Journal of Cotton Research

RESEARCH

Open Access

Genome-wide identification and analysis of the *CNGC* gene family in upland cotton under multiple stress conditions

KIRUNGU Joy Nyangasi[1†], MAGWANGA Richard Odongo[1,2†], SHIRAKU Margaret Linyerera[1], OKUTO Erick[2], CAI Xiaoyan[1], XU Yanchao[1], HOU Yuqing[1], AGONG' Stephen Gaya[2], WANG Kunbo[1], WANG Yuhong[1], ZHOU Zhongli[1*] and LIU Fang[1,3*]

Abstract

Background The cyclic nucleotide-gated channel (*CNGC*) gene family plays a significant role in the uptake of both essential and toxic cations, and has a role in enhancing tolerance to various forms of abiotic stresses as well as the modulation of the heavy metal toxicity to plant through the absorption of heavy metals.

Results A complete genome-wide identification and functional characterization of the cotton *CNGC* genes was carried out, in which 55, 28, and 29 *CNGC* genes were identified in *Gossypium hirsutum*, *G. raimondii*, and *G. arboreum*, respectively. The protein encoded by the *CNGC* genes exhibited GRAVY value below zero, indicating their hydrophilic property. *CNGC* genes were unevenly distributed in 19 out of 26 chromosomes, in which the highest density were observed on Ah05, with 8 genes. High gene coverage was observed among the diploid cotton species, with *CNGC* genes mapped on all A chromosomes and on 11 out of 13 of D chromosomes. The majority of CNGC proteins were localized in the endoplasmic reticulum, nucleus, and plasma membrane. Gene expression analysis revealed the up-regulation of *Gh_A01G0520* (*CNGC4*) and *Gh_D13G1974* (*CNGC5*) across various forms of abiotic stresses. Moreover, down-regulation of *Gh_A01G0520* (*CNGC4*) and *Gh_D13G1974* (*CNGC5*) in *CNGCs* silenced plants caused the significantly reduced ability to tolerate drought and salt stresses. All *CNGCs* silenced plants were recorded to have significantly low content of antioxidants but relatively higher content of oxidant, including MDA and H_2O_2. Furthermore, SPAD, CMS (cell membrane stability), ELWL (excised leaf water loss), SDW (shoot dry matter weight), and RDW (root dry matter weight) were all lower in *CNGCs* silenced plants compared with the wild type plants.

Conclusion Significant reduction in antioxidant content and negative effects of physiological and morphological characters in *CNGCs* silenced plants has revealed the novel role of *CNGC* genes in enhancing cell integrity under abiotic stress conditions. These results provide vital information that will expand our understanding of the *CNGC* gene family in cotton and other plants, thus promoting the integration of these genes in the development of the environmental resilient plants.

[†]Kirungu JN and Magwanga RO contributed equally to this work.

*Correspondence:
Zhou Zhongli
zhonglizhou@163.com
Liu Fang
liufcri@163.com
Full list of author information is available at the end of the article

Keywords Cyclic nucleotide-gated channel, Abiotic stress, Differential expression, VIGS-plants, Chromosome, Tetraploid cotton

Background

The cyclic nucleotide-gated channel (*CNGC*) gene family plays a vital role in plant physiological processes related to development, host-pathogen interactions, environmental stresses, and signaling pathways (Nawaz et al. 2019). The *CNGC* is believed to be involved in the uptake of both essential and toxic cations. Some of the essential cations include calcium, sodium, and potassium, while the toxic cations include cadmium, aluminum, and lead (Saand et al. 2015). In plants, *CNGC* proteins have a conserved structural component, composed of six transmembrane helices (S1–S6) with a pore-forming region between S5 and S6, a short cytosolic N-terminus, and a cytosolic C-terminus containing a *cNMP*-binding domain (CNBD) (Nawaz et al. 2014). Genome-wide studies have been done on several organisms such as tomato (Saand et al. 2015), maize (Hao and Qiao 2018), wheat (Guo et al. 2018), Chinese cabbage (Li et al. 2019a), tobacco (Nawaz et al. 2019), rice (Cui et al. 2020), pea (Chen et al. 2015), and *Brassica oleracea* (Kakar et al. 2017).

CNGCs have been reported to play a critical role in response to various abiotic stimuli, including salt, cold, light signaling, and metal stress conditions (Yuen and Christopher 2010). Furthermore, they have also been found associated with biotic stress signaling, such as pathogen defense signal transduction, hormone signal perception, gibberellic acid-induced signaling, and phytochrome signaling (Ma et al. 2010). CNGCs are cation transport channel proteins associated with sodium, calcium, and potassium transportation across cellular membranes. In Arabidopsis, it has been observed that AtCNGC2 is involved in jasmonic acid (JA)-induced apoplastic Ca^{2+} influx and is activated by cAMP. And the calcium ion (Ca^{2+}) is an essential secondary messenger in modulating multiple signaling pathways in plants.

Plants being sessile organisms, are constantly exposed to harsh environmental conditions, and have evolved and developed core stress signaling pathways to cope with these various environmental stress conditions (Verma et al. 2016). Stress signaling is essential in regulating proteins involved in homeostasis. The extreme environmental conditions include biotic stresses such as pathogens, and abiotic stresses, including salt, drought, cold, nutrient deficiency, heat, and heavy toxicity. Whole-genome sequencing of the tetraploid cotton species, including *Gossypium hirsutum* (Zhang et al. 2015), *G. arboreum* (Li et al. 2014), and *G. raimondii* (Lin et al. 2010), provides a platform for whole genome identification and functional characterizations of cotton CNGC proteins under drought and salt stresses.

Materials and methods

Identification of the *CNGC* gene family in the *Gossypium* genome

The various CNGCs were obtained by downloading from various genome databases. For the tetraploid cotton, the GhCNGC protein sequences were obtained from the Cotton Research Institute website (http://mascotton.njau.edu.cn, Nanjing, China), the diploid cotton CNGC sequences for *Gossypium raimondii* (*GrCNGC*) were downloaded from Phytozome 12 (http://www.phytozome.net/, Joint Genome Institute, Department of Energy), with the expected value $E < 0.01$, while the CGNC sequences for *Gossypium arboreum, GaCNGC* were downloaded from the Beijing Genome Institute (https://www.bgi.com/, Beijing, China). The conserved domain of CNGC proteins (PF00027) was downloaded from Pfam protein families (http://pfam.xfam.org/, European Molecular Biology Laboratory). For the identification of CNGC proteins in cotton, the hidden Markov model analysis (HMM) profile of the CNGC proteins was used as a query to carry out the HMMER search (http://hmmer.janelia.org/) against *G. hirsutum*, *G. raimondii*, and *G. arboreum*. Two online tools were later used to confirm the presence of the CNGC domain for further analysis: the Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/) and the ScanProsite tool (http://prosite.expasy.org/scanprosite/, Swiss Institute of Bioinformatics). SMART program and Protein family databases were used to verifying the presence of the *CNGC* domain. The physicochemical properties of CNGC proteins were determined through the ExPASy Server tool (http://www.web.xpasy.org/compute_pi/, Swiss Institute of Bioinformatics).

Phylogenetic analysis and gene structure

To investigate the evolutionary relationship among CNGC proteins, all of them were obtained to construct their phylogenetic relationship. The phylogenetic tree was constructed using MEGA 7.0, adopting the neighbor-joining (NJ) method. The bootstrap parameters were set as 1 000 times (Kumar et al. 2016). Multiple sequences alignment of CNGC sequences from cotton and other plants was done using the ClustalW version 2.0 (Higgins

et al. 1996). The exon/intron structures of CNGCs were generated using gene structure displayer software (http://gsds.cbi.pku.edu.cn) by aligning the coding domain sequences (CDS) and DNA sequences of *CNGC* genes.

Chromosomal location and subcellular localization

All the *CNGC* genes' chromosomal localization were obtained and then mapped to cotton chromosomes based on respective positions using MapChart 2.2 software (Voorrips 2002). The sub-cellular localization prediction for all the upland cotton CNGC proteins was determined through the online tool WoLF PSORT (Protein Sub-cellular Localization Prediction) (https://www.wolfpsort.hgc.jp/). Moreover, the sub-cellular prediction results were further confirmed using two other online tools, TargetP 1.1 server (Bodén and Hawkins 2005) and Protein Prowler Sub-cellular Localization Predictor version 1.2 (http://www.bioinf.scmb.uq.edu.au/pprowler_webapp_1-2/).

Quantitative real-time polymerase chain reaction (RT-qPCR) profiling of the cotton *CNGC* genes

The ribonucleic acid (RNA) was extracted from leaf samples using EASYspin plus plant RNA kit obtained from Aid Lab, China, following the manufacturer's instructions. The quality and quantity of all the extracted RNA samples were determined by Agarose gel electrophoresis. The RNA was then reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using Prime Script Reverse Transcript reagent Kit (TaKaRa, Kusatsu, Japan). The gene-specific primers (Supplementary Table S1) were used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis. The reaction was carried out as described by Magwanga et al. (2018).

Virus-induced gene silencing (VIGS) of the *GhCNGC* genes

Reverse genetic analysis was carried out via virus-induced gene silencing (VIGS) using tobacco rattle virus (TRV). The cloning efficiency was enhanced using the Cloning and Assembly Kit (Transgen, China). Primers designed for the PCR amplification had a 15-bp overlapped sequence with the pTRV vector and contained a BamHI and XBA1 restriction sites. The generation of a tobacco rattle virus (TRV) based VIGS vector for *Agrobacterium*-mediated inoculation and the recombinant pTRV vector for the silencing of *Gh_A01G0520* (2 130 bp) and *Gh_D13G1974* (2 232 bp) in cotton, were carried out as described by Zhao et al. (2013). The VIGS experiments used the *Agrobacterium tumefaciens* strain GV3101 containing pTRV1 or pTRV2. GV3101 containing the TRV-VIGS vectors was grown at 28 °C overnight in LB medium with 10 mmol·L^{-1} MES buffer (pH 5.6) and 20 mmol·L^{-1} acetosyringone, kanamycin, and rifampicin with a revolution set at 200 r·min^{-1}. The plants were inoculated by creating a small scar or wound on the leaf. And using an injection syringe, the inoculants were driven slowly into the plant (Broderick and Jones 2014).

Evaluation of the VIGS plants, wild type, and the empty vector transformed plants under abiotic stress conditions

To determine the effect of knockdown of *GhCNGC* genes in cotton, the *G. hirsutum* var. Marie-galante 85 (MAR85) was used. This variety is relatively tolerant of various forms of abiotic stresses such as drought, salt, and cold (Xu et al. 2019a). Seeds were planted on wet blotting papers, and transplanted in troughs filled with Hoagland solution for subsequent stress treatments. Cotton seedlings were planted in a growth chamber at 25 °C, with conditions set at 16 h light/8 h dark cycle. Plants were subjected to different abiotic stress treatments at three leaf stage. Seedlings were subjected to salt and drought stress conditions by supplementing Hoagland solution with 250 mmol·L^{-1} Sodium chloride (NaCl) or 17% polyethylene glycol-6000 (PEG-6000), respectively. After a period of stress exposure, analysis of morphological, physiological, and biochemical parameters were carried out. Morphological traits, including plant height (PH), root dry matter weight (RDW), and shoot dry matter weight (SDW) were evaluated. The physiological parameters evaluation included the cell membrane stability (CMS), chlorophyll content measured as the SPAD values, relative leaf water content (RLWC), and excised leaf water loss (ELWL). The RLWL was measured using the formula RLWC = [(FW-DW)/(SW-DW)]*100 (Soltys-Kalina et al. 2016), ELWL and CMS were conducted as described by Magwanga et al. (2020). The biochemical parameters of antioxidant and oxidant enzymes were evaluated. The measured antioxidants were superoxide dismutase (SOD), and proline, while for the oxidants, malondialdehyde (MDA), and the hydrogen peroxide (H_2O_2) contents were measured. All indexes were detected with three biological replicates and three experimental replicates.

Results

Identification and physicochemical analysis of *CNGC* genes in cotton

A total of 112 *CNGC* genes were obtained, with 55, 28, and 29 *CNGC* genes in *G. hirsutum* of AD genome, *G. raimondii* of D genome, and *G. arboreum* of A genome, respectively. The CNGC protein length in *G. hirsutum* ranged from 95 aa to 1 099 aa, and their molecular weight ranged from 10.539 kDa to 121.541 kDa. The pI values ranged from 4.6 to 9.7, the charge ranged

from −29.5 to 39, and the gravy value ranged between −0.673 and 0.044. The CNGC protein length of *G. raimondii* and *G. arboreum* ranged between 433 aa and 1 101 aa, the molecular weight ranged from 49.13 kDa to 121.80 kDa, pI values ranged from 4.89 to 10.10, the charge ranged from −31 to 32, and their gravy values ranged between −0.26 and 0.048 (Supplementary Table S2). The gravy values below zero indicated that these genes were mainly hydrophilic.

Chromosomal mapping of the *CNGC* genes in cotton

The *CNGC* genes were mapped against various chromosomes. In tetraploid cotton, genes were unevenly distributed. Only 19 out of 26 chromosomes harbored the *CNGC* genes. The highest gene density was observed in chromosome Ah05, Ah13, Dh05, and Dh13, with 8, 5, 5, and 5 genes, respectively. While the lowest gene density was noted in chromosomes Ah01, Ah08, Dh01, Dh02, and Dh10, with a single gene in each of chromosomes. Other chromosomes harbored 4 *CNGC* genes. However, chromosomes Ah02, Ah09, Ah10, Ah11, Dh09, Dh11, and Dh12 contained no *CNGC* gene (Fig. 1A). A unique observation was noted in chromosome Ah02 and Ah04, which harbored none of the CNGC gene, while chromosomes Dh02 and Dh04 harbored 2 and 3 *CNGC* genes, respectively. This showed that there could be the gene loss over time. The mapping of CNGC genes among the diploid cotton chromosomes, most A genome chromosomes harbored the *CNGC* genes except for Ah02 and Ah09, which had no *CNGC* gene. Moreover, the highest gene density was observed on Ah05 and Ah13, with 5 *CNGC* genes on each chromosome (Fig. 1C). In the D genome, a similar pattern was observed, only two chromosomes Dh06 and Dh08 harbored none of the *CNGC*

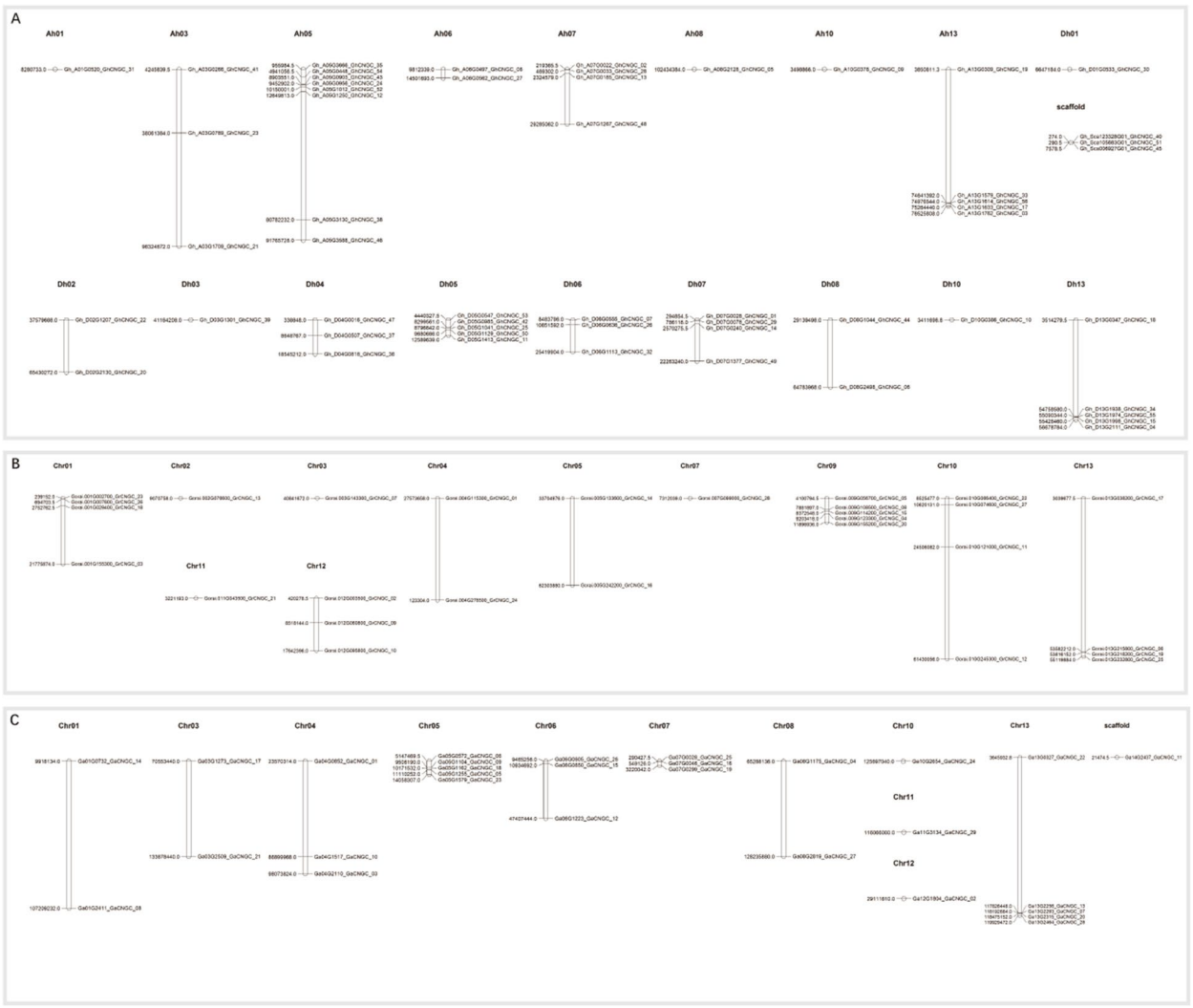

Fig. 1 Chromosome mapping of cotton *CNGC* genes. **A** *G. hirsutum*; **B** *G. raimondii*; **C** *G. arboreum*

gene, but more chromosomes were observed to have higher gene densities, for instance, chromosomes Dh01, Dh09, Dh10, and Dh13 had 4, 5, 4, and 4 *CNGC* genes, respectively (Fig. 1B).

Phylogenetic analysis and subcellular localization prediction of the proteins encoded by the *CNGC* genes in cotton

To determine the phylogenetic relationships of *CNGC* genes among *Gossypium spp* and *A. thaliana, O. sativa,* and other species, we performed phylogenetic analyses using CNGC protein sequences. The members of CNGCs were divided into three clades. All orthologous gene pairs of the cotton CNGCs were formed among the cotton species, which indicates a close relationship (Fig. 2).

To determine the possible subcellular localization, CNGC proteins' coding sequences from the three cotton species were analyzed through the online tool WoLF PSORT. Proteins were found to be located in various subcellular structures, namely endoplasmic reticulum (E.R.), cytoplasm (Cyto), mitochondrion (Mito), nucleus (nucl), plasma membrane (plas), and vacuole (vacu). Most CNGC proteins were predicted to be located in the endoplasmic reticulum (E.R.), with 20 (36.4%), 11(37.9%), and 11(39.3%) for the CNGC proteins obtained from *G. hirsutum*, *G. arboretum,* and *G. raimondii*, respectively (Fig. 3).

Gene structures and gene ontology analysis

The investigation of the exon-intron structure in upland cotton revealed that *GhCNGC* genes contained introns except for two genes located in the scaffold region, while the number of introns varied. The introns have a significant effect on the genes. The removal of the intron by the spliceosome were found to alter gene expression at several levels. Furthermore, phylogenetic tree analysis found similarities of *GhCNGC* gene subgroups' intron-exon structure (Fig. 4). Most *GhCNGC* genes clustered together showed high similarity in the exon-intron organization, in intron lengths and exon numbers.

In the GO analysis, a total of 55 genes were found to be associated with various gene ontology terms. The gene ontology terms were classified into three broad domains, including biological process, cellular component, and molecular function. Among the *CNGC* genes, higher number of the genes were involved in biological process (BP), including localization, biological regulation, regulation of the biological process, cellular process, response to stimuli, developmental process, multicellular organism process, and metabolic process. Plant response to abiotic stress includes modification of various metabolic process for instance amino acid metabolisms. Studies had shown that the amino acid derivatives increased in *Arabidopsis thaliana* post stress exposure, and increased the synthesis of amino acids precursors (Batista-Silva et al. 2019). The cellular component (CC) had four significant terms, namely the cell, cell part, membrane, and membrane part. In molecular functions (MF), three terms were observed, including cellular activity, binding, and transporter activities (Fig. 5). Some GO terms were found in almost all *CNGC* genes, such as GO:0016021 was associated with 51 genes, described as an integral component of membrane, GO:0071805 was linked to 42 genes, and described as potassium ion transmembrane transport, GO:0005249 as a voltage-gated potassium channel activity, and GO:0034765 was associated with regulation of ion transmembrane transport. (Fig. 5).

Gene expression analysis of *GhCNGC* gene under salt and drought stress

To evaluate the role of *CNGC* genes in cotton, the seeds of a relatively stress-tolerant upland cotton genotype, *G. hirsutum* var. Marie-galante 85 (MAR85) was used to analyze the gene expression profile (Yang et al. 2019). After 3 days of germination, the seedlings were transferred to a hydroponic setup. And at three leaf stage, the seedlings were exposed to salt and drought stress conditions. The drought and salt stress factors were imposed by adding 17% PEG-6000 and 250 mmol·L^{-1} of NaCl to the Hoagland solution. Samples were collected at 0 h, 3 h, 6 h, 12 h, and 24 h post stress exposure. RNA was extracted from leaf, stem, and root tissues and converted to cDNA for RT-qPCR analysis. The *CNGC* gene-specific primers were designed by primer premier six as outlined by You et al. (2005). The *GhActin* was used as an internal control. The expression pattern of the cotton *CNGC* genes exhibited a similar trend. It was observed that two genes showed significant upregulation of the *Gh_A01G0520* (*CNGC4*) cyclic nucleotide-gated ion channel 4 and *Gh_D13G1974 (CNGC5)*. Probable cyclic nucleotide-gated ion channel 5 under drought and salt stress conditions across all the tissues were analyzed (Fig. 6). The upregulation of these two *CNGC* genes demonstrated that the genes could be playing a vital role in enhancing drought and salt stress tolerance in cotton.

Silencing of GhCNGC4 and GhCNGC5 and RT-qPCR validation of the cotton *CNGC* genes under drought and salt stresses

In order to understand the role of the *GhCNGC* genes in salt and drought stresses, two of the upregulated genes,

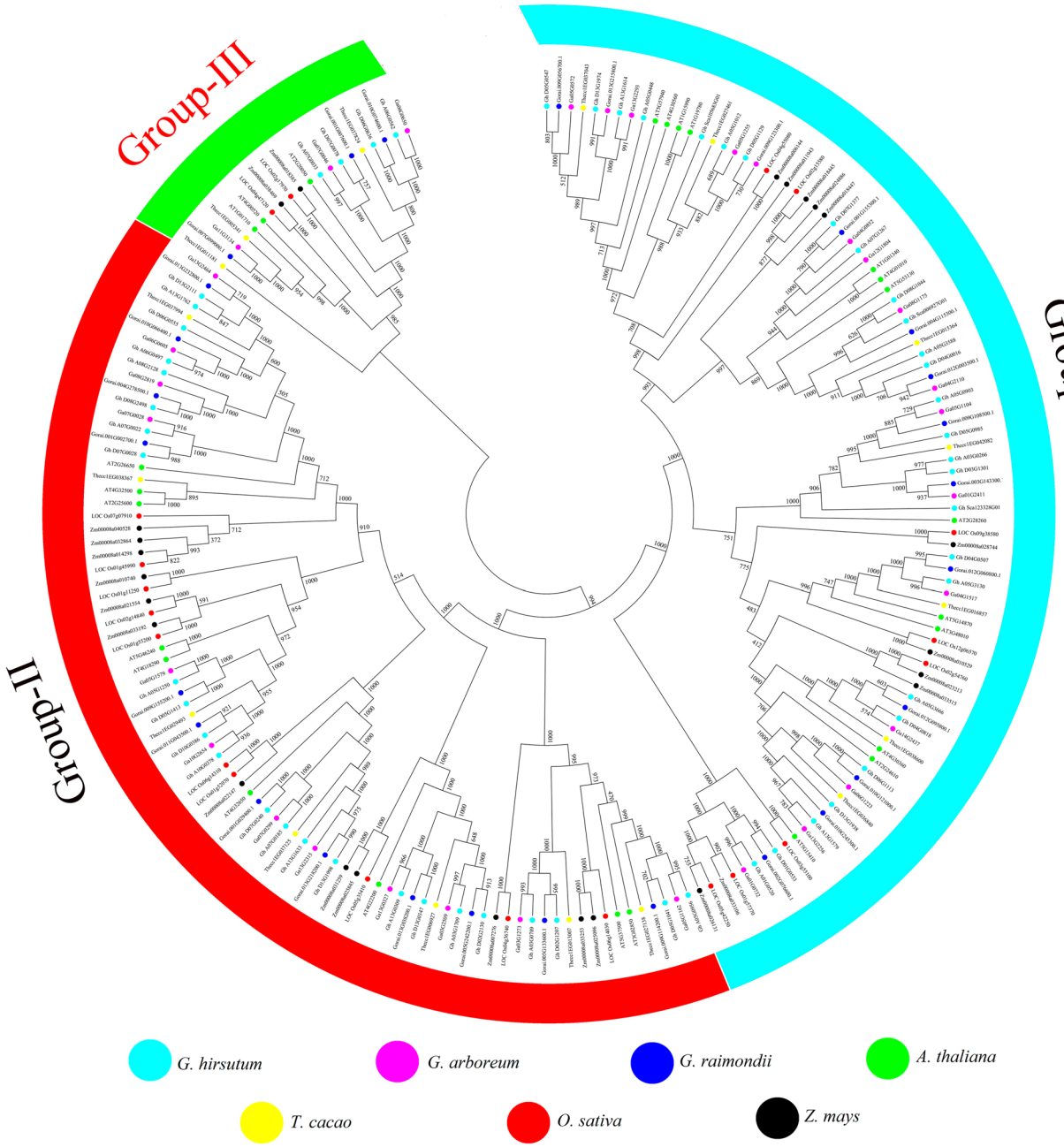

Fig. 2 Phylogenetic analysis of CNGCs. The tree comprised of *GhCNGCs*, *GaCNGCs*, *GrCNGCs*, *AtCNGCs*, *ThecCNGCs*, *LOCCNGCs,* and *ZMCNGCs*

Gh_A01G0520 (*CNGC4*) and *Gh_D13G1974* (*CNGC5*), were knocked down by the Virus-induced gene silencing (VIGS) method. We used the tobacco rattle virus (TRV)-based VIGS of PHYTOENE DESATURASE (PDS) to determine the genes' silencing effectiveness. PDS had been used in various experiments, and when expressed in plants, they exhibited photo-bleaching leaf phenotype (Xu et al. 2019b). The seedlings that were infused with *pTRV2:PDS* showed an albino appearance on the first true leaves after 7 days. The severity of the albino continued with the growth of seedlings, and over 99% of the bleaching young emerging leaves attained two weeks post inoculation (Fig. 7A). The silenced plants were adversely affected compared with the wild type plants (Fig. 7B and C). Environmental stresses caused osmotic stress in plants, affected plant growth and yield. The expression pattern of *GhCNGC* genes was examined in response to salt and drought stresses using RT-qPCR, and the analysis

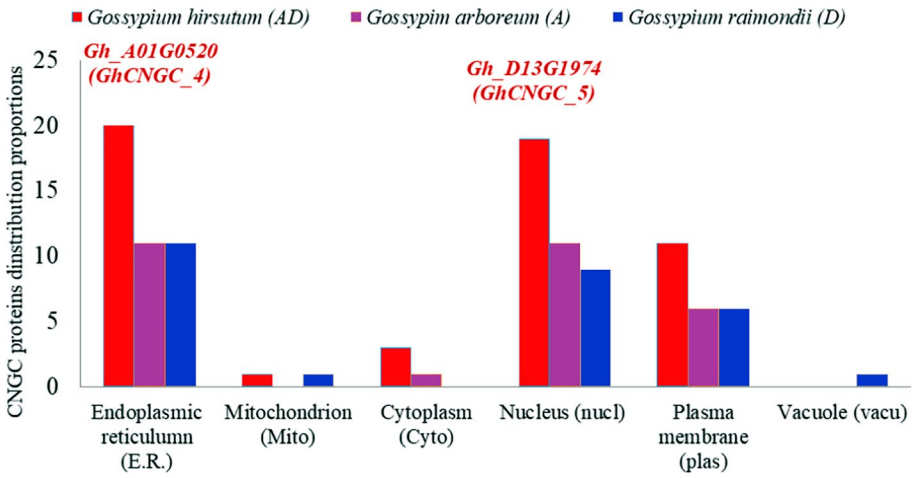

Fig. 3 Distribution of CNGC proteins in the three cotton species

was performed using the total RNA extracted from leaves of *G. hirsutum* var. Marie-galante 85 (MAR85). Seedlings were subjected to 24 h exposure to NaCl and PEG. Leaves were harvested. The expression pattern was determined through RT-qPCR, and the expression level of *CNGC* genes in the mutant cotton tissues showed significant down-regulation compared with its expression in the non-silenced cotton seedlings. This validated the role played by the novel silenced genes (Fig. 7D). These results concur with previous studies where genes of interest were knocked down, such as the knockdown of Dehydrin genes in *Capsicum annuum* L. (Jing et al. 2016), *GH3* genes in cotton (Kirungu et al. 2019), and cytochrome genes in cotton (Magwanga et al. 2019b). Knockdown of the two *CNGC* genes lowered the ability of the plant to withstand the stresses imposed on them. The plant height and the shoot fresh weight were greatly reduced in VIGS plants as compared with wild type plants and the positive control (Fig. 7E and F), this indicated that imposing the plants to the four stress conditions compromised the growth of plants. This showed the significant role of *CNGC* gene in promoting plant growth and development. Similar results have been observed in maize (Hao and Qiao 2018) where it promoted embryo development.

Biochemical analysis under drought and salt stress conditions

The antioxidants and oxidants enzymes activity in *CNGCs* silenced plants were assayed. The antioxidants evaluated in this study were superoxide dismutase (SOD) and proline, while the oxidants hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were assayed, too. The plants showed a significant reduction in the content of all the assayed antioxidants. However, there was a two-fold increase of oxidant enzyme concentration in the VIGS-plants compared with the wild type plant and the positive control plant under drought and salt stress conditions (Fig. 8). The antioxidants, such as SODs, were the first line of defense in plant against the damage caused by the excessive ROS when the equilibrium state of ROS was altered (Li et al. 2019b). The significant reduction of various antioxidants and increased level of oxidants demonstrated that knockdown of the two *GhCNGC* genes in cotton affected the plants' ability to tolerate drought and salt stresses.

Evaluation of physiological parameter

Some of the physiological traits were measured including excised leaf water loss (ELWL), saturated leaf weight (SLW), cell membrane stability (CMS), and chlorophyll content (SPAD value). In all the measured traits, there was a significant difference between the *CNGCs* silenced plants and wild type plant. There was a higher ion leakage in the *CNGCs* silenced plants compared with the wild type plants (Fig. 9C). The higher ion leakage was due to the compromised cell membrane which lead to extensive oxidative injury.

Discussion

Cotton farming is essential for many countries' economic growth. Textiles industries mostly rely on cotton as the primary source of natural fiber (Thyavihalli Girijappa et al. 2019), however its production has been affected by abiotic stresses such as salt, drought, cold, and heavy metals toxicity occasioned by poor disposal of industrial wastes. These stresses adversely affect plants and represent the output of complex interactions (Zhu 2016). In this study, we performed genome-wide identification

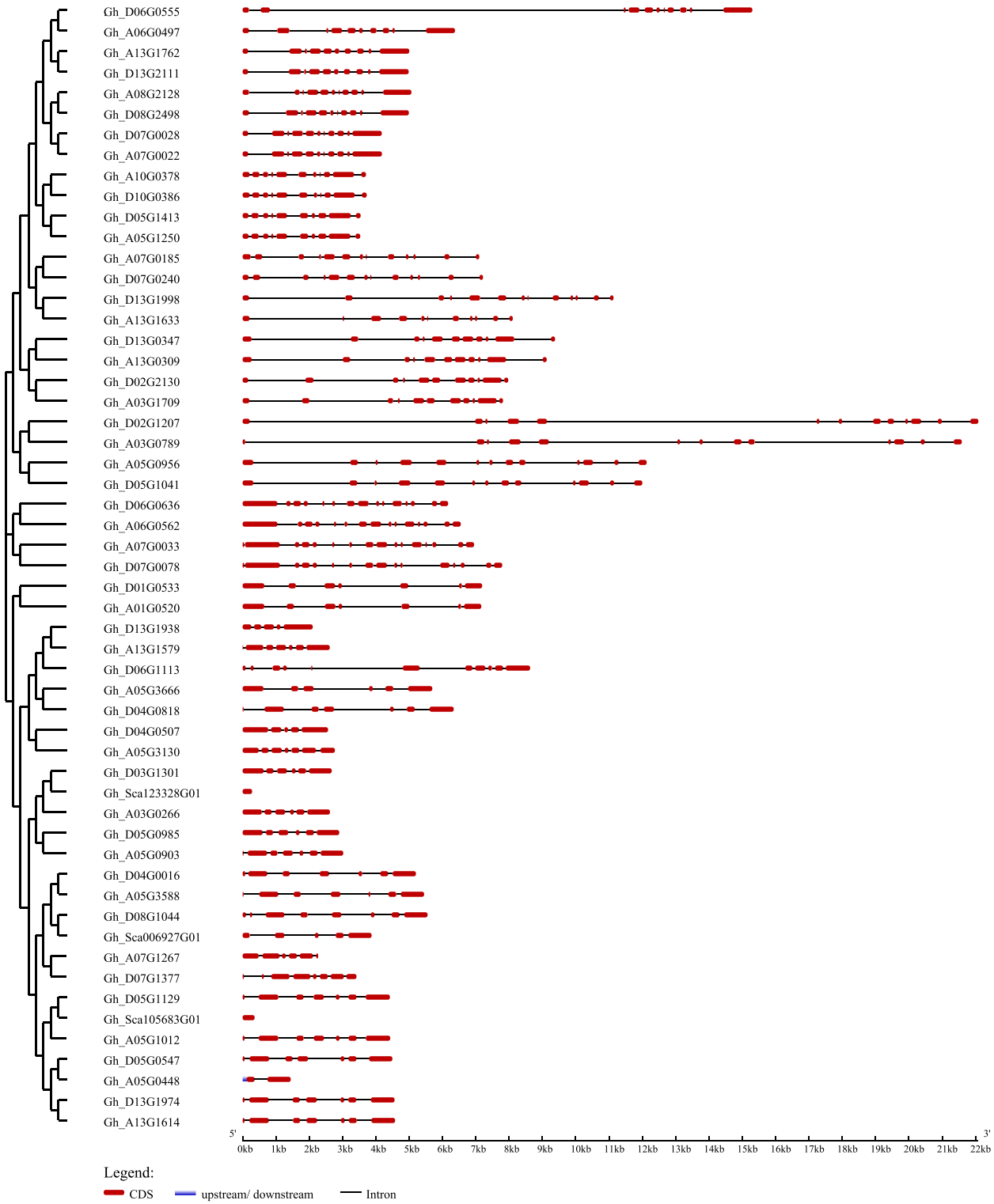

Fig. 4 Gene structure of *GhCNGC* genes in *G. hirsutum* (AD) genome

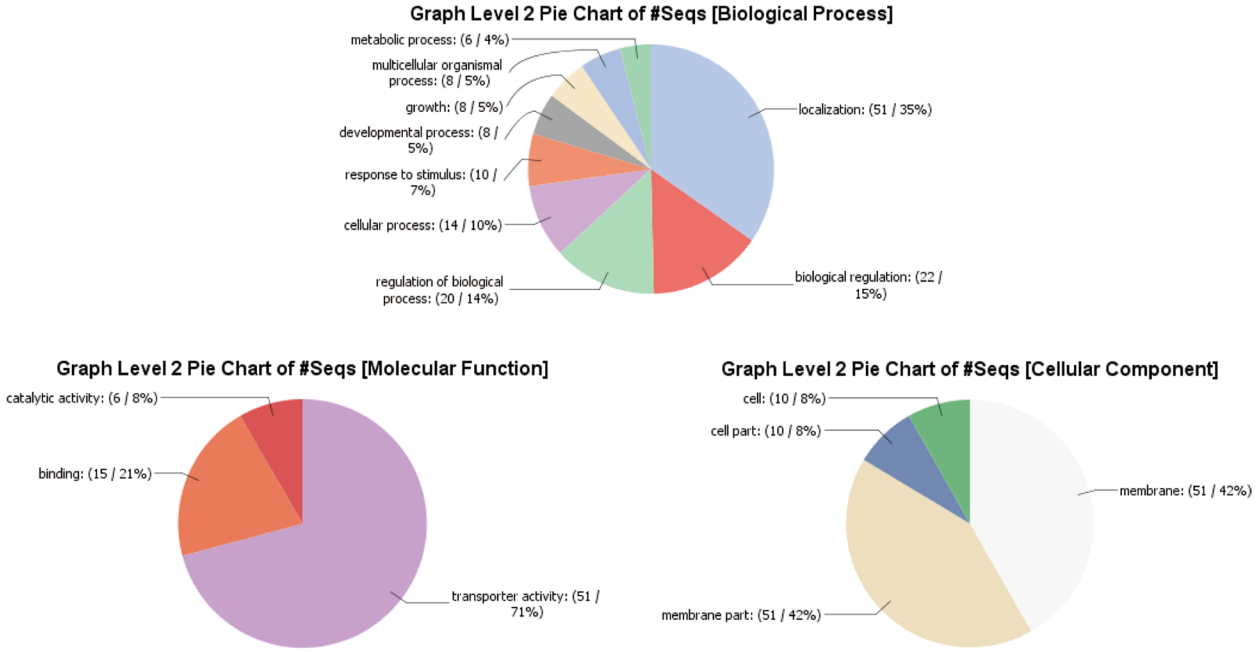

Fig. 5 Gene Ontology (GO) analysis of *GhCNGC* genes

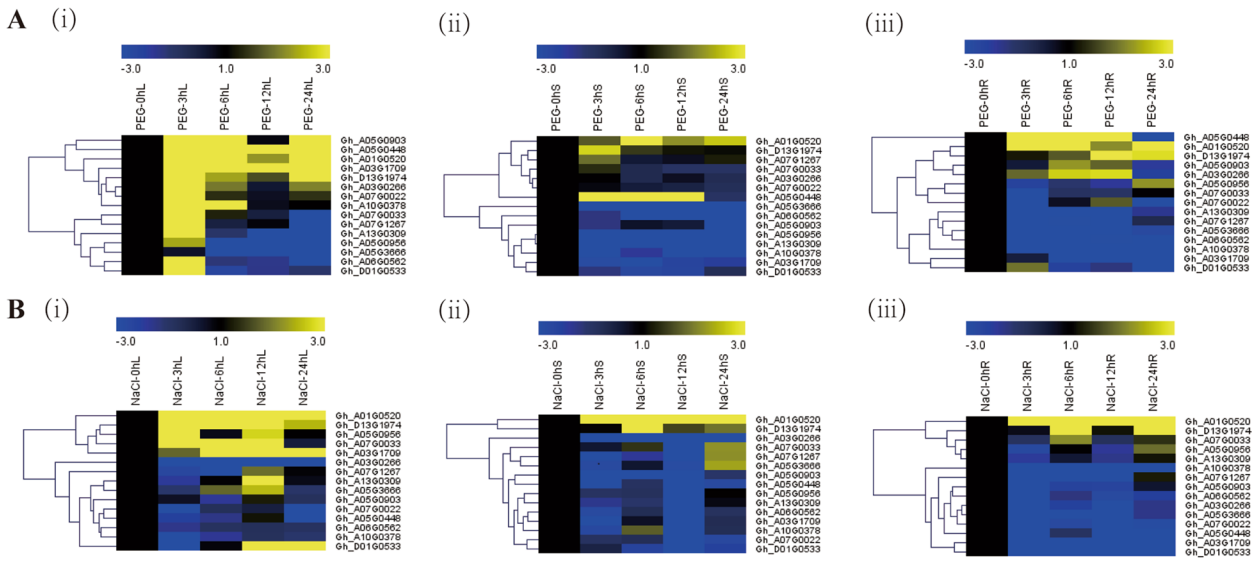

Fig. 6 RT-qPCR analysis of the cotton *GhCNGC* genes under drought and salt stress conditions. A treatments with PEG. B treatments with NaCl

and characterization of the *CNGC* gene family. Similar genome-wide studies have been carried out in other plants such as maize (Hao and Qiao 2018), wheat (Guo et al. 2018), and pear (Chen et al. 2015). We identified a total of 112 genes distributed in the three genomes, 55 *CNGC* genes in *G. hirsutum* of AD genome, 28 *CNGC* genes in *G. raimondii* of D genome, and 29 *CNGC* genes in *G. arboreum* of A genome. There is gene loss in upland cotton *G. hirsutum* compared with the total number of *CNGC* genes in D genomes and A genomes. Gene loss mainly occurs through pseudogenization or physical loss through gene deletion, resulting in a smaller genome

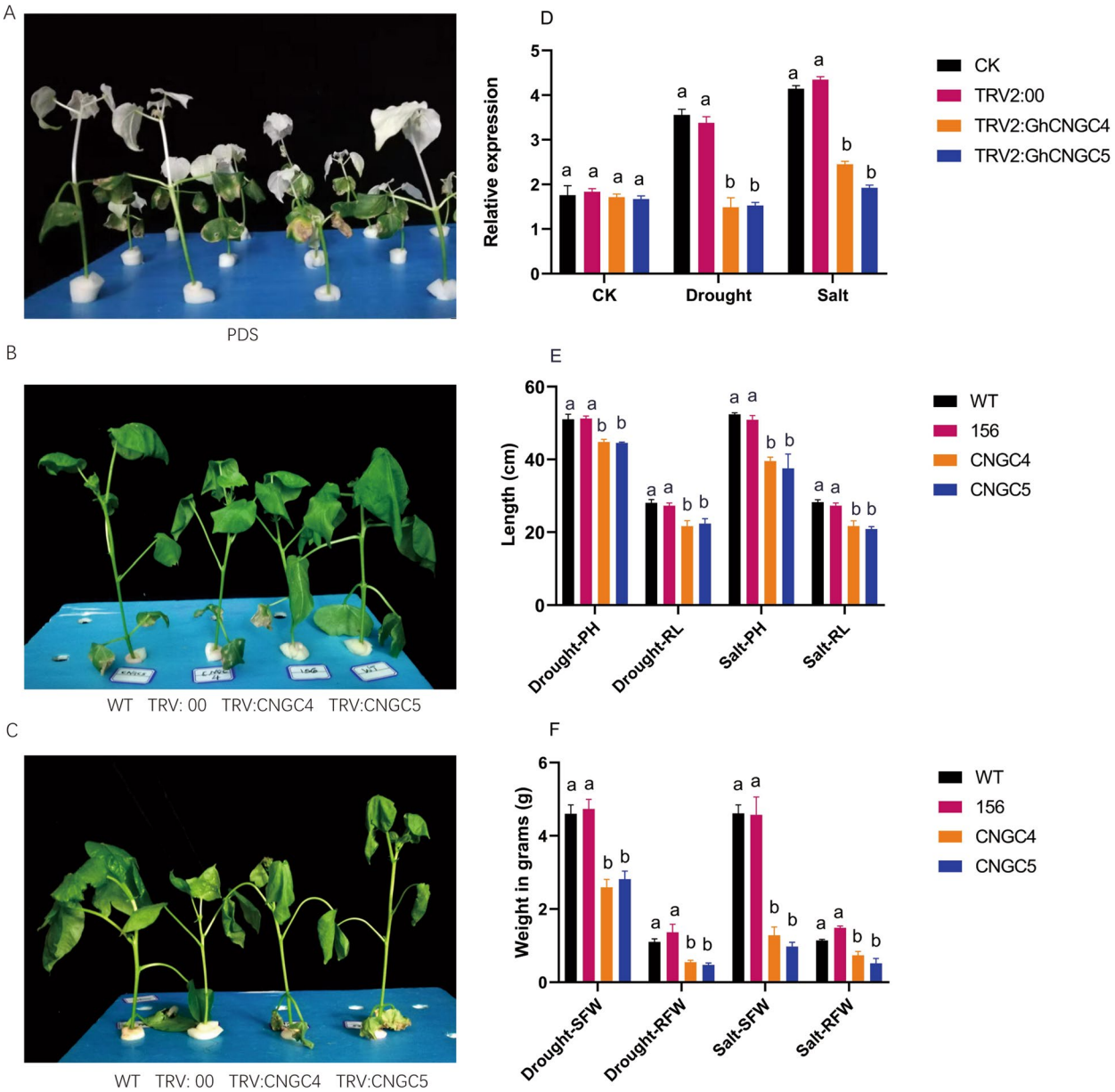

Fig. 7 Phenotype trait evaluation in the silenced plants with the TRV: 00 empty vector, wild type (WT) plants and the CNGCs silenced plant at 12 days post inoculation. **A** TRV: PDS infused plants. **B** Drought stress treatment in *CNGCs* silenced plant and WT. **C** Salt treatment in *CNGCs* silenced plant and WT. **D** RT-qPCR analysis of the change in the expression level of the *Gh_A01G0520 (CNGC4)* and *Gh_D13G1974 (CNGC5)* gene in cotton. TRV: 00 represented the plant carrying the empty TRV2 vector; TRV: *GhCNGC4* or TRV: *GhCNGC5* represents *Gh_A01G0520 (CNGC4* or *Gh_D13G1974 (CNGC5)* silenced plants. **E** Plant height and root length comparison of *CNGCs* silenced plant and WT after drought and salt stress. **F** Shoot fresh weight and root fresh weight comparison of *CNGCs* silenced plant and WT after drought and salt stress

size (Rong et al. 2010). Hence, it is most likely that such events occurred in the common ancestor of species that share the event. It has also been observed that cotton have the highest number of *CNGC* genes (112) than other plants, such as 20 in Arabidopsis, 16 in rice, and 12 in maize.

CNGC genes are distributed in various chromosomes in all three cotton genomes. In the tetraploid cotton *G. hirsutum*, genes are unevenly distributed in the chromosomes, only 19 out of 26 chromosomes harbored *CNGC* genes. A genome chromosomes harbore the *CNGC* genes except for Ah02 and Ah09. A similar pattern has been

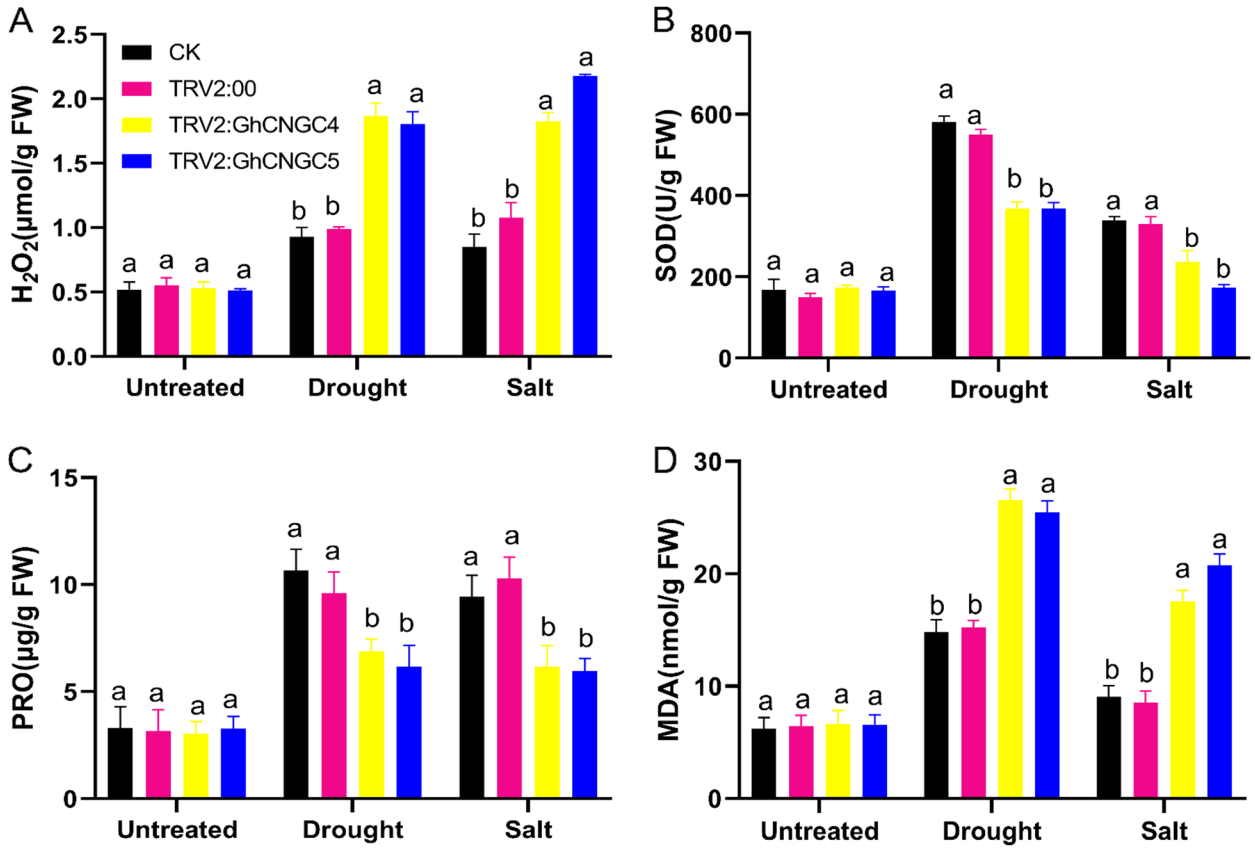

Fig. 8 Biochemical assays of the oxidant and antioxidant in *CNGCs* silenced plant under drought and salt stress conditions. **A** Quantitative determination of hydrogen peroxide (H_2O_2). **B** Quantitative determination of SOD concentration. **C** Quantitative determination of proline concentration. **D** Quantitative determination of MDA content in leaves of wild type and CNGCs silenced plants at 8 days post stress exposure. Each experiment was repeated three times. Error bar indicated the standard error (SE). Different letters indicated significant differences between wild type and *CNGCs* silenced plants (ANOVA; $p < 0.05$). Untreated indicated the normal growth condition

observed in D genome chromosomes, only two chromosomes Dh06 and Dh08 harbore none of the *CNGC* gene. Similar observations have been noted in several genome-wide studies in cotton, such as the *GH3* gene (Kirungu et al. 2019), the *AHL* gene family (Zhao et al. 2020), and the trihelix gene (Magwanga et al. 2019a). The phylogenetic tree analysis comprises 199 CNGCs genes including *GhCNGCs*, *GaCNGCs*, *GrCNGCs*, *AtCNGCs*, *ThecCNGCs*, *LOCCNGCs,* and *ZMCNGCs*. The cotton *CNGC* genes cluster into five groups (I, II, III, IVa, and IVb), with clade II forming orthologous pairs between the cotton gene family members, suggesting a closer relationship between the orthologs of *CNGC* genes from At-A or Dt-D in cotton. Proteins are found to be located in various subcellular structures, namely endoplasmic reticulum (E.R.), cytoplasm (Cyto), mitochondrion (Mito), nucleus (nucl), plasma membrane (plas), and vacuole (vacu). The majority of CNGC proteins are predicted to be located in the endoplasmic reticulum (E.R.), with 20 (36.4%), 11(37.9%), and 11(39.3%) of the CNGC proteins are obtained from *G. hirsutum*, *G. arboreum*, and *G. raimondii*, respectively. Moreover, three cellular structures, including endoplasmic reticulum, nucleus, and plasma membrane, are found to harbor the highest number of cotton CNGC proteins. The results are in agreement with previous findings in which Arabidopsis CNGC19 are experimentally determined to be located in the plasma membrane (Meena et al. 2019). Furthermore, maize ZmCNGCs are located within the plasma membrane (Hao and Qiao 2018). While in wheat, two TaCNGCs (TaCNGC2/3B and TaCNGC11B) are localized in the chloroplast thylakoid membrane, TaCNGC15a/b/c are localized in the nucleus, and the rest of the TaCNGCs are either embedded in the plasma membrane or endoplasmic reticulum (Guo et al. 2018).

We have conducted RT-qPCR analysis to validate effects of drought and salt stresses on *CNGC* genes. Some of *CNGC* genes are highly regulated under all forms of stress factors. It has been observed that two genes *Gh_A01G0520 (CNGC4)* and *Gh_D13G1974*

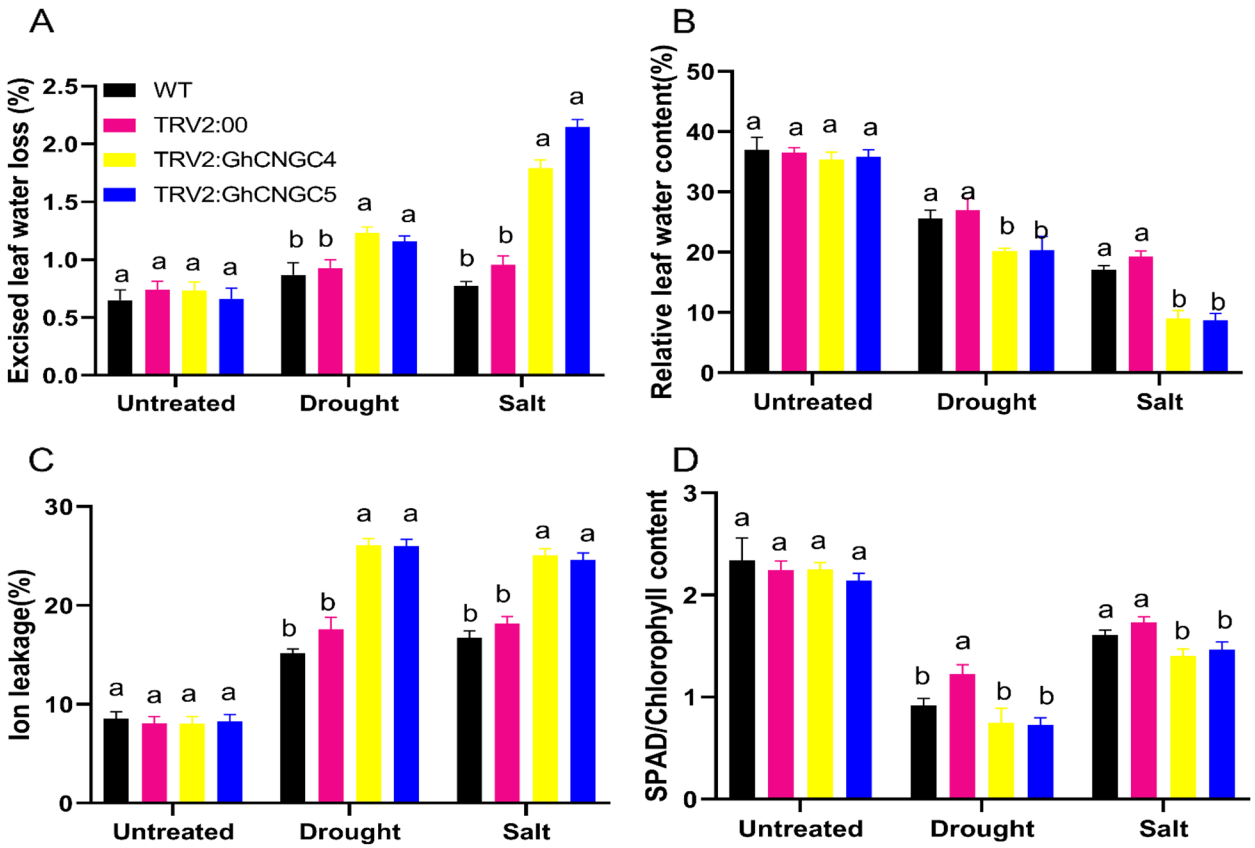

Fig. 9 Physiological trait measurement in Gh_A01G0520 (CNGC4) and Gh_D13G1974 (CNGC5) silenced cotton plants under drought and salt stresses (**A**). Quantitative determination of excised leaf water loss (ELWL), **B** Relative water content (RLWC), **C** Ion leakage concentration (evaluation of the cell membrane stability, CMS) **D** SPAD, representation of chlorophyll content. Each experiment was repeated three times. Error bars represent the standard deviation of three biological replicates

(CNGC5) show higher expression levels under all stress conditions. The higher expression levels show the significant biological roles these genes could play in enhancing tolerance towards salt and drought stresses in the cotton plant. The rest of genes showed differential expression patterns. CNGC proteins are mainly associated with the uptake of essential and toxic cations, Ca^{2+} signaling, pathogen defense, and plant thermo-tolerance. They have also implicated in plant growth and development, including hormonal, abiotic, and biotic stress responses. To determine the functional role of *CNGC* genes, we have silenced *Gh_A01G0520 (CNGC4)* and *Gh_D13G1974 (CNGC5)* which are up-regulated under stress conditions by using a hydroponic system. At 7 days post inoculation, the TRV: PDS plants showed albino-like phenotypes on the first foliage leaves, indicating that the silencing was effective. The RT-qPCR analysis has then been performed to validate the effect of gene silencing. The two genes' expression levels are significantly reduced in the silenced plants than in the wild type plants under drought and salt stresses. The silencing of these genes compromises the ability of plants to tolerate the stresses imposed on them.

The morphological, physiological, and biochemical parameters were then analyzed. The VIGS-plants showed higher content of MDA and H_2O_2, and reduced content of proline and SOD. The increased oxidant enzyme activity showed that the VIGS-plants had higher levels of damage under oxidative stress than the WT and the positive control plants. The induction of salt and drought stress response in plants initiate the overproduction of reactive oxygen species (ROS), which leads to the destruction to plant cell structures and cellular components, and finally the apotosis. The higher levels of proline and MDA suggest that the silenced plants have a reduced ability to properly scavenge the ROS, leading to the destruction of cell membrane and reducing chlorophyll content. When plants are exposed to abiotic stresses, the delicate balance between production and elimination of ROS shifts, leading to the excessive accumulation of ROS within plant cell (Khan and Khan 2017). When ROS accumulates

in plant cells, it causes extensive damage to plant cell due to oxidative stress. The *CNGCs* silenced plants are unable to mobilize the antioxidants has showed that the downregulation of the *CNGC* genes could have affected the antioxidant pathways. The root and shoot length was also significantly reduced in *CNGCs* silenced plants compared with WT, suggesting the compromised growth in both shoot and root, which leads to reduced plant biomass.

Conclusion

A total of 112 genes distribute across three genomes, 55 *CNGC* genes in *G. hirsutum* of AD genome, 28 *CNGC* genes in *G. raimondii* of D genome, and 29 *CNGC* genes in *G. arboreum* of A genome. Their GRAVY values are negative, indicating that the proteins were hydrophilic, which is the shared feature among the proteins encoded by several stress-responsive genes. The majority of CNGC proteins are predicted to be located in the endoplasmic reticulum (E.R.), nucleus, and plasma membrane. These structures play a significant role in the cell. Introns are found in all *CNGC* genes except for the two genes located in the scaffold region Gh_Sca105683G01 and Gh_Sca123328G01. The RT-qPCR analysis has revealed that two genes, *Gh_A01G0520 (CNGC4)* and *Gh_D13G1974 (CNGC5)* show higher expression levels under all stress conditions. The two genes are then silenced through VIGS, and cause higher concentrations of MDA and H_2O_2, and reduced content of proline and SOD. This study provides preliminary analysis on the role of *CNGC* genes in enhancing abiotic stress tolerance in plants.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42397-023-00152-z.

Additional file 1: Supplementary table S1. CNGC gene primers for RT-qPCR analysis.

Additional file 2: Supplementary table S2. Physicochemical properties of the *CNGC* genes in *G. hirsutum*, *G. raimondii* and *G. arboretum*.

Additional file 3: Supplementary table S3. GO analysis of the upland cotton, *G. hirsutum CNGC* genes.

Acknowledgements

The authors would wish to sincerely thank the students, laboratory staff and mentors for their valuable contributions in the entire process of the research work.

Authors' contributions

Kirungu JN and Magwanga RO conceived the ideas, designed the methodology, participated in the field works, and wrote the manuscript. Shiraku ML, Cai X, Xu Y, Hou Y, Wang K designed the methodology and organized the manuscript. Kirungu JN, Magwanga RO, Liu F and Wang K participated in the field works and indoor experiments. Kirungu JN, Magwanga RO and Okuto E analyzed the data and results. Kirungu JN, Magwanga RO, Agong' SG, Hou Y, and Zhou ZA produced the figures and tables. All authors contributed critically to the article and approved the final version.

Funding

This research was funded by the National Natural Science Foundation of China (31621005, 32072023), National Key R&D Program of China ((2021YFE0101200), and PSF/CRP/18thProtocol (07).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Consent for publication

No consent was sought.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author details

[1]State Key Laboratory of Cotton Biology/Institute of Cotton Research, Chinese Academy of Agricultural Science (ICR-CAAS), Anyang, Henan 455000, China. [2]School of Biological, Physical, Mathematics and Actuarial Sciences (SBPMAS), Jaramogi Oginga Odinga University of Science and Technology (JOOUST), Bondo, 210-40601, Kenya. [3]School of Agricultural Sciences, Zhengzhou University, Zhengzhou, Henan 450001, China.

Received: 11 January 2023 Accepted: 20 August 2023
Published online: 08 October 2023

References

Batista-Silva W, Heinemann B, Rugen N, et al. The role of amino acid metabolism during abiotic stress release. Plant Cell Environ. 2019;42:1630–44. https://doi.org/10.1111/pce.13518.

Bodén M, Hawkins J. Prediction of subcellular localization using sequence-biased recurrent networks. Bioinformatics. 2005;21:2279–86. https://doi.org/10.1093/bioinformatics/bti372.

Broderick SR, Jones ML. An optimized protocol to increase virus-induced gene silencing efficiency and minimize viral symptoms in Petunia. Plant Mol Biol Report. 2014;32:219–33. https://doi.org/10.1007/s11105-013-0647-3.

Chen J, Yin H, Gu J, et al. Genomic characterization, phylogenetic comparison and differential expression of the cyclic nucleotide-gated channels gene family in pear (*Pyrus bretchneideri* Rehd.). Genomics. 2015;105:39–52. https://doi.org/10.1016/j.ygeno.2014.11.006.

Cui Y, Lu S, Li Z, et al. CYCLIC NUCLEOTIDE-GATED ION CHANNELs 14 and 16 promote tolerance to heat and chilling in rice. Plant Physiol. 2020;183:1794–808. https://doi.org/10.1104/pp.20.00591.

Guo J, Islam MA, Lin H, et al. Genome-wide identification of cyclic nucleotide-gated ion channel gene family in wheat and functional analyses of *TaCNGC14* and *TaCNGC16*. Front Plant Sci. 2018;9:18. https://doi.org/10.3389/fpls.2018.00018.

Hao L, Qiao X. Genome-wide identification and analysis of the *CNGC* gene family in maize. PeerJ. 2018;6:e5816. https://doi.org/10.7717/peerj.5816.

Higgins DG, Thompson JD, Gibson TJ. [22] Using CLUSTAL for multiple sequence alignments. Methods Enzymol. 1996;26:383–402.

Jing H, Li C, Ma F, et al. Genome-wide identification, expression diversication of dehydrin gene family and characterization of *CaDHN3* in pepper (*Capsicum annuum* L.). PLoS One. 2016;11(8):e0161073. https://doi.org/10.1371/journal.pone.0161073.

Kakar KU, Nawaz Z, Kakar K, et al. Comprehensive genomic analysis of the *CNGC* gene family in *Brassica oleracea*: novel insights into synteny, structures, and transcript profiles. BMC Genomics. 2017;18(1):869. https://doi.org/10.1186/s12864-017-4244-y.

Khan MIR, Khan NA. Reactive oxygen species and antioxidant systems in plants: role and regulation under abiotic stress. Singapore: Springer; 2017. https://doi.org/10.1007/978-981-10-5254-5.

Kirungu JN, Magwanga RO, Lu P, et al. Functional characterization of Gh-A08G1120 (GH3.5) gene reveal their significant role in enhancing drought

and salt stress tolerance in cotton. BMC Genet. 2019;20. https://doi.org/10.1186/s12863-019-0756-6.
Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4. https://doi.org/10.1093/molbev/msw054.
Li F, Fan G, Wang K, et al. Genome sequence of the cultivated cotton *Gossypium arboreum*. Nat Genet. 2014;46:567–72. https://doi.org/10.1038/ng.2987.
Li J, Yang Y, Sun K, et al. Exogenous melatonin enhances cold, salt and drought stress tolerance by improving antioxidant defense in tea plant (*Camellia sinensis* (L.) O. Kuntze). Molecules. 2019b;24:1826. https://doi.org/10.3390/molecules24091826.
Lin L, Pierce GJ, Bowers JE, et al. A draft physical map of a D-genome cotton species (*Gossypium raimondii*). BMC Genomics. 2010;11:395. https://doi.org/10.1186/1471-2164-11-395.
Li Q, Yang S, Ren J, et al. Genome-wide identification and functional analysis of the cyclic nucleotide-gated channel gene family in Chinese cabbage. 3 Biotech. 2019a;9:114. https://doi.org/10.1007/s13205-019-1647-2.
Ma W, Yoshioka K, Gehring C, Berkowitz GA. The function of cyclic nucleotide-gated channels in biotic stress. In: Demidchik V, Maathuis F, editors. Ion channels and plant stress responses. Signaling and communication in plants. Berlin, Heidelberg: Springer; 2010. p. 159–74. https://doi.org/10.1007/978-3-642-10494-7_8.
Magwanga RO, Kirungu JN, Lu P, et al. Genome wide identification of the trihelix transcription factors and overexpression of *Gh_A05G2067* (*GT-2*), a novel gene contributing to increased drought and salt stresses tolerance in cotton. Physiol Plant. 2019a;167:447–64. https://doi.org/10.1111/ppl.12920.
Magwanga RO, Lu P, Kirungu JN, et al. Identification of QTLs and candidate genes for physiological traits associated with drought tolerance in cotton. J Cotton Res. 2020;3:3. https://doi.org/10.1186/s42397-020-0043-0.
Magwanga RO, Lu P, Kirungu JN, et al. GBS mapping and analysis of genes conserved between *Gossypium tomentosum* and *Gossypium hirsutum* cotton cultivars that respond to drought stress at the seedling stage of the BC_2F_2 generation. Int J Mol Sci. 2018;19:1614. https://doi.org/10.3390/ijms19061614.
Magwanga RO, Lu P, Kirungu JN, et al. Knockdown of cytochrome P450 genes *Gh_D07G1197* and *Gh_A13G2057* on chromosomes D07 and A13 reveals their putative role in enhancing drought and salt stress tolerance in *Gossypium hirsutum*. Genes. 2019b;10:226. https://doi.org/10.3390/genes10030226.
Meena MK, Prajapati R, Krishna D, et al. The Ca^{2+} channel CNGC19 regulates Arabidopsis defense against Spodoptera Herbivory. The Plant Cell. 2019;31:1539–62. https://doi.org/10.1105/tpc.19.00057.
Nawaz Z, Kakar KU, Saand MA, Shu QY. Cyclic nucleotide-gated ion channel gene family in rice, identification, characterization and experimental analysis of expression response to plant hormones, biotic and abiotic stresses. BMC Genomics. 2014;15(1):853. https://doi.org/10.1186/1471-2164-15-853.
Nawaz Z, Kakar KU, Ullah R, et al. Genome-wide identification, evolution and expression analysis of cyclic nucleotide-gated channels in tobacco (*Nicotiana tabacum* L.). Genomics. 2019;111:142–58. https://doi.org/10.1016/j.ygeno.2018.01.010.
Rong J, Feltus FA, Liu L, et al. Gene copy number evolution during tetraploid cotton radiation. Heredity. 2010;105:463–72. https://doi.org/10.1038/hdy.2009.192.
Saand MA, Xu YP, Li W, et al. Cyclic nucleotide gated channel gene family in tomato: genome-wide identification and functional analyses in disease resistance. Front Plant Sci. 2015;6:303. https://doi.org/10.3389/fpls.2015.00303.
Soltys-Kalina D, Plich J, Strzelczyk-Żyta D, et al. The effect of drought stress on the leaf relative water content and tuber yield of a half-sib family of 'Katahdin'-derived potato cultivars. Breed Sci. 2016;66:328–31. https://doi.org/10.1270/jsbbs.66.328.
Thyavihalli Girijappa YG, Mavinkere Rangappa S, Parameswaranpillai J, Siengchin S. Natural fibers as sustainable and renewable resource for development of eco-friendly composites: a comprehensive review. Front Mater. 2019;6:226. https://doi.org/10.3389/fmats.2019.00226.
Verma V, Ravindran P, Kumar PP. Plant hormone-mediated regulation of stress responses. BMC Plant Biol. 2016;16:86. https://doi.org/10.1186/s12870-016-0771-y.
Voorrips RE. MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered. 2002;93:77–8. https://doi.org/10.1093/jhered/93.1.77.
Xu H, Xu L, Yang P, et al. Virus-induced *Phytoene Desaturase* (*PDS*) gene silencing using *tobacco rattle virus in Lilium* × *formolongi*. Hortic Plant J. 2019b;5(1):31–8. https://doi.org/10.1016/j.hpj.2018.10.002.
Xu Y, Magwanga RO, Cai X, et al. Deep transcriptome analysis reveals reactive oxygen species (ROS) network evolution, response to abiotic stress, and regulation of fiber development in cotton. Int J Mol Sci. 2019a;20:1863. https://doi.org/10.3390/ijms20081863.
Yang X, Xu YC, Yang FF, et al. Genome-wide identification of *OSCA* gene family and their potential function in the regulation of dehydration and salt stress in *Gossypium hirsutum*. J Cotton Res. 2019;2:11. https://doi.org/10.1186/s42397-019-0028-z.
You M, Boersma JG, Buirchell BJ, et al. A PCR-based molecular marker applicable for marker-assisted selection for anthracnose disease resistance in lupin breeding. Cell Mol Biol Lett. 2005;10:123–34.
Yuen CYL, Christopher DA. The role of cyclic nucleotide-gated channels in cation nutrition and abiotic stress. In: Demidchik V, Maathuis F, editors. Ion channels and plant stress responses. Signaling and communication in plants. Berlin, Heidelberg: Springer; 2010. p. 137–57. https://doi.org/10.1007/978-3-642-10494-7_7.
Zhang T, Hu Y, Jiang W, et al. Sequencing of allotetraploid cotton (*Gossypium hirsutum* L. acc. TM-1) provides a resource for fiber improvement. Nat Biotechnol. 2015;33:531–7. https://doi.org/10.1038/nbt.3207.
Zhao J, Gao Y, Zhang Z, et al. A receptor-like kinase gene (*GbRLK*) from *Gossypium barbadense* enhances salinity and drought-stress tolerance in *Arabidopsis*. BMC Plant Biol. 2013;13:110. https://doi.org/10.1186/1471-2229-13-110.
Zhao L, Lü Y, Chen W, et al. Genome-wide identification and analyses of the *AHL* gene family in cotton (*Gossypium*). BMC Genomics. 2020;21:1–14. https://doi.org/10.1186/s12864-019-6406-6.
Zhu JK. Abiotic stress signaling and responses in plants. Cell. 2016;167:313–24. https://doi.org/10.1016/j.cell.2016.08.029.