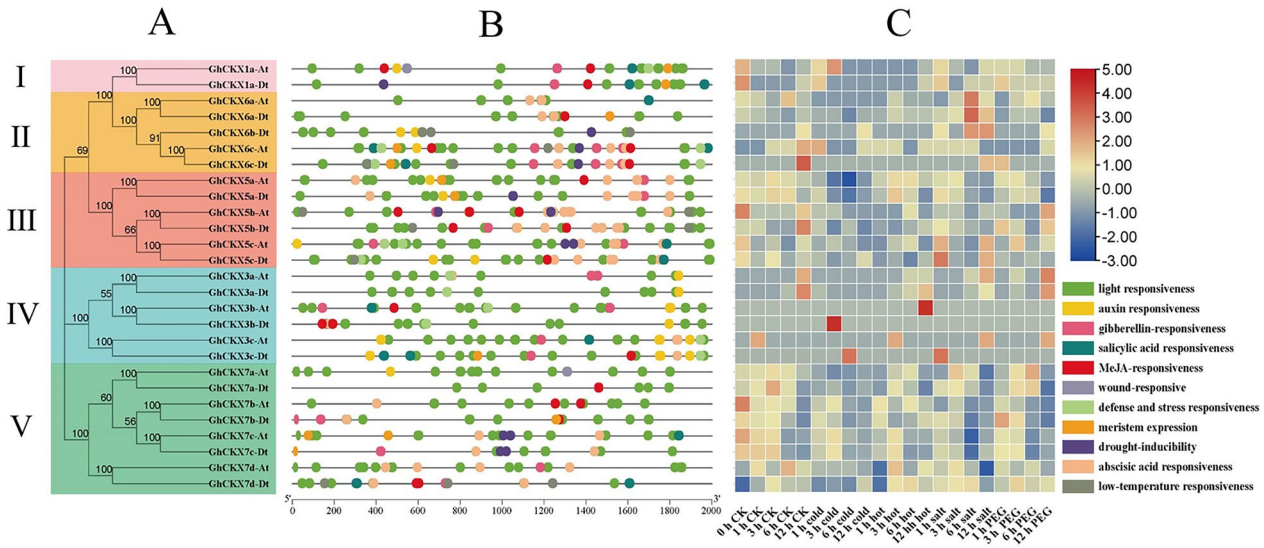

Fig. 3 Expression patterns and promoter analysis of the *GhCKXs*. **A** Phylogenetic relationship of *GhCKXs*, the 5 major phylogenetic subgroups, designated as I to V. **B** Cis-acting elements in promoters of *GhCKXs*. **C** Heatmap of the expression of *GhCKXs* under different abiotic stresses at different times of stress (cold, heat, salt and PEG)

with slightly different expression patterns. *GhCKX6a-At*, *GhCKX6a-Dt*, *GhCKX5c-At* and *GhCKX5c-Dt* were highly expressed in root; *GhCKX1a-Dt*, *GhCKX6b-Dt*, *GhCKX6c-At* and *GhCKX6c-At* were highly expressed in leaves. *GhCKX5b-Dt* was the most highly expressed in the torus; *GhCKX7b-At*, *GhCKX7b-Dt*, *GhCKX3b-At* and *GhCKX3b-Dt* were highly expressed in the petal; *GhCKX3a-At* and *GhCKX3c-Dt* were highly expressed in the stamen; in the calycle, *GhCKX5a-At*, *GhCKX5a-Dt* and *GhCKX3c-At* were the most highly expressed, and *GhCKX5b-At* was almost not expressed. The expression of *GhCKXs* were lower in both stem and pistil. As with *Brassica oleracea* L., the diversity of expression patterns showed that *CKXs* have a widespread biological function in the growth and development of cotton [40].

Gene ontology (GO) annotation analysis of *GhCKXs*

GO enrichment consists of Molecular Function, Biological Process, and Cellular Component. GO enrichment of *GhCKXs* by Cotton FGD showed that *GhCKXs* embody the properties of genes in terms of both molecular functions and biological processes (Fig. 5). *GhCKXs* are the most involved in molecular functions, including catalytic activity (GO:0003824), oxidoreductase activity (GO:0016491), oxidoreductase activity, acting on CH-OH group of donors (GO:0016614), cytokinin dehydrogenase activity (GO:0019139), flavin adenine dinucleotide binding (GO:0050660) and UDP-*N*-acetylmuramate dehydrogenase activity (GO:0008762). *GhCKXs* also participates in cytokinin metabolic process (GO:0009690) and obsolete oxidation–reduction process (GO:0055114).

Expression of *GhCKXs vis-à-vis* salt stress at different durations

To verify the potential role of *GhCKXs* in response to salt stress, cotton seedlings were treated with 100 mmol/L NaCl solution for different time of stress. And the expression levels of *GhCKXs* in leaves at NaCl solution for different durations were examined by qRT-PCR (Fig. 6). It was found that except for *GhCKX3a-Dt*, all 26 *GhCKXs* responded to salt stress in leaves of seedlings subjected to different levels and the tendency of expression changes. With the change in duration of stress, the expression levels of two genes in most A\D group showed the same trend. At 6 h of 100 mmol/L NaCl stress, 19 *CKXs* were shown to be significantly different in expression from 0 h. At 12 h of 100 mmol/L NaCl stress, 20 *CKXs* were shown to be significantly different in expression from 0 h. At 24 h of 100 mmol/L NaCl stress, 24 *CKXs* were shown to be significantly different in expression from 0 h. The differences in the expression of 13 *CKXs* (*GhCKX1a-At*, *GhCKX1a-Dt*, *GhCKX3a-At*, *GhCKX3b-At*, *GhCKX3c-At*, *GhCKX5b-Dt*, *GhCKX5c-Dt*, *GhCKX6a-Dt*, *GhCKX6b-Dt*, *GhCKX6c-At*, *GhCKX7a-At*, *GhCKX7a-Dt*, and *GhCKX7b-At*) were significant at 6 h, 12 h, and 24 h compared with 0 h, and most of them were extremely significant. Consequently, it is speculated that CKX genes are participated in the regulation of salt stress.

Effect of silencing *GhCKX6b-Dt* on NaCl stress in cotton

To verify whether the *CKX* genes responded to salt stress, the gene *GhCKX6b-Dt*, whose expression level of gene was significantly up-regulated at 6 h, 12 h and 24 h after

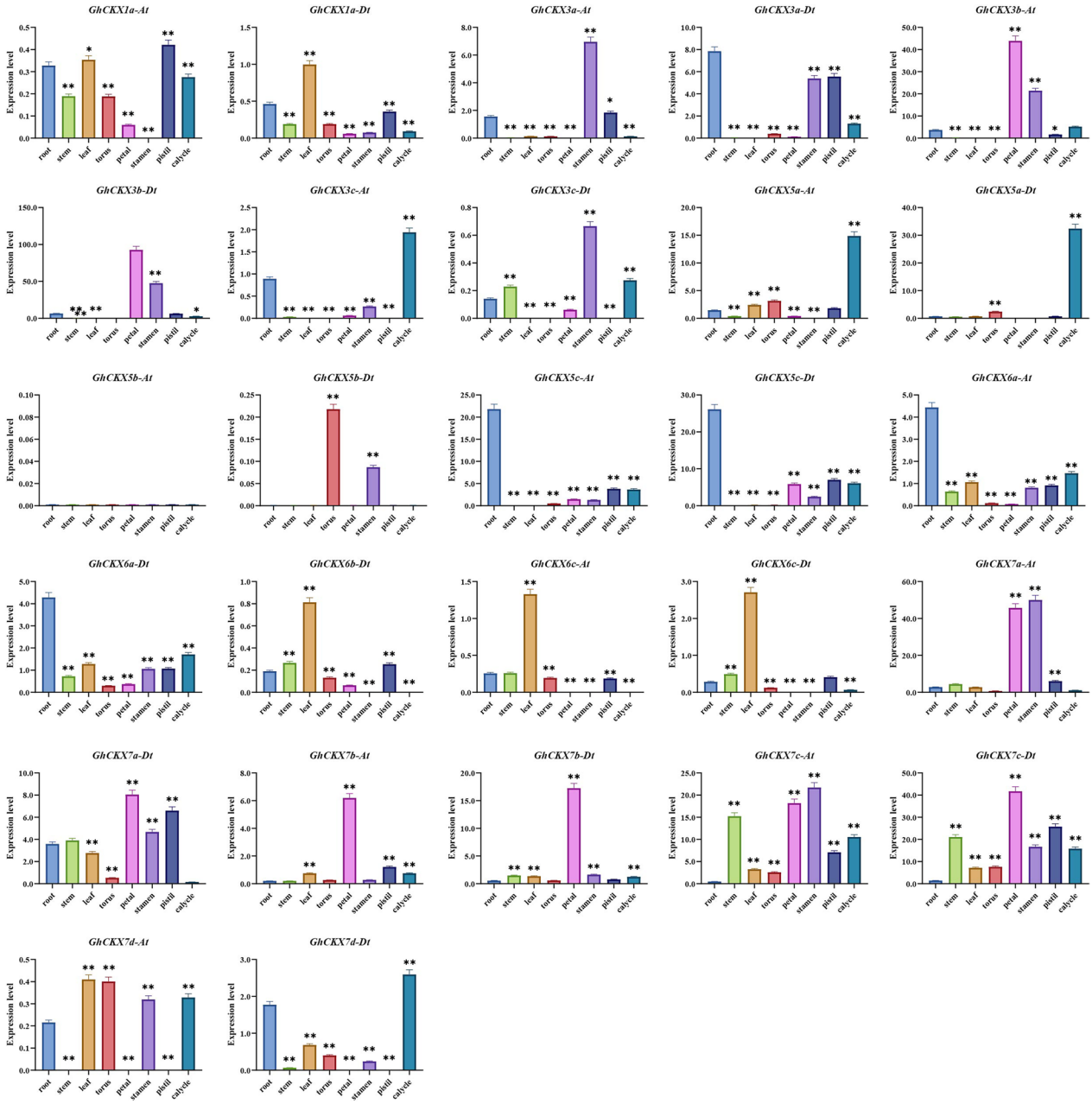

Fig. 4 Expression of *GhCKXs* in different tissues. Different tissues were represented as columns of different colors (root, stem, leaf, torus, petal, stamen, pistil and calycle), significance analysis of different tissues compared to root (*: $0.01 < p < 0.05$; **: $p < 0.01$)

treatment with 100 mmol/L NaCl solution, was silenced. As shown (Fig. 7A), pYL156: PDS plants showed albinism, indicating successful gene silencing. After treatment with 100 mmol/L NaCl, cotton leaves lost their luster and wilted after *GhCKX6b-Dt* silencing compared with pYL156. The expression of *GhCKX6b-Dt* in cotton leaves was detected by qRT-PCR. And the results showed (Fig. 7B) that the expression of *GhCKX6b-Dt* was significantly decreased in the latter compared with pYL156 and pYL156: *GhCKX6b-Dt*, which illustrated the good effect of *GhCKX6b-Dt* silencing. After NaCl stress, leaves of *GhCKX6b-Dt* silenced plants showed dark brown spots after DAB staining, showing that *GhCKX6b-Dt* was more severely injured after silencing (Fig. 7C). Consistent with the above results (Fig. 7D, E), both PRO content and MDA content were highly significantly elevated in *GhCKX6b-Dt* silenced plants after stress compared to pYL156 plants. In contrast, the SOD activity of silenced

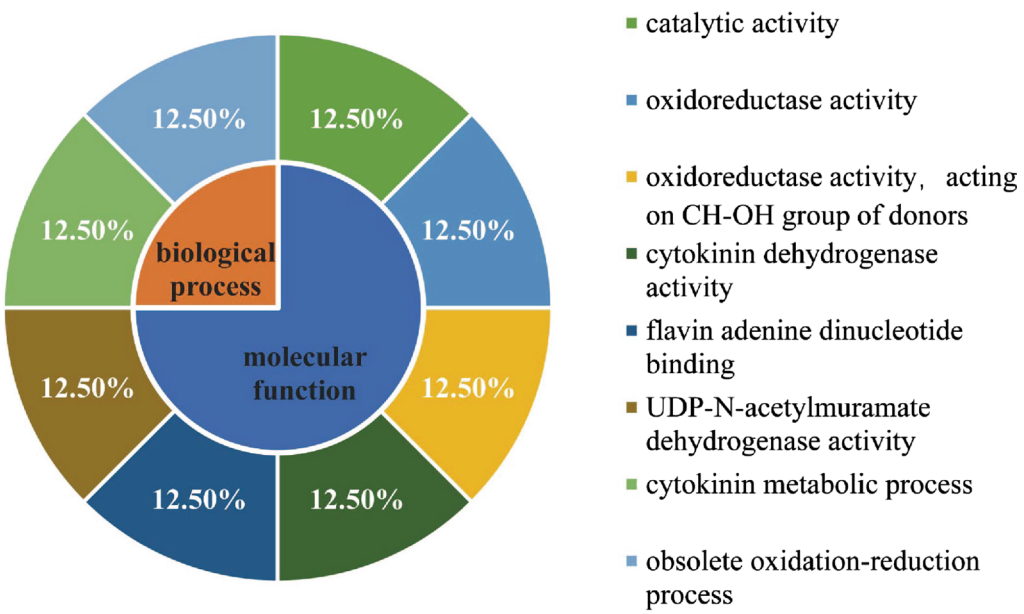

Fig. 5 Gene ontology (GO) analysis of *GhCKXs*

plants after stress was significantly declined compared to pYL156 (Fig. 7F), illustrating the decline in antioxidant capacity after *GhCKX6b-Dt* silencing.

Interaction network of GhCKX6b-Dt protein

Based on the homologous gene *AtCKX6*, which has the highest homology with GhCKX6b-Dt in *Arabidopsis*, the STRING database was used to construct an interaction network of CKX protein functions. The results showed that both *AtCKX6* and Polyprenyltransferase 1 (PPT1), Glycerol-3-phosphate dehydrogenase SDP6 (SDP6), RNA dimethylallyltransferase 2 (IPT2), Adenylate dimethylallyltransferase (cytokinin synthase) (IPT1), CHASE domain containing histidine kinase protein(WOL), Peroxisomal (*S*)-2-hydroxy-acid oxidase GLO4 (HAOX1), Peroxisomal (*S*)-2-hydroxy-acid oxidase GLO3(HAOX2), Aldolase-type TIM barrel family protein (GOX2), Aldolase-type TIM barrel family protein (GOX1) and Peroxisomal (*S*)-2-hydroxy-acid oxidase GLO5 (GOX3) interacted with each other (Fig. 8A).

After analysis, the synthesis enzymes IPT associated with CK and the signaling molecule WOL were noticed. Therefore, the expression of each gene was determined by qRT-PCR in cotton seedlings after *GhCKX6b-Dt* silencing. As shown in Fig. 8B, the results demonstrated that there was no significant difference in the expression of each gene in pYL156 and pYL156:*GhCKX6b-Dt* plants before stress. However, the differences in the expression of *IPT1*, *IPT2* and *WOL* were significant after NaCl treatment, and the expression of *IPT1*, *IPT2* and *WOL* were significantly increased in *GhCKX6b-Dt* silenced plants compared with pYL156 control plants.

Discussion

Cytokinin is an essential plant hormone that regulates various developmental and physiological processes [41, 42]. CKX, a key regulatory enzyme for the irreversible degradation of the plant hormone CK, is indispensable for maintaining CK homeostasis [43]. Although the biological functions of the *CKXs* have been identified in a variety of plants [27, 44, 45] and were particularly prominent in adversity stresses [46–48], functional studies of *CKX* in cotton are still limited. In this study, 27 *GhCKXs*, 28 *GbCKXs* and 14 each of *GaCKXs* and *GrCKXs* were identified. And chromosomal locations, *cis*-acting elements and expression patterns under different abiotic stresses were analyzed, then the *CKX*s functions were characterized by VIGS technique. A review of the literatures revealed that the genomes of other species closely related to cotton contain much smaller numbers of CKX members, for example, 7 *CKX*s in the *Arabidopsis* [49]; 11 *CKX*s in the rice [50]; 12 *CKXs* in the *Sorghum bicolor*; 11 *CKX*s in the *Setaria italica*; and 15 *CKX*s in the maize [51]. The *CKX* members has more genes in polyploid plants compared to haploids, *CKX* members contain 20 genes in *Eleusine coracana* [52]; 16 *CKXs* in *Glycine max* [53]; and more *CKX*s in *Triticum* (31) [54].

The main reason for family gene amplification is gene duplication which can diversify gene functions to facilitate rapid adaptation of organisms to different environments [55, 56]. The main source of gene duplication in

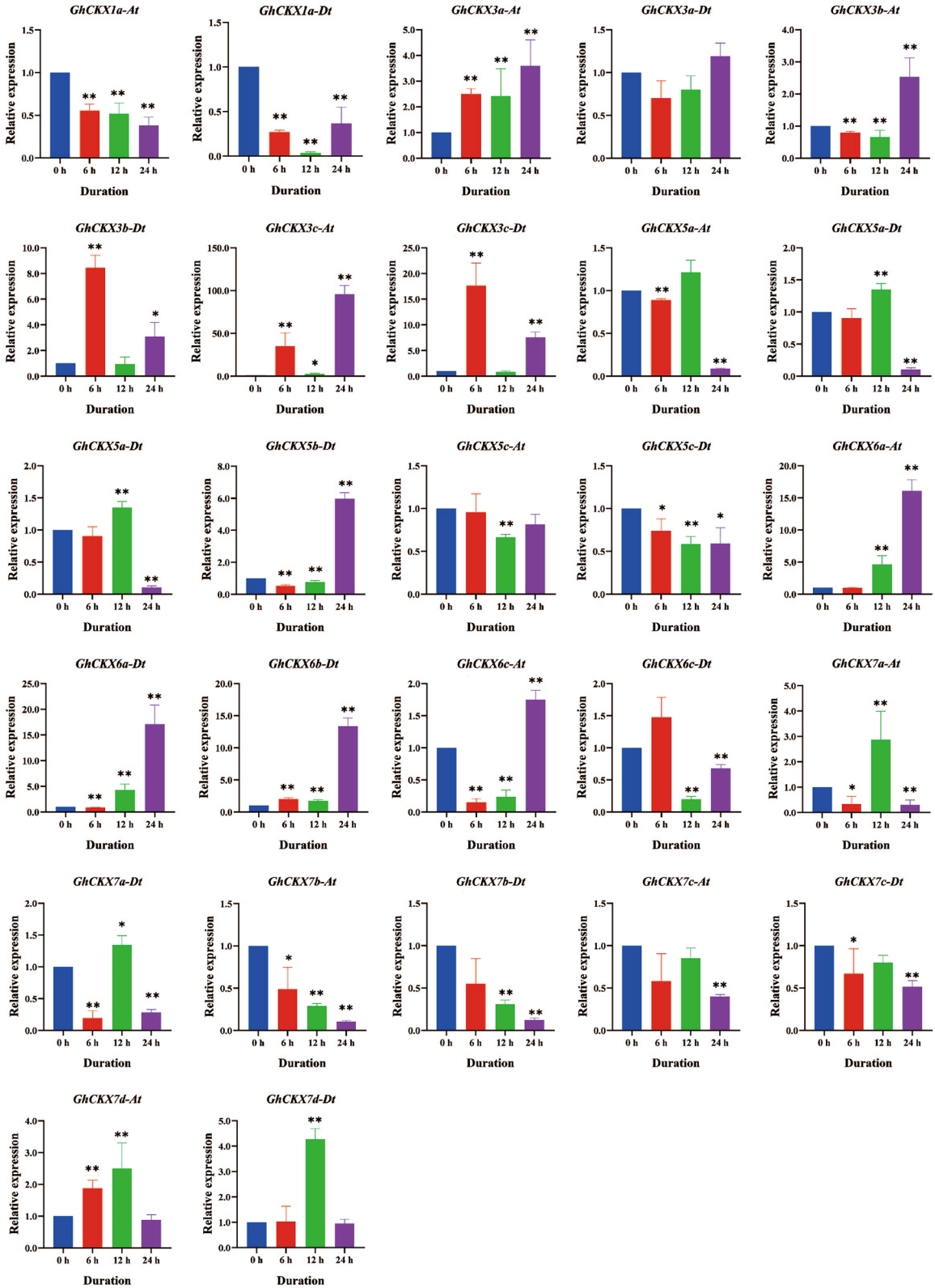

Fig. 6 Expression of *GhCKXs* at different durations of salt stress in leaves using qRT-PCR. Column indicate the relative expression levels of *GhCKXs* in leaves under 100 mmol/L NaCl stress for 0 h, 6 h, 12 h, and 24 h (*: $0.01 < p < 0.05$; **: $p < 0.01$). The mean values were from three independent biological replicates

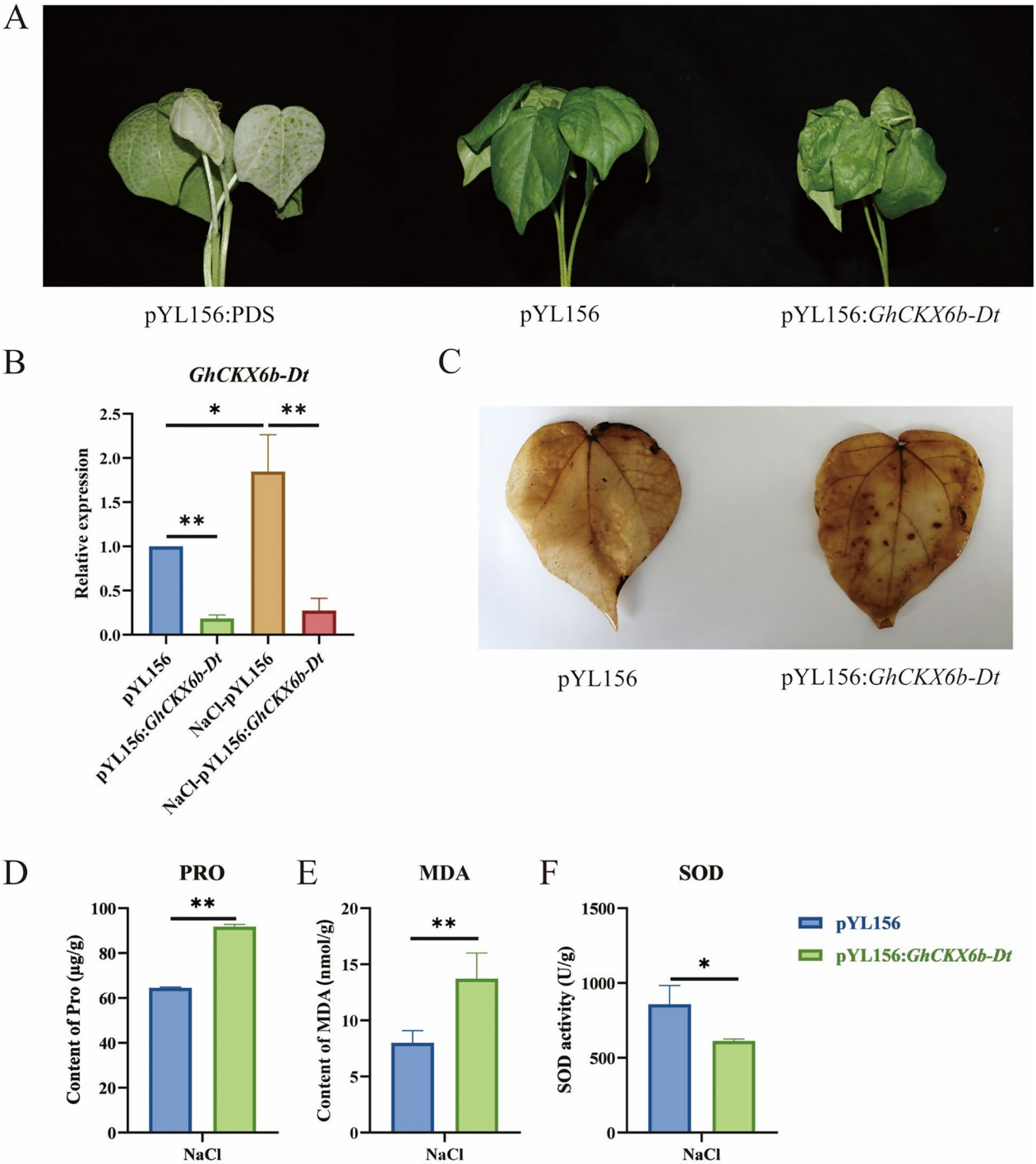

Fig. 7 Effect of silencing *GhCKX6b-Dt* on NaCl stress in cotton. **A** The phenotype of cotton after *GhCKX6b-Dt* silencing under NaCl stress. pYL156: PDS as a positive control, pYL156 was an empty vector as control, and pYL156: *GhCKX6b-Dt* was *GhCKX6b-Dt* silenced lines. **B** The relative expression level of *GhCKX6b-Dt* under NaCl stress. **C** DAB staining. **D** PRO content of empty control and VIGS plants under NaCl stress. **E** MDA content of empty control and VIGS plants under NaCl stress. **F** SOD activity of empty control and VIGS plants under NaCl stress. *$0.01 < p < 0.05$, **$p < 0.01$

eukaryotic genomes is interchromosomal duplication [57]. During the evolution of diploids to tetraploids, it can be noticed that the number of *CKX* genes in each branch of tetraploid cotton was almost twice as much as that of diploid cotton from the four *Gossypium* species evolutionary tree (Fig. 1B). However, it can be found

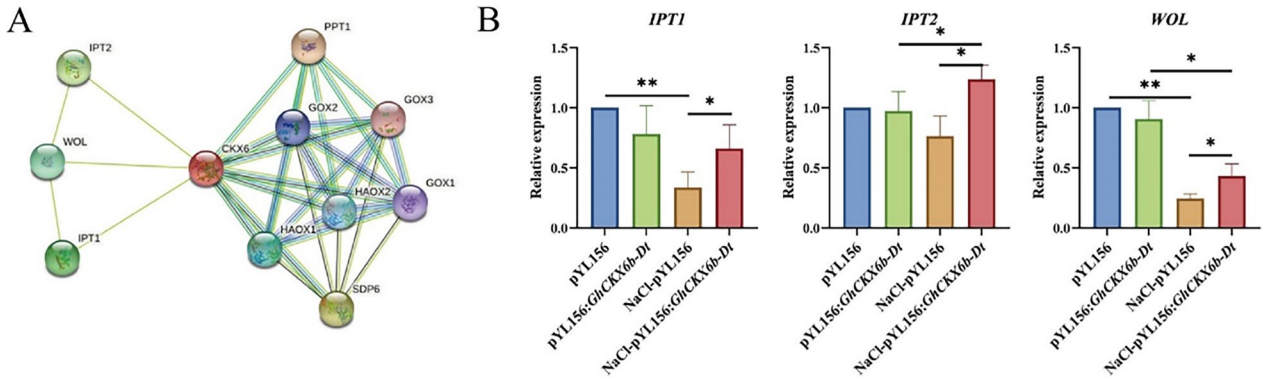

Fig. 8 Interaction network of *GhCKX6b-Dt* protein and expression of related genes after *GhCKX6b-Dt* silencing. **A** The CKX6 represented the protein *AtCKX6* corresponding to the protein in *Arabidopsis* with the highest homology to GhCKX6b-Dt. **B** Relative expression levels of *IPT1*, *IPT2* and *WOL* in *GhCKX6b-Dt* silenced plants before and after NaCl stress. pYL156: PDS as a positive control, pYL156 was an empty vector as control, and pYL156: *GhCKX6b-Dt* was *GhCKX6b-Dt* silenced lines. *$0.01 < p < 0.05$, **$p < 0.01$

that one gene of *GhCKXs* was absent in branch II, which may be due to the loss of genes during evolution. Meanwhile, as seen from the chromosome position (Fig. 2), chromosome GaA04 evolved to tetraploid Gb translocated to chromosome A05, but no gene was found on chromosome GhA05, indicating that the same translocation did not occur in *G. hirsutum*, so it was speculated that the loss of the gene may have occurred here. In addition, the presence of a gene on chromosome GbD07 and the absence of a gene on chromosome GhD07 suggests that the gene has been altered during the evolution of the gene. Upon scrutiny, it was found that two genes appeared in *G. hirsutum* that were not annotated on any chromosome, which it was possible that A06 and D07 chromosomes were the result of translocation, and there were multiple genes in the GrD group of chromosomes that did not correspond to Gh and Gb. These may be due to the translocation of *CKXs* through fragmentary or whole genome replication events and did not replicate in tandem during evolution of four *Gossypium* species, which in turn diversified the *CKXs*, which was consistent with the results of the *Brassica oleracea* L. [40].

The results of *cis*-acting elements in the promoter indicate (Fig. 3B) that *GhCKXs* are involved in light response [58], hormone response and regulation of abiotic stress and also play an active role in plant growth and development. Multiple plant hormone *cis*-acting elements were predicted in the promoter region of *GhCKXs*, such as auxin responsiveness element, gibberellin-responsiveness element, salicylic acid responsiveness element, MeJA-responsiveness element and abscisic acid responsiveness element. It was demonstrated in a number of studies that CK interacted with multiple hormones through the *CKXs*, thereby regulating plant growth and development [59, 60]. *OsCKX4* integrated cytokinin and auxin signaling to control crown root formation in rice [44]. In addition to the promoter region of *GhCKXs* containing various phytohormone *cis*-acting elements, there were also various stress-related *cis*-acting elements such as wound-responsiveness element, defense and stress responsiveness element, drought-inducibility element and low-temperature responsiveness, which indicated that *GhCKXs* can respond to various abiotic stresses, and this is in accordance with previous reports. Reduced expression of *OsCKX2*, a cytokinin oxidase specific to inflorescence meristem tissue in rice, enhanced tolerance to salt stress [61]; overexpression of *CKX1* in tobacco and barley improved drought tolerance and heat tolerance in plants [46, 62]. Nishiyama et al. showed that CK-deficient *CKX* overexpression plants (*35S:CKX1-35S:CKX4*) became more tolerant to salt and drought in *Arabidopsis* compared with WT plants [23]. Heat map analysis in this study revealed the same results (Fig. 3C), that *CKX* genes play an important role in response to abiotic stresses (cold, heat, salt and PEG). In addition, we found that a few *GhCKXs* contained meristem expression elements, and most *GhCKXs* were expressed in various tissues (Fig. 4), indicating that *GhCKXs* play a role in plant growth and development [63].

The salt tolerance mechanism is extremely complex involving ion transport, osmoregulation and oxidative stress, each of which is in turn regulated by multiple components [64–66]. As we all know, cytokines can regulate salt tolerance in plants [67, 68], with a positive or negative effect [68, 69]. Here, it was found that most of the *GhCKXs* were significantly distinct under NaCl stress during different time, indicating that *GhCKXs* responded to salt stress. One *CKX* gene, *GhCKX6b-Dt*, was found to respond to salt stress as revealed from RNA-Seq data,

and its expression gradually increased with time and the difference was highly significant, indicating that this gene responded positively to salt stress, but negatively regulated salt stress. This's why *GhCKX6b-Dt* was selected for performing virus induced gene silencing experiments. Abiotic stresses contribute to the excessive production of ROS in plant cells, leading to oxidative damage to biomolecules [70]. Damaged biomolecules include products of protein oxidation, enzyme inactivation, lipid peroxidation, increased membrane fluidity, chlorophyll degradation, nucleic acid damage, and apoptotic pathways, and these damages can affect plant growth and development [70, 71]. In this study, compared with control plants, *GhCKX6b-Dt* silenced plants wilted after stress, in which both PRO and MDA contents were highly significantly increased (Fig. 7D, E), demonstrating that *GhCKX6b-Dt* silenced plants produced excessive ROS to expose the plants to severe salt stress, and vice versa, indicating that *GhCKX6b-Dt* positively regulates salt stress and CK negatively regulates salt stress, which is consistent with the results of previous studies [72]. SOD is the most effective scavenger of ROS and is the first line of defense against ROS-induced damage under abiotic stresses [73]. Compared with control plants, the SOD activity of *GhCKX6b-Dt* silenced plants was significantly decreased after stress (Fig. 7F), presumably GhCKX6b-Dt-silenced plants failed to produce an appropriate amount of SOD to scavenge reactive oxygen species, resulting in wilting of the plants and reduced salt tolerance.

After GO analysis, the biological processes involved in the *GhCKXs* were revealed to be CK metabolic process and obsolete oxidation–reduction process. And the molecular functions were catalytic oxidoreductase activity (oxidoreductase activity and cytokinin dehydrogenase activity) and UDP-*N*-acetylmuramate dehydrogenase activity and flavin adenine dinucleotide binding, which illustrate the involvement of *GhCKXs* in the regulation of redox dynamics under stress. Protein interactions predicted by homologs of GhCKX6b-Dt in *Arabidopsis* thaliana revealed that this gene is strongly associated with CK synthase (IPT1 and IPT2), CK receptor (WOL) and redox reaction-related enzymes (GOX1, GOX2, GOX3, HOX1 and HOX2). Therefore, it was hypothesized that *CKXs* regulate the CK content in cotton by interacting with IPT and WOL. Seedlings after *GhCKX6b-Dt* silencing wilted more heavily than the negative control. It was hypothesized that *GhCKX6b-Dt* might play an important role in response to NaCl stress (Fig. 9). When *GhCKX6b-Dt* silenced plants were subjected to salt stress resulting in cellular damage, excessive ROS were produced,

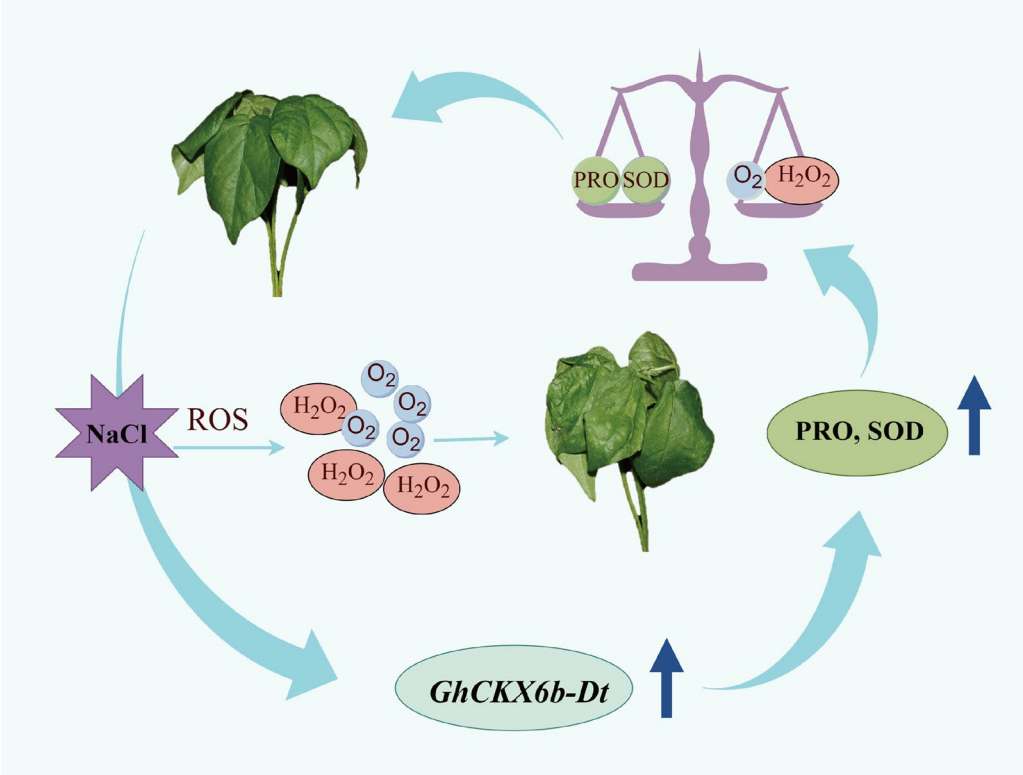

Fig. 9 Mechanism model of *GhCKX6b-Dt* responding to NaCl stress in cotton. The figure was drawn using the software Figdraw

causing an imbalance of redox reactions in the plant, and the plants showed wilting. It has been shown in the literature that the ion stress and osmotic stress caused by salt pollution can be alleviated by osmotic regulating substances such as proline [74, 75]. Salt stress can affect CK content, and genes related to biosynthesis and signal transduction were significantly up-regulated or down-regulated, including *CKXs* and *IPTs* [76]. The results show that *GhCKX6b-Dt* regulates CK and thus regulates the content of proline, which enables antioxidant enzymes to clear ROS and relieve the damage of plant cells caused by salt stress [21], resisting salt stress and normalizing plant growth.

Conclusion

In this study, the *CKXs* were characterized based on the results of phylogenetic relationships, gene chromosome localization and *cis*-acting element analysis. In addition, the expression patterns of *GhCKX*s were investigated under different abiotic stresses, while using VIGS technology to understand the response of *GhCKXs* to salt stress as a function. *GhCKX6b-Dt* silenced plants were found to have increased PRO and MDA contents, decreased SOD activity, and reduced ROS scavenging capacity after stress, and thus were severely injured. Combining GO analysis and the gene interaction network of *GhCKX* proteins, it is hypothesized that *GhCKX6b-Dt* alleviates salt stress by scavenging reactive oxygen species through the antioxidant system. The results revealed that *GhCKX6b-Dt* positively regulates salt stress, while CK negatively regulates salt stress. The results of this study provide a basis for further studies on the response of *CKXs* to regulate CK homeostasis and to abiotic stresses during plant development.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-023-00788-3.

Additional file 1. Renaming of CKXs and Gene-specific primers for qRT-PCR.

Additional file 2. Construction of VIGS experimental vector and cloning strategy.

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Author contributions
ML, YC, FP, SW and RC performed the experiments and analyzed data. XL, YZ, HH, YF, TJ, XF, YL, KN, MH, WC, YM, JW, XC, XL, DW, LG, and LZ analyzed data and provided critical feedback. ML, YC, FP, SW, RC, JJ and WY revised and edited the final version of the manuscript. All authors read and approved the final manuscript.

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All data supporting the conclusions of this article are provided in the article and its additional files. The sequences of the genomics can be found in CottonFGD (https://cottonfgd.org/).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors agreed to publish the paper.

Competing interests
The authors declare that they have no competing interests.

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References

1. Yang Y, Guo Y (2018) Unraveling salt stress signaling in plants. J Integr Plant Biol 60(9):796–804
2. Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651
3. Ouhibi C, Attia H, Rebah F, Msilini N, Chebbi M, Aarrouf J, Urban L, Lachaal M (2014) Salt stress mitigation by seed priming with UV-C in lettuce plants: growth, antioxidant activity and phenolic compounds. Plant Physiol Biochem 83:126–133
4. Chen Y, Han Y, Kong X, Kang H, Wang W (2017) Ectopic expression of wheat expansin gene *TaEXPA2* improved the salt tolerance of transgenic tobacco by regulating Na^+/K^+ and antioxidant competence. Physiol Plant 159(2):161–177
5. Meifang Li, Shangjing G, Ying Xu, Qingwei M, Gang Li (2014) Glycine betaine-mediated potentiation of HSP gene expression involves calcium signaling pathways in tobacco exposed to NaCl stress. Physiol Plant 150(1):63–75
6. Shi XP, Ren JJ, Yu Q, Zhou SM, Wang XL (2017) Overexpression of *SDH* confers tolerance to salt and osmotic stress, but decreases ABA sensitivity in *Arabidopsis*. Plant Biol (Stuttg) 20(2):327–337
7. Lu C, Chen MX, Liu R, Zhang L, Liu YG (2019) Abscisic acid regulates auxin distribution to mediatemaize lateral root development under salt stress. Front Plant Sci 10:716
8. Wei D, Zhang W, Wang C, Meng Q, Li G, Chen T, Yang X (2017) Genetic engineering of the biosynthesis of glycinebetaine leads to alleviate salt-induced potassium efflux and enhances salt tolerance in tomato plants. Plant Sci 257:74–83
9. Zhang QY, Wang LY, Kong FY, Deng YS, Li B, Meng QW (2012) Constitutive accumulation of zeaxanthin in tomato alleviates salt stress-induced photoinhibition and photooxidation. Physiol Plant 146(3):363–373
10. Roper SD, Chaudhari N (2017) Taste buds: cells, signals and synapses. Nat Rev Neurosci 18(8):485–497
11. Chang YN, Zhu C, Jiang J, Zhang H, Zhu JK, Duan CG (2020) Epigenetic regulation in plant abiotic stress responses. J Integr Plant Biol 62(5):563–580
12. Liu Y, Zhang M, Meng Z, Wang B, Chen M (2020) Research progress on the roles of cytokinin in plant response to stress. Int J Mol Sci 21(18):6574
13. Zhang H, Zhu J, Gong Z, Zhu JK (2022) Abiotic stress responses in plants. Nat Rev Genet 23(2):104–119
14. Kiba T, Takei K, Kojima M, Sakakibara H (2013) Side-chain modification of cytokinins controls shoot growth in *Arabidopsis*. Dev Cell 27(4):452–461

15. Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol 57:431–449
16. Mboene Noah A, Casanova-Sáez R, Makondy Ango RE, Antoniadi I, Karady M, Novák O, Niemenak N, Ljung K (2021) Dynamics of auxin and cytokinin metabolism during early root and hypocotyl growth in *Theobroma cacao*. Plants (Basel) 10(5):967
17. Ullah A, Manghwar H, Shaban M, Khan AH, Akbar A, Ali U, Ali E, Fahad S (2018) Phytohormones enhanced drought tolerance in plants: a coping strategy. Environ Sci Pollut Res 25(33):33103–33118
18. Li SM, Zheng HX, Zhang XS, Sui N (2020) Cytokinins as central regulators during plant growth and stress response. Plant Cell Rep 40(2):271–282
19. Schmülling T, Werner T, Riefler M, Krupková E, Manns IBY (2003) Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. J Plant Res 116(3):241–252
20. Kowalska M, Galuszka P, Frébortová J, Šebela M, Béres T, Hluska T, Šmehilová M, Bilyeu KD, Frébort I (2010) Vacuolar and cytosolic cytokinin dehydrogenases of *Arabidopsis thaliana*: heterologous expression, purification and properties. Phytochemistry 71(17–18):1970–1978
21. Werner T, Schmülling T (2009) Cytokinin action in plant development. Curr Opin Plant Biol 12(5):527–538
22. Paces VC, Werstiuk E, Hall RH (1971) Conversion of N^6-(Δ^2-isopentenyl) adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol 48(6):775–778
23. Nishiyama R, Watanabe Y, Fujita Y, Le D, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T et al (2011) Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. Plant Cell 23(6):2169–2183
24. Zhao J, Bai W, Zeng Q, Song S, Zhang M, Li X, Hou L, Xiao Y, Luo M, Li D (2015) Moderately enhancing cytokinin level by down-regulation of *GhCKX* expression in cotton concurrently increases fiber and seed yield. Mol Breed 35(2):60
25. Zeng J, Yan X, Bai W, Zhang M, Chen Y, Li X, Hou L, Zhao J, Ding X, Liu R (2022) Carpel-specific down-regulation of *GhCKXs* in cotton significantly enhances seed and fiber yield. J Exp Bot 73(19):6758–6772
26. Li S, An Y, Hailati S, Zhang J, Yang P (2019) Overexpression of the cytokinin oxidase/dehydrogenase (CKX) from *Medicago sativa* enhanced salt stress tolerance of *Arabidopsis*. J Plant Biol 62(5):374–386
27. Zhang W, Peng K, Cui F, Wang D, Zhao J, Zhang Y, Yu N, Wang Y, Zeng D, Wang Y (2021) Cytokinin oxidase/dehydrogenase *OsCKX11* coordinates source and sink relationship in rice by simultaneous regulation of leaf senescence and grain number. Plant Biotechnol J 19(2):335–350
28. Huang P, Zhao J, Hong J, Zhu B, Xia S, Zhu E, Han P, Zhang K (2023) Cytokinins regulate rice lamina joint development and leaf angle. Plant Physiol 191(1):56–69
29. Abdelraheem A, Esmaeili N, O'Connell M, Zhang J (2019) Progress and perspective on drought and salt stress tolerance in cotton. Ind Crops Prod 130:118–129
30. Yu K, Yu Y, Bian L, Ni P, Ji X, Guo D, Zhang G, Yang Y (2021) Genome-wide identification of cytokinin oxidases/dehydrogenase (*CKXs*) in grape and expression during berry set. Sci Hortic 280:109917
31. Wang X, Lu X, Malik WA, Chen X, Wang J, Wang D, Wang S, Chen C, Guo L, Ye W (2020) Differentially expressed bZIP transcription factors confer multi-tolerances in *Gossypium hirsutum* L. Int J Biol Macromol 146:569–578
32. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R (2020) TBtools: an integrative toolkit developed for interactive analyses of big biological data. Mol Plant 13(8):1194–1202
33. Hu Y, Chen J, Fang L, Zhang Z, Ma W, Niu Y, Ju L, Deng J, Zhao T, Lian J (2019) *Gossypium barbadense* and *Gossypium hirsutum* genomes provide insights into the origin and evolution of allotetraploid cotton. Nat Genet 51(4):739–748
34. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408
35. Ji J, Shi Z, Xie T, Zhang X, Chen W, Du C, Sun J, Yue J, Zhao X, Jiang Z et al (2020) Responses of GABA shunt coupled with carbon and nitrogen metabolism in poplar under NaCl and $CdCl_2$ stresses. Ecotoxicol Environ Saf 193:110322
36. Zhang Y, Rui C, Fan Y, Xu N, Zhang H, Wang J, Sun L, Dai M, Ni K, Chen X (2022) Identification of SNAT family genes suggests *GhSNAT3D* functional reponse to melatonin synthesis under salinity stress in cotton. Front Mol Biosci 9:843814
37. Liu P, Zhang C, Ma J-Q, Zhang L-Y, Yang B, Tang X-Y, Huang L, Zhou X-T, Lu K, Li J-N (2018) 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 9(3):168
38. Wang C, Wang H, Zhu H, Ji W, Hou Y, Meng Y, Wen J, Mysore KS, Li X, Lin H (2021) Genome-wide identification and characterization of cytokinin oxidase/dehydrogenase family genes in *Medicago truncatula*. J Plant Physiol 256:153308
39. Huang F, Shi C, Zhang Y, Hou X (2022) Genome-wide Identification and characterization of TCP family genes in pak-choi [*Brassica campestris* (syn. *Brassica rapa*) ssp. chinensis var. communis]. Front Plant Sci 13:854171
40. Zhu M, Wang Y, Lu S, Yang L, Zhuang M, Zhang Y, Lv H, Fang Z, Hou X (2022) Genome-wide identification and analysis of cytokinin dehydrogenase/oxidase (CKX) family genes in *Brassica oleracea* L. reveals their involvement in response to *Plasmodiophora brassicae* infections. Hortic Plant J 8(1):68–80
41. Ferreira FJ, Kieber JJ (2005) Cytokinin signaling. Curr Opin Plant Biol 8(5):518–525
42. Leibfried A, To JP, Busch W, Stehling S, Kehle A, Demar M, Kieber JJ, Lohmann JU (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature 438(7071):1172–1175
43. Avalbaev AM, Somov KA, Yuldashev RA, Shakirova FM (2012) Cytokinin oxidase is key enzyme of cytokinin degradation. Biochemistry 77(12):1354–1361
44. Gao S, Fang J, Xu F, Wang W, Sun X, Chu J, Cai B, Feng Y, Chu C (2014) *CYTOKININ OXIDASE/DEHYDROGENASE4* integrates cytokinin and auxin signaling to control rice crown root formation. Plant Physiol 165(3):1035–1046
45. Rong C, Liu Y, Chang Z, Liu Z, Ding Y, Ding C (2022) Cytokinin oxidase/dehydrogenase family genes exhibit functional divergence and overlap in rice growth and development, especially in control of tillering. J Exp Bot 73(11):3552–3568
46. Pospíšilová H, Jiskrová E, Vojta P, Mrízová K, Kokáš F, Čudejková MM, Bergougnoux V, Plíhal O, Klimešová J, Novák O et al (2016) Transgenic barley overexpressing a cytokinin dehydrogenase gene shows greater tolerance to drought stress. N Biotechnol 33(5 Pt B):692–705
47. Macková H, Hronková M, Dobrá J, Turečková V, Novák O, Lubovská Z, Motyka V, Haisel D, Hájek T, Prášil IT et al (2013) Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. J Exp Bot 64(10):2805–2815
48. Sharma A, Prakash S, Chattopadhyay D (2022) Killing two birds with a single stone-genetic manipulation of cytokinin oxidase/dehydrogenase (*CKX*) genes for enhancing crop productivity and amelioration of drought stress response. Front Genet 13:941595
49. Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) 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 15(11):2532–2550
50. Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q, Kitano H, Matsuoka M (2005) Cytokinin oxidase regulates rice grain production. Science 309(5735):741–745
51. Mameaux S, Cockram J, Thiel T, Steuernagel B, Stein N, Taudien S, Jack P, Werner P, Gray JC, Greenland AJ et al (2012) Molecular, phylogenetic and comparative genomic analysis of the cytokinin oxidase/dehydrogenase gene family in the *Poaceae*. Plant Biotechnol J 10(1):67–82
52. Blume R, Yemets A, Korkhovyi V, Radchuk V, Rakhmetov D, Blume Y (2022) Genome-wide identification and analysis of the cytokinin oxidase/dehydrogenase (*ckx*) gene family in finger millet (*Eleusine coracana*). Front Genet 13:963789
53. Liu LM, Zhang HQ, Cheng K, Zhang YM (2021) Integrated bioinformatics analyses of *PIN1*, *CKX*, and yield-related genes reveals the molecular mechanisms for the difference of seed number per pod between soybean and cowpea. Front Plant Sci 12:749902
54. Jain P, Singh A, Iquebal MA, Jaiswal S, Kumar S, Kumar D, Rai A (2022) Genome-wide analysis and evolutionary perspective of the cytokinin

dehydrogenase gene family in wheat (*Triticum aestivum* L). Front Genet 13:931659
55. Cannon SB, Mitra A, Baumgarten A, Young ND, May G (2004) The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. BMC Plant Biol 4(1):1–21
56. Vision TJ, Brown DG, Tanksley SD (2000) The origins of genomic duplications in *Arabidopsis*. Science 290(5499):2114–2117
57. Friedman R, Hughes AL (2001) Gene duplication and the structure of eukaryotic genomes. Genome Res 11(3):373–381
58. Schlüter T, Leide J, Conrad K (2011) Light promotes an increase of cytokinin oxidase/dehydrogenase activity during senescence of barley leaf segments. J Plant Physiol 168(7):694–698
59. Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T (2006) New insights into the biology of cytokinin degradation. Plant Biol (Stuttg) 8(03):371–381
60. Todorova D, Vaseva I, Malbeck J, Trávníčková A, Macháčková I, Karanov E (2007) Cytokinin oxidase/dehydrogenase activity as a tool in gibberellic acid/cytokinin cross talk. Biol Plant 51(3):579–583
61. Joshi R, Sahoo KK, Tripathi AK, Kumar R, Gupta BK, Pareek A, Singla-Pareek SL (2018) Knockdown of an inflorescence meristem-specific cytokinin oxidase–*OsCKX2* in rice reduces yield penalty under salinity stress condition. Plant Cell Environ 41(5):936–946
62. Lubovská Z, Dobrá J, Štorchová H, Wilhelmová N, Vanková R (2014) Cytokinin oxidase/dehydrogenase overexpression modifies antioxidant defense against heat, drought and their combination in *Nicotiana tabacum* plants. J Plant Physiol 171(17):1625–1633
63. Nguyen HN, Kambhampati S, Kisiala A, Seegobin M, Emery RJN (2021) The soybean (*Glycine max* L.) cytokinin oxidase/dehydrogenase multigene family; identification of natural variations for altered cytokinin content and seed yield. Plant Sci 5(2):e00308
64. Bose J, Rodrigo-Moreno A, Shabala S (2014) ROS homeostasis in halophytes in the context of salinity stress tolerance. J Exp Bot 65(5):1241–1257
65. Feng J, Li J, Gao Z, Lu Y, Yu J, Zheng Q, Yan S, Zhang W, He H, Ma L et al (2015) SKIP confers osmotic tolerance during salt stress by controlling alternative gene splicing in *Arabidopsis*. Mol Plant 8(7):1038–1052
66. Yin P, Liang X, Zhao H, Xu Z, Chen L, Yang X, Qin F, Zhang J, Jiang C (2023) Cytokinin signaling promotes salt tolerance by modulating shoot chloride exclusion in maize. Mol Plant S1674–2052(23):00109–01100
67. Li Y, Liu F, Li P, Wang T, Zheng C, Hou B (2020) An *Arabidopsis* cytokinin-modifying glycosyltransferase *UGT76C2* improves drought and salt tolerance in rice. Front Plant Sci 11:560696
68. Yan Z, Wang J, Wang F, Xie C, Lv B, Yu Z, Dai S, Liu X, Xia G, Tian H et al (2021) MPK3/6-induced degradation of ARR1/10/12 promotes salt tolerance in *Arabidopsis*. EMBO Rep 22(10):e52457
69. Sun L, Zhang Q, Wu J, Zhang L, Jiao X, Zhang S, Zhang Z, Sun D, Lu T, Sun Y (2014) Two rice authentic histidine phosphotransfer proteins, *OsAHP1* and *OsAHP2*, mediate cytokinin signaling and stress responses in rice. Plant Physiol 165(1):335–345
70. Sachdev S, Ansari SA, Ansari MI, Fujita M, Hasanuzzaman M (2021) Abiotic stress and reactive oxygen species: generation, signaling, and defense mechanisms. Antioxidants 10(2):277
71. Petrov V, Hille J, Mueller-Roeber B, Gechev TS (2015) ROS-mediated abiotic stress-induced programmed cell death in plants. Front Plant Sci 6:69
72. Wang Y, Shen W, Chan Z, Wu Y (2015) Endogenous cytokinin overproduction modulates ROS homeostasis and decreases salt stress resistance in *Arabidopsis thaliana*. Front Plant Sci 6:1004
73. Das K, Roychoudhury A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front Environ Sci 2:53
74. van Zelm E, Zhang Y, Testerink C (2020) Salt tolerance mechanisms of plants. Annu Rev Plant Biol 71:403–433
75. Naliwajski M, Skłodowska M (2021) The relationship between the antioxidant system and proline metabolism in the leaves of cucumber plants acclimated to salt stress. Cells 10(3):609
76. Wang B, Wang J, Yang T, Wang J, Dai Q, Zhang F, Xi R, Yu Q, Li N (2023) The transcriptional regulatory network of hormones and genes under salt stress in tomato plants (*Solanum lycopersicum* L.). Front Plant Sci 14:1115593

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