



# GhCLCg-1, a Vacuolar Chloride Channel, Contributes to Salt Tolerance by Regulating Ion Accumulation in Upland Cotton

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Soil and freshwater salinization is increasingly becoming a problem worldwide and has adversely affected plant growth. However, most of the related studies have focused on sodium ion (Na<sup>+</sup>) stress, with relatively little research on chloride ion (Cl<sup>-</sup>) stress. Here, we found that upland cotton (*Gossypium hirsutum*) plants accumulated Cl<sup>-</sup> and exhibited strong growth inhibition under NaCl or KCl treatment. Then, a chloride channel gene (*GhCLCg-1*) was cloned from upland cotton. Phylogenetic and sequence analyses indicated that *GhCLCg-1* was highly homologous to *AtCLCg* and also have conserved voltage\_CLC and CBS domains. The subcellular localization assay showed that GhCLCg-1 was localized on the vacuolar membrane. Gene expression analyses revealed that the expression of *GhCLCg-1* increased rapidly in cotton in response to chloride stress (NaCl or KCl), and the transcript levels increased as the chloride stress intensified. The overexpression of *GhCLCg-1* in *Arabidopsis thaliana* changed the uptake of ions with a decrease of the Na<sup>+</sup>/K<sup>+</sup> ratios in the roots, stems, and leaves, and enhanced salt tolerance. In contrast, silencing *GhCLCg-1* in cotton plants increased the Cl<sup>-</sup> contents in the roots, stems, and leaves and the Na<sup>+</sup>/K<sup>+</sup> ratios in the stems and leaves, resulting in compromised salt tolerance. These results provide important insights into the toxicity of chloride to plants and also indicate that *GhCLCg-1* can positively regulate salt tolerance by adjusting ion accumulation in upland cotton.

**Keywords:** chloride channel, *Gossypium hirsutum*, vacuolar, ion content, salt tolerance

## INTRODUCTION

Salinization is a serious environmental problem worldwide. During the last few decades, many fresh water regions have been affected by increasing salinity (Kaushal, 2016). The causes of salinization include alterations to freshwater flows, irrigation, wastewater treatment, sea level increases, and the use of salt for deicing roads (Herbert et al., 2015). The application of deicing salts, such as NaCl, CaCl<sub>2</sub>, and MgCl<sub>2</sub>, has increased the salinization of water and soil, which has directly and negatively affected natural environments (Buss et al., 2020). Because chloride salts are commonly used, the chloride ion (Cl<sup>-</sup>) may be useful as a tracer for deicing salts (Thunqvist, 2004). Recent studies proved that the widespread use of road salt has resulted in increased chloride concentrations in

lakes and rivers (Thunqvist, 2004; Gutchess et al., 2016; Lacey et al., 2019). Vehicular traffic may be responsible for transferring salt into the adjacent roadside, where the salt may seep into the soil and the underlying water (Fay and Shi, 2012).

Salinization is extremely detrimental to sustainable agricultural production worldwide (Saghir et al., 2002). Sodium chloride (NaCl), which is the most ubiquitous salt, induces cellular osmotic and ionic stresses (Deinlein et al., 2014; Nie et al., 2015). To date, studies on the physiological and molecular mechanisms underlying plant salt tolerance have mostly focused on Na<sup>+</sup> toxicity and adaptations, but there has been relatively little research on salt damage caused by Cl<sup>-</sup> (Teakle and Tyerman, 2010; Li et al., 2016). The previous studies have confirmed that sodium/proton (Na<sup>+</sup>/H<sup>+</sup>) antiporters (NHXs) are involved in the regulation and stabilization of Na<sup>+</sup> under salt stress in plants and improved the salt tolerance of plants (Ma et al., 2020; Feng et al., 2021).

The chloride ion is one of the essential trace elements in plants. More specifically, it enhances plant growth and development (Broyer et al., 1954; Wei et al., 2019), while also affecting stomatal movement, cell osmotic pressure, electrical charge balance, cell turgor pressure, and intracellular pH (Franco Navarro et al., 2015). Despite its beneficial effects on plants, under salt stress conditions, Cl<sup>-</sup> is a major toxic element in the cytosol of plant cells, where it accumulates excessively, especially in shoots, thereby adversely affecting growth (Munns and Tester, 2008; Weg et al., 2017). Plants counter these stresses *via* the elimination of excess ions across the plasma membrane or the intracellular vacuolar compartmentalization to decrease the effective Cl<sup>-</sup> levels inside cells, particularly in the aerial tissues (Zhang et al., 2011). Earlier research revealed that the vacuolar chloride channel CLCg selectively transports chloride across the vacuolar membrane to enhance the tolerance to chloride salts (Nguyen et al., 2015). A recent study indicated *GhCLC4/15* expression is up-regulated in the roots and leaves of cotton seedlings under salt stress (Liu et al., 2020). Additionally, the vacuolar chloride channel CLCc maintains chloride homeostasis. The *Arabidopsis thaliana* mutant *atclc-c* reportedly exhibits increased sensitivity to NaCl (Jossier et al., 2010). In tobacco (*Nicotiana tabacum*), the Cl<sup>-</sup> content is obviously lower in *NtCLC2*-silenced plants than in control plants (Zhang et al., 2018). The overexpression of the vacuolar chloride channel gene *GmCLC1* in soybean can increase the segregation of Cl<sup>-</sup> in the roots and decrease the transport of Cl<sup>-</sup> to the shoots (Wei et al., 2016). These findings suggest that CLCs can take up Cl<sup>-</sup> to increase salt tolerance at high salt concentrations, and the resulting accumulation of Cl<sup>-</sup> in plants may minimize the effects of chloride on the environment.

Upland cotton (*Gossypium hirsutum*) is a widely grown fiber crop and has been used as a pioneer crop in regions with saline and alkaline land (Wang N. et al., 2016; Wang X.G. et al., 2016). In this study, the potential effects of chloride concentrations on cotton plants were analyzed. We treated cotton plants with different chloride salts (NaCl or KCl) and observed the resulting damage. And we also found the vacuolar chloride channel *GhCLCg-1* was activated in plants exposed to chloride salt concentrations by quantitative real-time polymerase chain reaction (PCR) (qRT-PCR). Furthermore, overexpression in

*A. thaliana* and virus-induced gene silencing (VIGS) technology in cotton were used to characterize the function of *GhCLCg-1* in cotton response to chloride salt stress. The objectives of this study were to determine the toxicity of chloride in cotton plants and prove the important role of *GhCLCg-1* in cotton resistance to salinity.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

*Gossypium hirsutum* L. acc. TM-1 seeds were soaked in sterile water to promote germination. After a 16-h incubation at 30°C, the germinating seeds were transferred to vermiculite-filled pots in a greenhouse (16-h light/8-h dark photoperiod at 23°C). When the cotyledon expanded, uniformly growing seedlings were selected, rinsed clean with running water, and transferred to a hydroponic tank containing Hoagland solution, which was refreshed weekly. To induce salinity stress, the cotton seedlings were grown to the three-leaf stage and then treated with Hoagland solution containing 50, 100, 150, or 200 mM NaCl or 200 mM KCl (treatment group). The seedlings in the control group were treated with Hoagland solution lacking NaCl or KCl. Root, stem, and leaf samples were collected at 0, 1, 3, and 6 h after treatments. The samples were rinsed with distilled water 3–5 times and then immediately frozen in liquid nitrogen and stored at -80°C for the subsequent total RNA extraction. Each treatment was completed with three biological replicates.

The wild-type (WT) *A. thaliana* plants used in this study were Columbia (Col-0). After a 2-day vernalization at 4°C, the *A. thaliana* seeds were surface sterilized by soaking in 75% ethanol for 3–5 min. After they were washed with sterile distilled water six times, the seeds were placed in plates containing Murashige and Skoog (MS) medium supplemented with 3% sucrose and 0.7% agar (pH adjusted to 5.8). The plates were incubated at 21°C with a 16-h light/8-h dark photoperiod. After 2 weeks, the *A. thaliana* seedlings were transferred to pots filled with vermiculite and nutritive soil (1:1, v/v) and placed in a greenhouse (16-h light/8-h dark photoperiod at 21°C).

### Gene Cloning and Sequencing

Total RNA was isolated from TM-1 seedlings using the RNAprep Pure Polysaccharide Polyphenol Plant Total RNA Extraction Kit (Tiangen, Beijing, China). The RNA concentration was determined using the NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), whereas RNA integrity was assessed by 1% agarose gel electrophoresis. First-strand cDNA was synthesized from 1 µg RNA using the PrimeScript<sup>TM</sup> II first Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). To clone the vacuolar chloride channel gene in upland cotton, the BlastP and tBlastN programs were performed against the *G. hirsutum* genome database<sup>1</sup> using the *A. thaliana* CLCg amino acid sequence as the query. The coding sequences of gene IDs GH\_A06G0574 and GH\_D06G0541 as the best match were retrieved and gene-specific primers were

<sup>1</sup><https://www.cottongen.org/>

designed. Full-length cDNAs were amplified using the GhCLCg-1F/R primer pair (**Supplementary Table 1**) and the KOD-Plus-Neo DNA polymerase (TaKaRa, Dalian, China). The PCR products were purified using the FastPure Gel DNA Extraction Mini Kit (Vazemy Biotech Co., Ltd.) and then cloned using the pEASY®-Blunt Cloning Kit for the subsequent transformation of *Trans1-T1 Escherichia coli* competent cells (TransGen Biotech). Positive clones cultured at 37°C were analyzed by sequencing to verify they were correctly transformed.

## Phylogenetic Tree Construction and Sequence Analysis

*Arabidopsis thaliana* CLC amino acid sequences were downloaded from TAIR database<sup>2</sup>. Seven AtCLC sequences along with *G. hirsutum* GhCLCg-1A and GhCLCg-1D (**Supplementary Table 2**) were used to construct a phylogenetic tree according to the neighbor-joining method of the MEGA-X software, with pairwise deletion and 1,000 bootstrap replicates. Conserved domains were detected using InterPro<sup>3</sup> and gene structures were examined using TBtools (Chen et al., 2020). Amino acid sequences were aligned using the DNAMAN program.

## Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from the roots, stems, and leaves using the RNA Extraction kit (Tiangen). The HiScript® II Q Select RT SuperMix for qPCR (+gDNA wiper) (Vazemy Biotech Co., Ltd.) was used to synthesize cDNA from approximately 1 µg total RNA. Gene-specific primers were designed according to conserved sequences in *GhCLCg-1A* and *GhCLCg-1D* (**Supplementary Table 1**). The qRT-PCR analysis was performed using the LightCycler 480 system (Roche, Switzerland) and the ChamQ Universal SYBR qPCR Master Mix (Vazemy Biotech Co., Ltd.). The *GhHIS3* gene served as an internal control (Ma et al., 2020). Three biological replicates were analyzed for each sample. Relative gene expression levels were calculated according to the  $2^{-\Delta CT}$  method (Livak and Schmittgen, 2001).

## Gene Overexpression Plasmid Construction

The full-length *GhCLCg-1A* and *GhCLCg-1D* open reading frames without a stop codon were amplified by PCR using primers with *Bam*HI and *Sma*I sites (**Supplementary Table 1**) and then inserted into the *Bam*HI/*Sma*I-digested pCAMBIA2300-35S-GFP-HA plasmid, which contains the CaMV 35S promoter. The resulting recombinant plasmids were inserted into *Trans1-T1* competent cells (TransGen Biotech). The accuracy of the inserted sequences in the transformants was confirmed by sequencing. The recombinant plasmids carrying the GhCLCg-1A-GFP and GhCLCg-1D-GFP constructs were inserted into *Agrobacterium tumefaciens* strain GV3101 cells

via a heat shock protocol. The *E. coli* and *A. tumefaciens* strains containing the overexpression plasmids were stored at -80°C.

## Subcellular Localization of GhCLCg-1A/D

A transient expression system using *A. thaliana* mesophyll protoplasts was prepared as previously described (Sheen, 2001; Feng et al., 2021). Protoplasts were co-transfected with 10 µg GhCLCg-1A-GFP/GhCLCg-1D-GFP recombinant plasmids and 10 µg vacuolar marker protein δ-TIP-RFP (Jauh et al., 1998) according to a polyethylene glycol-mediated transfection method (Yoo et al., 2007). After incubating the protoplasts at room temperature for 12–20 h in darkness, the green fluorescent protein (GFP) and red fluorescent protein (RFP) signals were detected using the FV1200 confocal laser scanning microscope (Olympus, Japan).

## Virus-Induced GhCLCg-1 Silencing in Upland Cotton

Based on the *GhCLCg-1A* and *GhCLCg-1D* conserved sequences, a 430-bp fragment was amplified by PCR from cotton cDNA using the GhCLCg-1-p-F/R primers (**Supplementary Table 1**) and inserted between the *Sac*I and *Eco*RI restriction sites in the VIGS vector pTRV2 of the ClonExpress® Ultra One Step Cloning Kit (Vazemy Biotech Co., Ltd.). Plasmids from the positive transformants carrying pTRV1 (helper plasmid) and *TRV:GhCLCg-1* were inserted into *A. tumefaciens* strain GV3101 cells via a heat shock protocol. Similarly, we constructed a visual marker (*TRV:CLA*, where CLA is chloroplasts alterados) to monitor the silencing efficiency. Additionally, *TRV:00* (empty vector) was used as the negative control. The VIGS experiments were performed by the tobacco rattle virus (TRV) system as previously described (Pang et al., 2013).

Two fully expanded cotyledons of cotton seedlings were used for the *A. tumefaciens* infiltration. At 10 days after the transformation, the true leaves of *TRV:CLA*-silenced cotton plants exhibited signs of albinism. The second true leaves, stems, and roots were harvested from at least three randomly selected *TRV:00* and *TRV:GhCLCg-1* cotton plants for an RNA isolation. The efficiency of *GhCLCg-1* silencing was evaluated by qRT-PCR, with *GhHIS3* used as the internal control (Ma et al., 2020). The *TRV:00* and *TRV:GhCLCg-1* plants were then treated with 200 mM NaCl for 3-day, the plants were photographed and their roots, stems, and leaves were collected for an analysis of their Cl<sup>-</sup>, Na<sup>+</sup>, and K<sup>+</sup> contents. The assays were completed with three biological replicates.

## GhCLCg-1 Overexpression in Arabidopsis thaliana

Because *GhCLCg-1A* and *GhCLCg-1D* have highly similar sequences, we used *GhCLCg-1A* to generate transgenic *A. thaliana* plants overexpressing *GhCLCg-1*. The recombinant plasmid carrying the GhCLCg-1A-GFP fusion construct was inserted into *A. thaliana* plants using the floral dip transformation method (Clough and Bent, 1998). Homozygous plants were selected on MS medium containing 50 µg/ml

<sup>2</sup><https://www.arabidopsis.org/>

<sup>3</sup><http://www.ebi.ac.uk/interpro/>

kanamycin. The T<sub>1</sub> generation plants were examined by PCR to confirm the positive lines were transformed correctly. Homozygous T<sub>3</sub> generation progenies were analyzed by reverse transcription PCR using the GhCLCg-1-Q-F/R primers (**Supplementary Table 1**). Seeds from homozygous T<sub>3</sub> plants were placed on MS agar medium (control group) or MS agar medium supplemented with 100 mM NaCl (salt treatment group). The seedlings were photographed after 3 weeks. Additionally, the taproot length was measured using a vernier caliper. Nine plants per line were examined and their tissues were harvested for an analysis of the Cl<sup>-</sup>, Na<sup>+</sup>, and K<sup>+</sup> contents.

## Analysis of Ion Contents

The VIGS plants and transgenic *A. thaliana* plants overexpressing GhCLCg-1 were collected and incubated at 105°C for 10 min to denature enzymes and then at 75°C to achieve a constant weight. The samples were subsequently ground to a powder. To measure the Cl<sup>-</sup> content, a 50-ml Erlenmeyer flask was washed with 5% HNO<sub>3</sub>. After drying, 0.1 g powder was added to the flask and resuspended with 15 ml boiled deionized water. The solution was cooled to room temperature and then filtered. The filtrate was collected in a 25-ml volumetric flask to a constant volume. The solution was analyzed using an ion chromatography system (ICS-5000) (Thermo Fisher Scientific, Waltham, MA, United States) to measure the Cl<sup>-</sup> content. The experiment was repeated three times.

To measure the Na<sup>+</sup> and K<sup>+</sup> contents, 0.1 g powder was treated with nitric acid (HNO<sub>3</sub>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydrofluoric acid (HF) and then microwaved (i.e., digested) before being analyzed using an inductively coupled plasma optical emission spectrometry system (ICAP-7400) (Thermo Fisher Scientific, Waltham, MA, United States). Three biological replicates were analyzed for each sample.

## RESULTS

### Uptake and Effect of Chloride in Upland Cotton Plants

Upland cotton plants were treated with 200 mM NaCl or KCl to assess the effects of chloride. After 10 days, compared with the mock controls, the growth of the plants treated with NaCl or KCl was significantly inhibited. Additionally, the salt-treated plants had shriveled and wilted leaves, some of which had fallen off the plants (**Figure 1A**).

An analysis of the plant ion contents indicated that the chloride (Cl<sup>-</sup>), sodium (Na<sup>+</sup>), and potassium (K<sup>+</sup>) contents were higher in the salt-treated samples than in the mock controls (**Figures 1B,C** and **Supplementary Figure 1**). After the NaCl and KCl treatments, cotton plants had extremely high Cl<sup>-</sup> contents in the leaves, roots, and stems (**Figures 1B,C**). As expected, the Na<sup>+</sup> or K<sup>+</sup> contents also increased in the leaves, stems, and roots of plants treated with NaCl or KCl (**Supplementary Figure 1**). These results suggest that Cl<sup>-</sup> can accumulate in cotton tissues, thereby affecting plant growth and development.

### Phylogenetic Analysis and Conserved Domains of GhCLCg-1

The vacuolar chloride channel (CLCg) can protect plant cells from ion-induced damages (Nguyen et al., 2015). Using a homology-based cloning strategy, we cloned the full-length coding sequences of two CLCg-encoding genes in *G. hirsutum*, which are homoeologs from D-subgenome and A-subgenome of the allotetraploid upland cotton and named GhCLCg-1A/D (suffixes D and A represented the D and A subgenomes, respectively). The GhCLCg-1A and GhCLCg-1D sequences were 2,325 bp long and encoded 774 amino acids. To further characterize GhCLCg-1A/D, we used the MEGA-X program to construct a phylogenetic tree that included seven AtCLCs. In the constructed tree, the CLC family was divided into two clades, with GhCLCg belonging to class I and highly homologous to AtCLCg (**Figure 2A**).

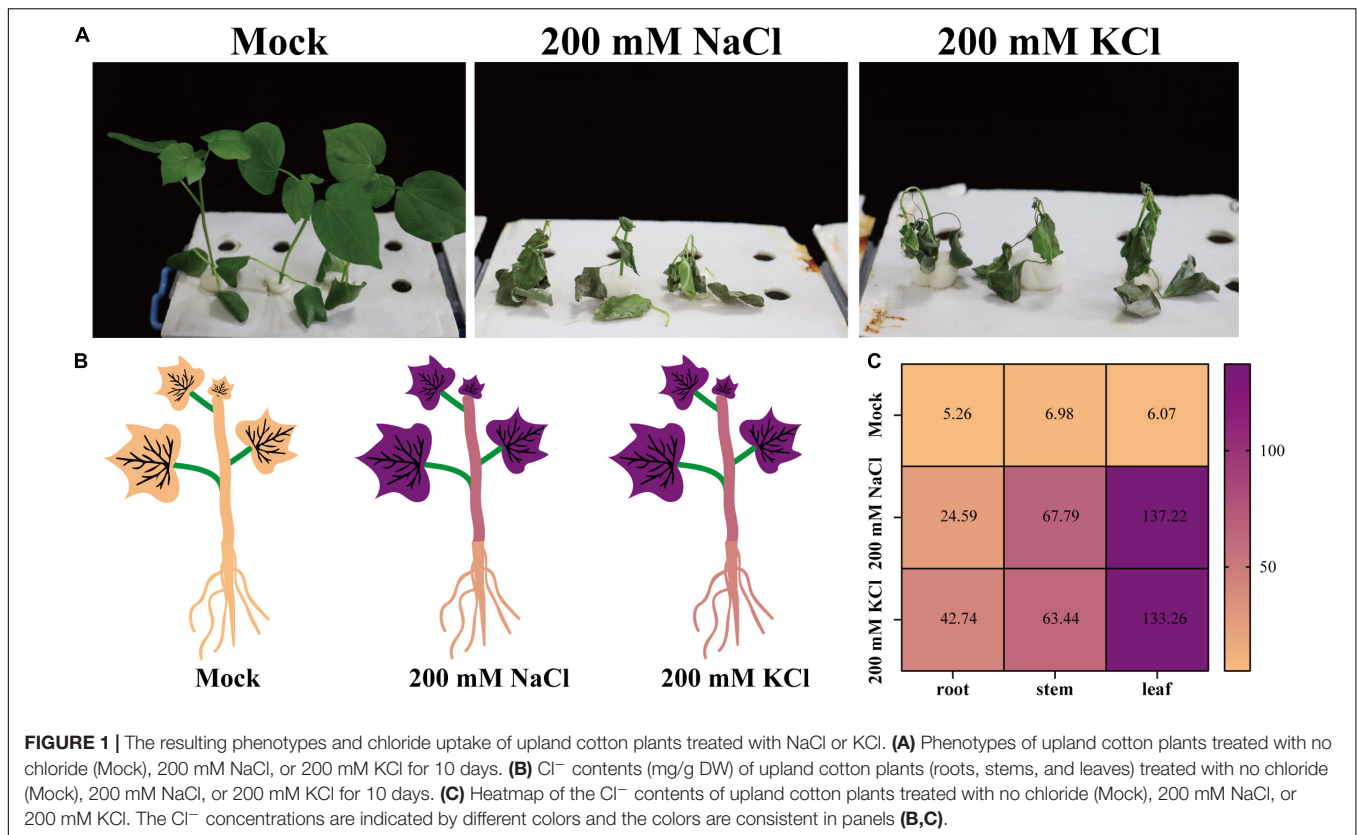
The results of the GhCLCg-1A and GhCLCg-1D gene structural analysis revealed these two genes have a similar intron-exon arrangement (**Figure 2B**). Specifically, they both comprise seven exons and six introns as well as domains unique to the CLC family (i.e., voltage\_CLC and CBS). To further investigate the similarity in the GhCLCg-1A and GhCLCg-1D sequences, their amino acid sequences were aligned to the AtCLCg amino acid sequence (**Supplementary Figure 2**). The multiple sequence alignment confirmed that GhCLCg-1A and GhCLCg-1D are highly conserved (98.19% identity) and are similar to AtCLCg at the amino acid level (91.13% identity). The multiple sequence alignment also indicated that GhCLCg-1A and GhCLCg-1D have three regions that are conserved in AtCLCg, namely GxGIPE, GKxGPxxH, and PxxGxLF. In the GxGIPE sequence, x was a serine (S), implying GhCLCg-1A/D are specific for Cl<sup>-</sup> (**Supplementary Figure 2**) (Barbier-Brygoo et al., 2000; Dutzler et al., 2002; Zifarelli and Pusch, 2010). These results imply that GhCLCg-1A and GhCLCg-1D are functionally similar to AtCLCg.

### Subcellular Localization of GhCLCg-1

The localization of proteins provides clues regarding their functions (Long et al., 2020). The subcellular localization of GhCLCg-1A/D was determined using fusion proteins with a C-terminal GFP. The fusion proteins were transiently expressed in *A. thaliana* leaf protoplasts that also contained the vacuolar marker protein δ-TIP with a C-terminal RFP (**Figure 3**). In the control protoplasts with GFP alone and δ-TIP-RFP, the GFP signal was detected throughout, whereas the RFP fluorescence was restricted to the tonoplast. The GhCLCg-1A/D-GFP fusion proteins co-localized with δ-TIP-RFP in the protoplast, with red and green fluorescent signals overlapping, which confirmed that GhCLCg-1A/D are localized to the vacuolar membrane.

### Activation of GhCLCg-1 by Chloride in Upland Cotton Plants

To determine whether GhCLCg-1A/D are involved in salt stress responses, we analyzed the expression of the corresponding genes following an exposure to chloride salts. The substantial similarity in the GhCLCg-1A and GhCLCg-1D sequences makes it very

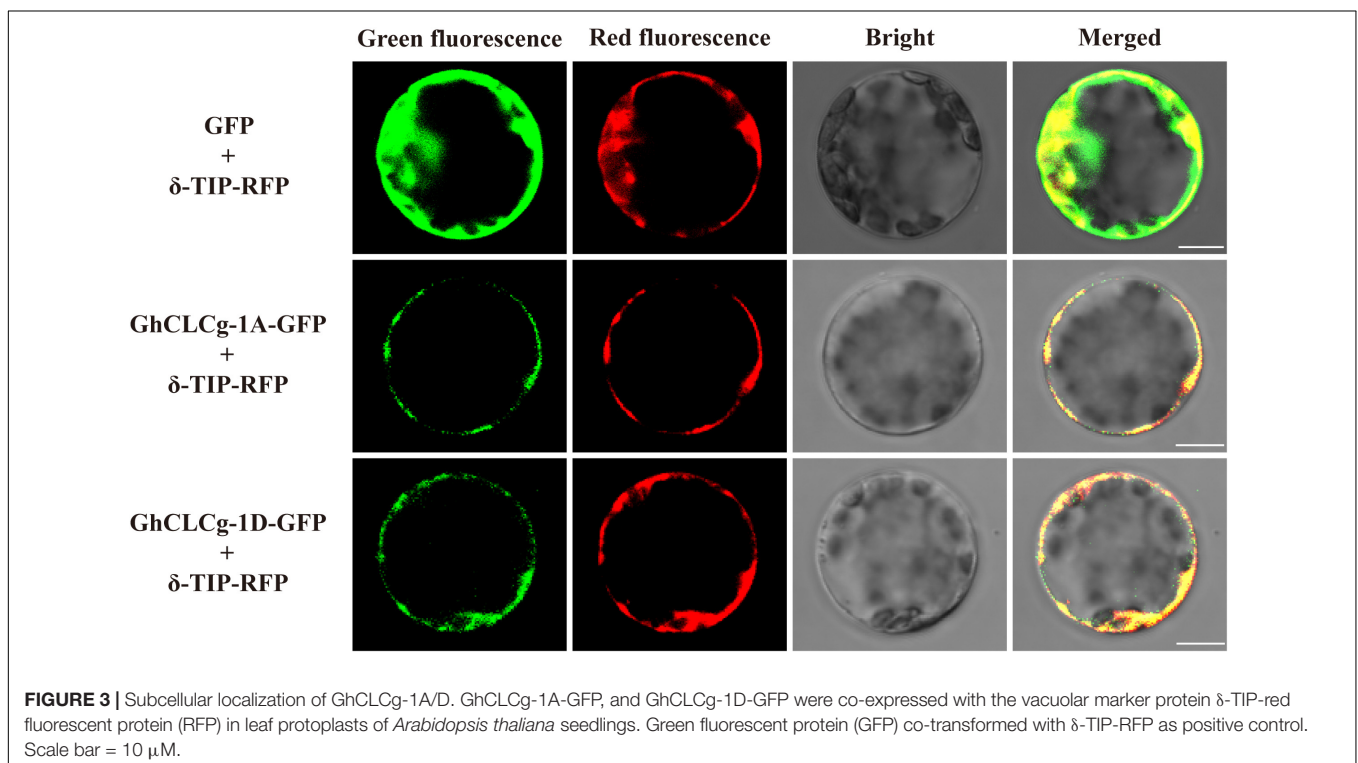
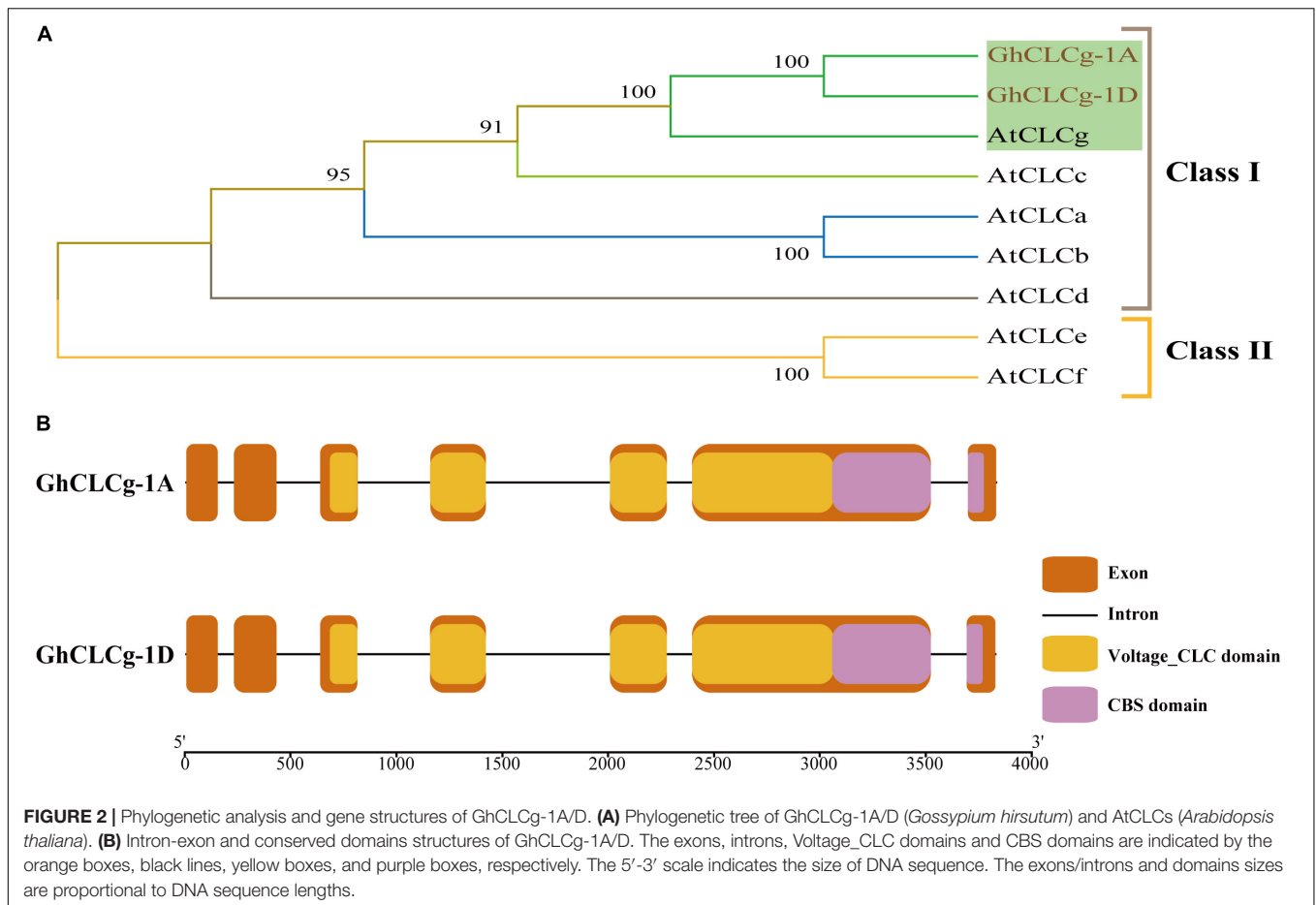


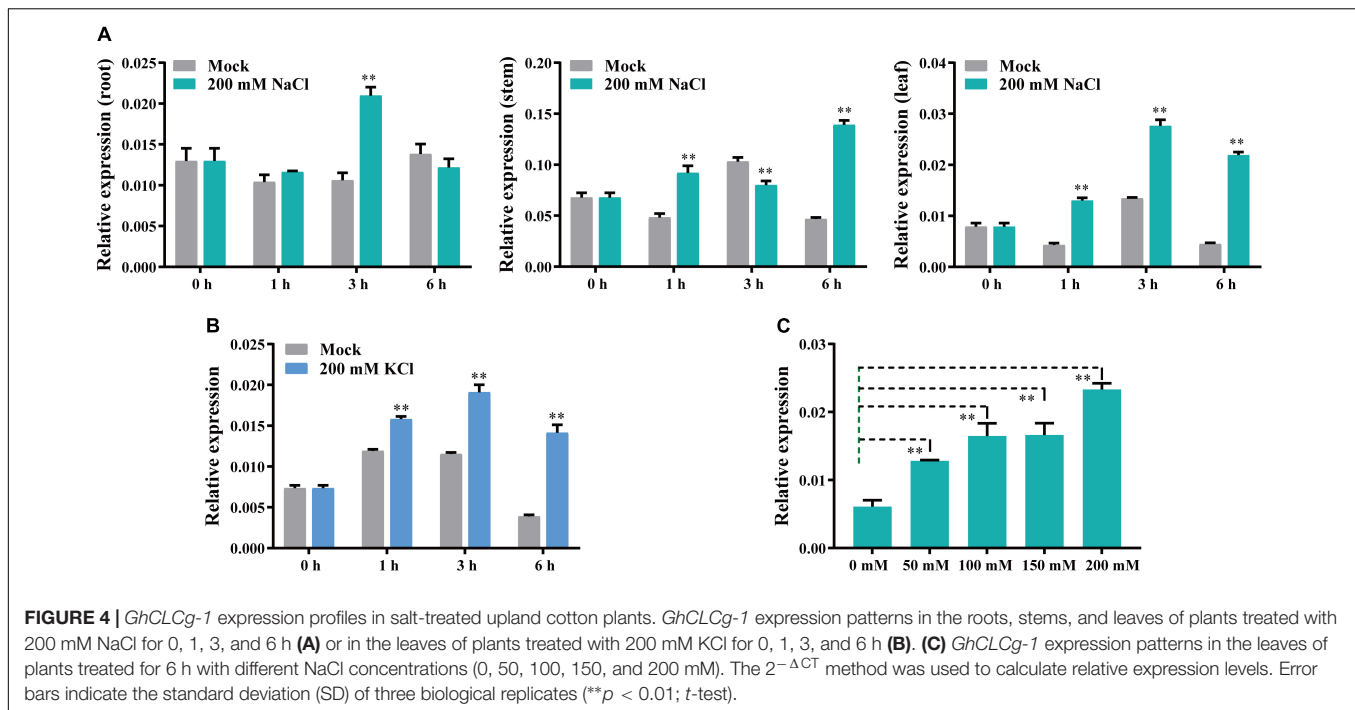
difficult to study the expression of these two genes by qRT-PCR. In this study, the universal qRT-PCR primers GhCLCg-1-Q-F/R were designed to analyze *GhCLCg-1A/D* expression in the roots, stems, and leaves at various time-points (**Figure 4**). Following the 200 mM NaCl treatment, *GhCLCg-1* expression was up-regulated at 3 h in the roots (approximately two times higher than expression level of the mock control). In the stems, *GhCLCg-1* expression was about 2–3 times higher in the NaCl-treated plants than in the mock controls at 1 and 6 h. Unexpectedly, the expression level of *GhCLCg-1* at 3 h in stems of the 200 mM NaCl treated plants was significantly lower than in stems of the mock plants. The *GhCLCg-1* transcript levels in stems treated with different salt concentration at 3 h were analyzed. The results showed that the expression of *GhCLCg-1* at different salt concentration is lower than the control (0 mM NaCl), but the expression is increased by high salt concentration, and the expression level under 200 mM NaCl is higher than under 50, 100, and 150 mM NaCl (**Supplementary Figure 3**). In the leaves, *GhCLCg-1* expression was up-regulated by the salt treatment at 1, 3, and 6 h. More specifically, the *GhCLCg-1* transcript level was highest at 3 h, but the increase (relative to expression level of the mock control) was greatest at 6 h (five times higher) (**Figure 4A**). To further demonstrate that *GhCLCg-1* is involved in plant responses to chloride-mediated salt stress, cotton plants were treated with 200 mM KCl (**Figure 4B**). The changes in the leaf *GhCLCg-1* expression levels under 200 mM KCl were similar to those resulting from the 200 mM NaCl treatment. Given the up-regulated *GhCLCg-1* expression induced by 200 mM NaCl,

the leaf *GhCLCg-1* expression levels following 6 h treatments with various NaCl concentrations (0, 50, 100, 150, and 200 mM) were analyzed. The relative *GhCLCg-1* expression level increased as the salt concentration increased, peaking at 200 mM NaCl (**Figure 4C**). Thus, *GhCLCg-1* expression in leaves was induced and regulated by chloride-mediated salt stress. Moreover, this expression was positively related to the chloride concentration.

### Overexpression of *GhCLCg-1* in *Arabidopsis thaliana* Enhanced Salt Tolerance

To further clarify the *GhCLCg-1* function related to chloride tolerance, *GhCLCg-1*-overexpressing (OE) transgenic *A. thaliana* plants were generated and examined (**Figure 5**). The WT, OE1, OE2, and OE3 *A. thaliana* plants had similar growth phenotypes on MS medium (Mock), with no significant differences in the root length and shoot growth. However, when treated with 100 mM NaCl, the transgenic *A. thaliana* plants (OE1, OE2, and OE3) had larger leaves and longer roots than the WT plants (**Figure 5A**). A quantitative analysis of the root length under salt stress conditions confirmed that the transgenic *A. thaliana* plants had significantly longer roots than the WT plants (**Figure 5C**). The *GhCLCg-1* expression level increased significantly in the transgenic *A. thaliana* plants, whereas *GhCLCg-1* expression was undetectable in the WT controls (**Figure 5B**). The changes in the Cl<sup>-</sup> contents in *A. thaliana* plants (WT, OE1, OE2, and OE3) were analyzed. Under normal conditions, there were no





significant differences in the  $\text{Cl}^-$  contents between the WT and transgenic plants. However, the  $\text{Cl}^-$  contents were significantly higher in the transgenic plants than in the WT controls under salt stress conditions (Figure 5D). These results indicate that *GhCLCg-1* confers salt tolerance, while also altering the uptake of  $\text{Cl}^-$  in plants.

High environmental  $\text{Na}^+$  and  $\text{Cl}^-$  levels alter the ion homeostasis of plant cells, resulting in an ionic imbalance and toxicity. Thus, re-establishing cellular ion homeostasis is critical for metabolic functions and growth (Niu et al., 1995). The changes in  $\text{Na}^+$  and  $\text{K}^+$  contents as well as the  $\text{Na}^+/\text{K}^+$  ratio in WT and transgenic *A. thaliana* plants (OE1, OE2, and OE3) were analyzed (Figure 6). There were no significant differences in the  $\text{Na}^+$  and  $\text{K}^+$  contents or the  $\text{Na}^+/\text{K}^+$  ratio among the WT, OE1, OE2, and OE3 plants on MS medium (Mock). An examination of the effects of the 100 mM NaCl treatment revealed that compared with the WT controls, the  $\text{Na}^+$  and  $\text{K}^+$  contents were significantly higher in the OE1, OE2, and OE3 plants, whereas the  $\text{Na}^+/\text{K}^+$  ratios were significantly lower in the OE1, OE2, and OE3 plants. These findings suggest that overexpression of *GhCLCg-1* can affect the accumulation of  $\text{Na}^+$  and  $\text{K}^+$ , then leads to a decrease of the  $\text{Na}^+/\text{K}^+$  ratios under the NaCl treatment, which is beneficial for plant tolerance to salt.

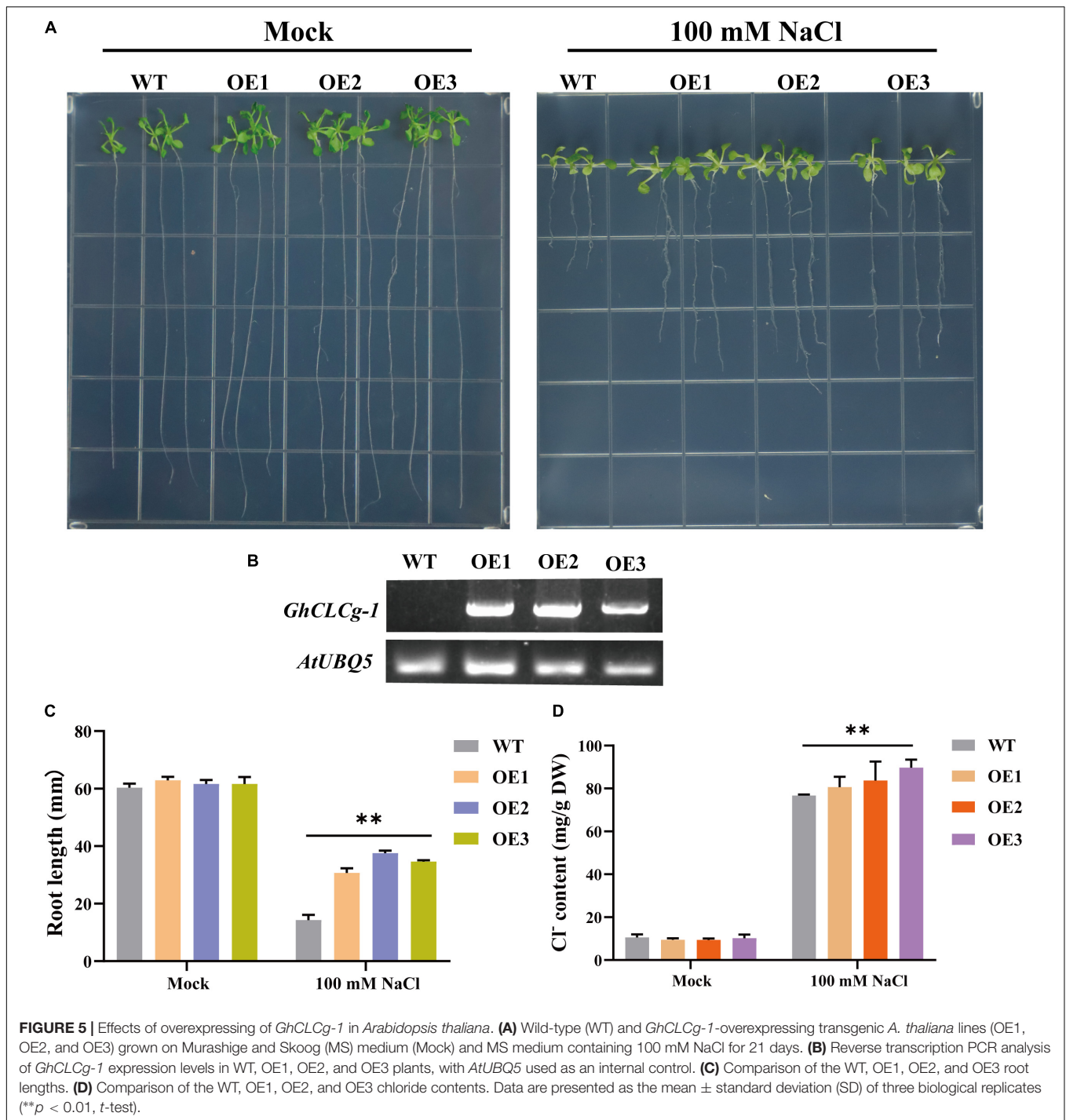
## Silencing of *GhCLCg-1* in Upland Cotton Compromised Salt Tolerance

To more thoroughly investigate the roles of *GhCLCg-1* related to the salt tolerance of upland cotton plants, we used the TRV-based VIGS system to generate *GhCLCg-1* knockdown cotton plants (Figure 7). At 10 days after the *A. tumefaciens* infiltration, the cotton seedlings transformed with *TRV:CLA* exhibited

an albino phenotype (Figure 7A). The *GhCLCg-1* expression levels were determined by qRT-PCR to assess how efficiently the gene was silenced in the cotton plants. The *GhCLCg-1* expression levels in the roots, stems, and leaves were obviously lower in the *TRV:GhCLCg-1* plants than in the *TRV:00* plants (Figure 7B). Accordingly, *GhCLCg-1* was efficiently silenced in the *TRV:GhCLCg-1* cotton plants.

There were no observable differences in the growth of *TRV:00* and *TRV:GhCLCg-1* plants under normal conditions (Mock). However, after a 3-day 200 mM NaCl treatment, several symptoms consistent with salt stress were detected on the *TRV:GhCLCg-1* plants (Figure 7C). Specifically, the leaves were shrunk and wilted and some of the leaves even fell from the plants. A decrease in the *GhCLCg-1* expression level increased the sensitivity of plants to salt stress, suggesting *GhCLCg-1* influences cotton salt tolerance. Furthermore, we analyzed the  $\text{Cl}^-$  contents in the roots, stems, and leaves of the *TRV:00* and *TRV:GhCLCg-1* cotton plants (Figure 7D). As expected, in the absence of chloride stress, there were no significant differences in the  $\text{Cl}^-$  contents of *TRV:GhCLCg-1* and *TRV:00* plants. However, following the 200 mM NaCl treatment, the root, stem, and leaf  $\text{Cl}^-$  contents were significantly higher in the *TRV:GhCLCg-1* plants than in the *TRV:00* plants. A comparison of these three tissues in *TRV:GhCLCg-1* plants revealed that the  $\text{Cl}^-$  contents were highest in the leaves, followed by the stems and then the roots. Compared with the *TRV:00* plants, the *GhCLCg-1*-silenced plants accumulated more  $\text{Cl}^-$ , especially in the leaves, making them more sensitive to salt stress.

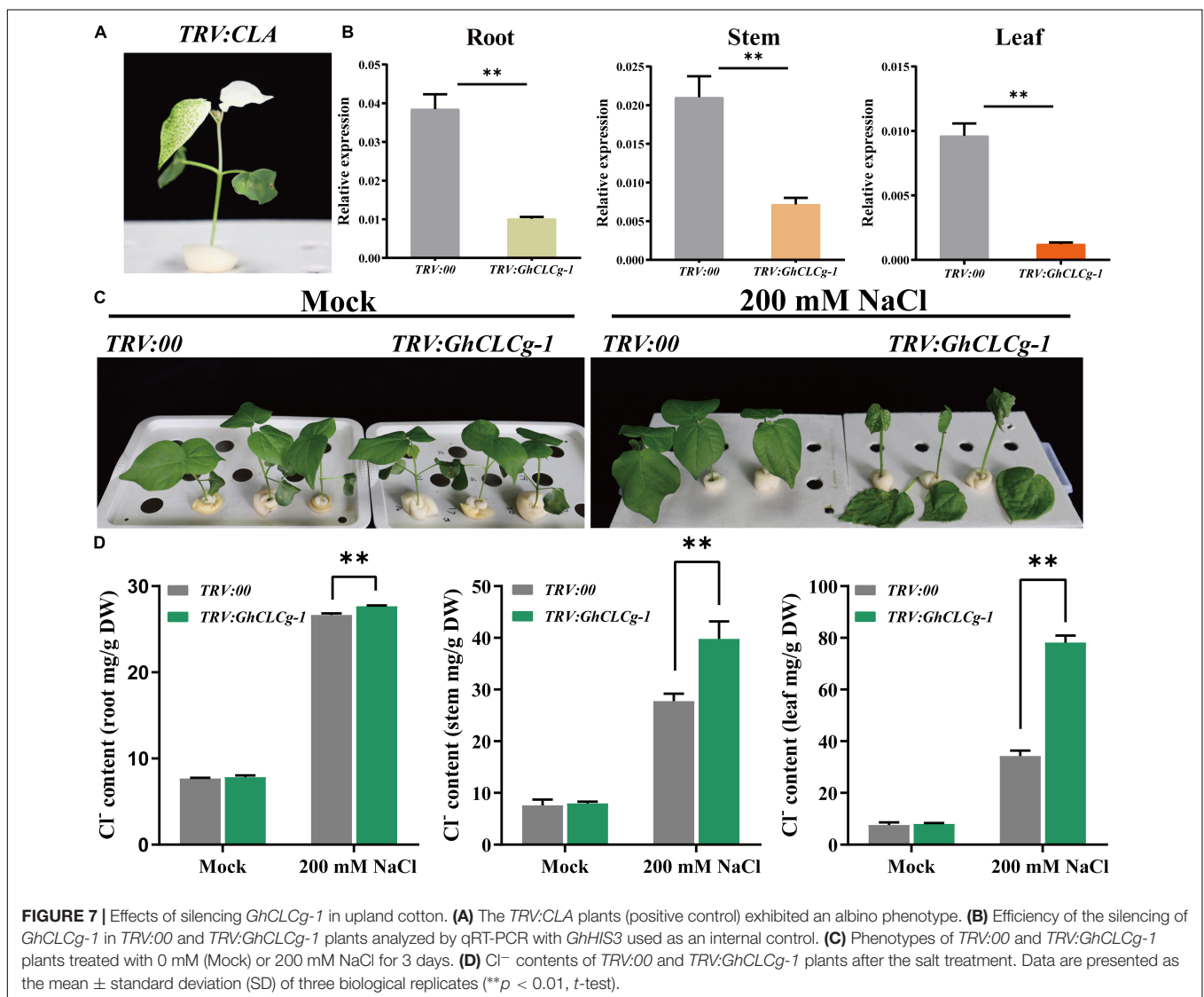
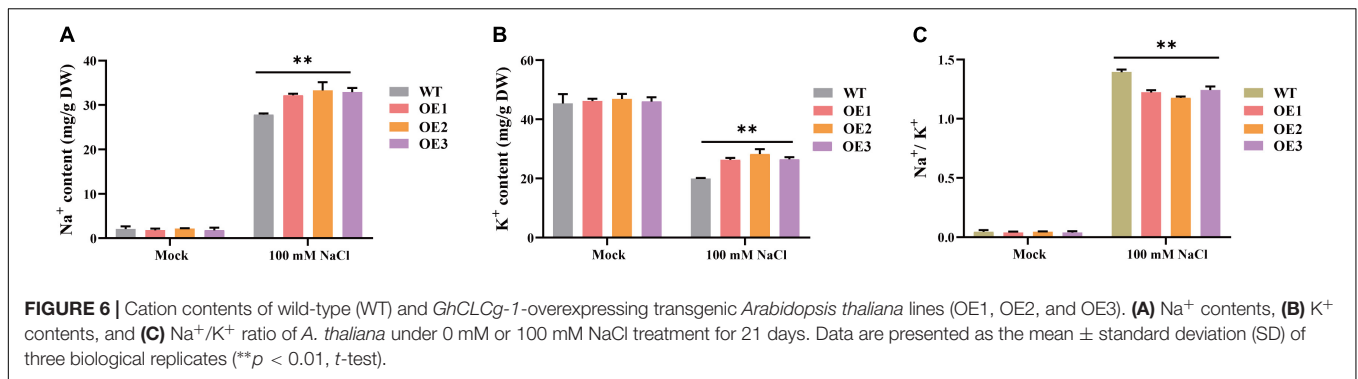
The  $\text{Na}^+$  and  $\text{K}^+$  contents as well as the  $\text{Na}^+/\text{K}^+$  ratio in *TRV:00* and *TRV:GhCLCg-1* plants were measured. There were no significant differences in the  $\text{Na}^+$  and  $\text{K}^+$  contents or the  $\text{Na}^+/\text{K}^+$  ratio between the *TRV:00* and *TRV:GhCLCg-1* plants in



the absence of salt stress (Figure 8). In contrast, after the 200 mM NaCl treatment, the shoot (stems and leaves) Na<sup>+</sup> content was higher in the *TRV:GhCLCg-1* plants than in the *TRV:00* plants, and the Na<sup>+</sup> content was highest in the *TRV:GhCLCg-1* leaves (Figure 8A). The NaCl treatment did not significantly affect the K<sup>+</sup> contents of the *TRV:00* and *TRV:GhCLCg-1* shoots, but it obviously decreased the K<sup>+</sup> contents of the *TRV:00* and *TRV:GhCLCg-1* roots (Figure 8B). Interestingly,

the Na<sup>+</sup>/K<sup>+</sup> ratios sharply and significantly increased in the stems and leaves of *TRV:GhCLCg-1* plants in response to the salt treatment, and the Na<sup>+</sup>/K<sup>+</sup> ratio was highest in the *TRV:GhCLCg-1* leaves (Figure 8C). These results indicate that silencing *GhCLCg-1* causes cotton plants to absorb Na<sup>+</sup> and accumulate in shoots, then leads to an increased Na<sup>+</sup>/K<sup>+</sup> ratio in stems and leaves under NaCl treatment, which increases sensitivity to salinity stress.

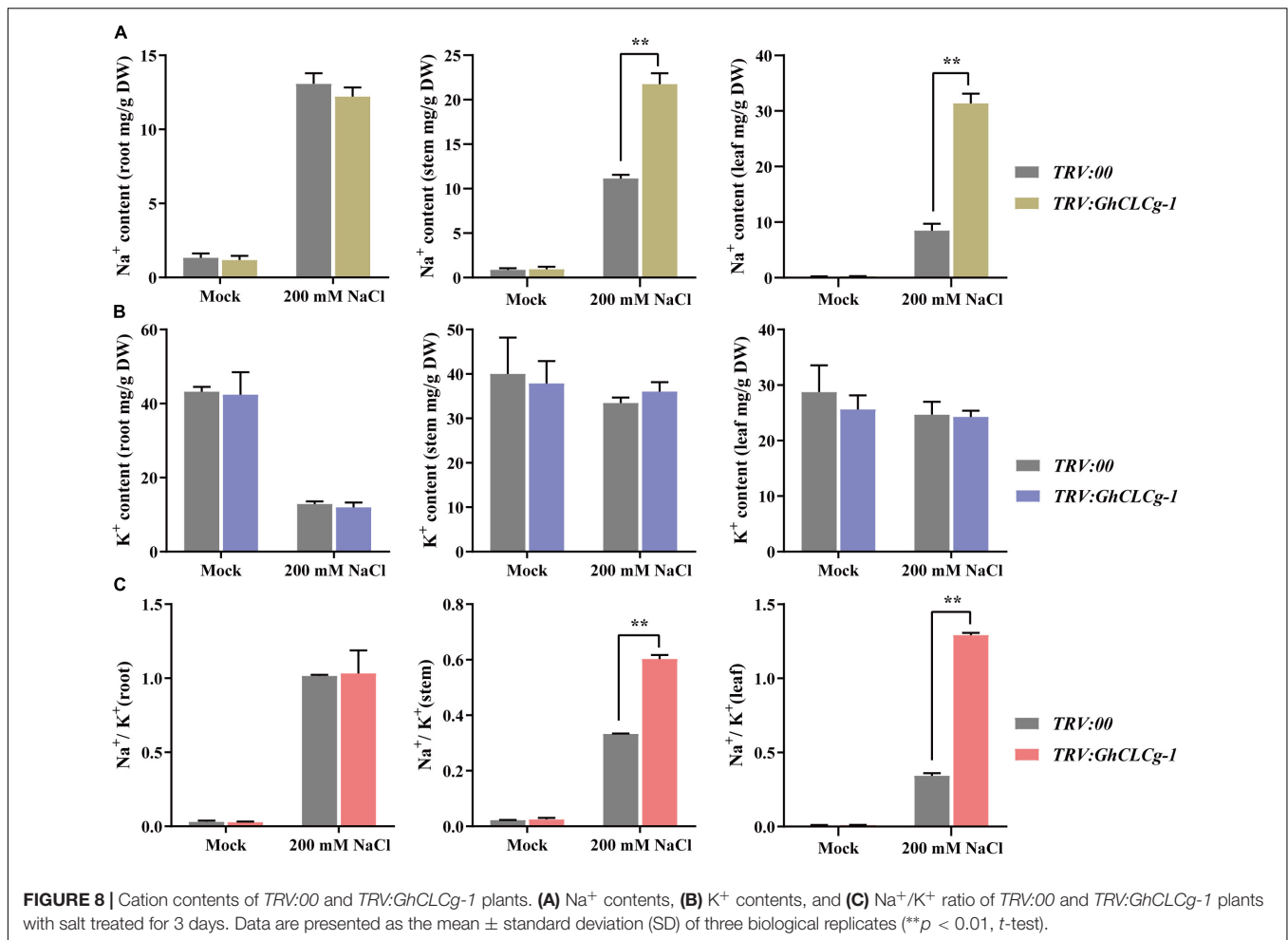




## DISCUSSION

Many studies showed that salt ions can induce osmotic stress and ion toxicity in plants and restrict their growth (Munns and Tester, 2008). Moreover, soil salinity interferes with

water and nutrient uptake by the roots, and salt ions accumulate more in leaves than in roots (Tester and Davenport, 2003). In the current study, we proved that chloride salt stress causes cotton plants to accumulate more salt ions ( $\text{Cl}^-$ ,  $\text{Na}^+$ , and  $\text{K}^+$ ), especially in the leaves. Cotton plant growth and development



were significantly inhibited by high salinity (Figure 1 and Supplementary Figure 1).

Previous studies revealed that *AtCLCg* in *A. thaliana* is localized to the tonoplast and modulates chloride homeostasis in response to chloride stress, with shoot chloride levels increasing in *atclcg* knockout mutants (Nguyen et al., 2015). In the current study, we cloned a chloride channel gene (*GhCLCg-1A/D*) in upland cotton. Analysis of amino acid sequences revealed *GhCLCg-1A/D* belong to the *CLCg* subfamily and contain the conserved amino acid sequence motif (GSGIPE) associated with chloride selectivity (Atsuko et al., 2006; Li et al., 2006; Zifarelli and Pusch, 2010). The qRT-PCR data verified that *GhCLCg-1* is responsive to chloride salt stress in leaves. At the transcriptional level, *GhCLCg-1* was similarly affected by 200 mM KCl and 200 mM NaCl (Figures 4A,B). The leaf *GhCLCg-1* transcription level increased as the treatment duration and chloride concentration increased (Figures 4A,C). These results imply *GhCLCg-1* is involved in the chloride-mediated salt stress response in upland cotton plants.

Plants can minimize the effects of salt stress by eliminating excess salt ions through plasma membranes or by sequestering them in vacuoles (Zelm et al., 2020). The biological roles of plant proteins are closely related to their subcellular localization

(Long et al., 2020). Our study confirmed that *GhCLCg-1A/D* are localized to the tonoplast (Figure 3), suggesting they may contribute to the transport of  $\text{Cl}^-$  into vacuoles to participate in plant response to chloride salt. In a previous study, *GmCLC1*-overexpressing soybean plants sequestered more  $\text{Cl}^-$  in the roots and transported less  $\text{Cl}^-$  to the shoots than the WT controls, which enhanced the salt tolerance of the transgenic plants (Wei et al., 2016). The overexpression of the vacuolar chloride channel gene *GsCLC-c2* influences  $\text{Cl}^-$  homeostasis. More specifically, it improves salt tolerance by increasing the accumulation of  $\text{Cl}^-$  in the roots, thereby decreasing the amount of  $\text{Cl}^-$  transported to the shoots. Using two-electrode voltage clamps and *Xenopus laevis* oocytes, a previous study proved that *GsCLC-c2* mediates the transport of  $\text{Cl}^-$  (Wei et al., 2019). The insertion of the vacuolar chloride channel gene *OsCLC-1* into *gef1* yeast cells, in which the single putative chloride channel is mutated, decreases the relative sensitivity of yeast to various chloride salts (Atsuko et al., 2006).

In this study, our qRT-PCR analysis suggested that tonoplast-localized *GhCLCg-1* is involved in chloride stress responses (Figure 4), which is consistent with the findings of a recent investigation (Liu et al., 2020). The overexpression of *GhCLCg-1* in *A. thaliana* can enhance salt tolerance and alter  $\text{Cl}^-$

accumulation (Figure 5). During an exposure to NaCl stress, most of the  $\text{Cl}^-$  is not in vacuoles, but is in the cytoplasm or the apoplast, where it inhibits enzyme activities or increases water loss, respectively, in the absence of AtCLCg (Flowers et al., 2010; Teakle and Tyerman, 2010). The overexpression of the chloride channel gene *CsCLC* in *A. thaliana* increases the salt tolerance of the transgenic plants. Moreover, the root and shoot  $\text{Cl}^-$  contents are lower in the transgenic plants than in the mutant or WT plants (Wei et al., 2013). In this study, the transgenic *A. thaliana* plants overexpressing *GhCLCg-1* accumulated more  $\text{Cl}^-$  than the WT controls (Figure 5C). This is in contrast to the  $\text{Cl}^-$  accumulation in the *CsCLC*-overexpressing *A. thaliana* plants examined in an earlier investigation (Wei et al., 2013), but it is similar to the reported changes to the  $\text{Cl}^-$  contents in *A. thaliana atclcg* mutants (Nguyen et al., 2015). It could be deduced that the results might be related to the role of CLCg. In *A. thaliana atclcg* mutants, loss of function of *AtCLCg* would result in accumulating more  $\text{Cl}^-$  in the cytoplasm, because  $\text{Cl}^-$  transport to the vacuole was disrupted, which increased  $\text{Cl}^-$  contents and compromised salt tolerance. However, in the *A. thaliana* plants overexpressing *GhCLCg-1*, the extra  $\text{Cl}^-$  was transported and accumulated in the vacuole by *GhCLCg-1* to reduce its toxic effects in the cytoplasm, which also increased  $\text{Cl}^-$  contents, but enhanced salt tolerance. Similar to the *A. thaliana atclcg* mutants, silencing *GhCLCg-1* expression via VIGS also increased the  $\text{Cl}^-$  contents and the sensitivity of cotton plants to chloride salt (Figure 7). Notably, *TRV:GhCLCg-1* plants accumulated more  $\text{Cl}^-$  in the leaves than in the roots under salt stress conditions, which was consistent with previous studies that *A. thaliana clc* mutants have more  $\text{Cl}^-$  in the shoots than in other tissues (Jossier et al., 2010; Nguyen et al., 2015). The ion homeostasis is crucial for the salt tolerance of higher plants (Li et al., 2006). Furthermore, a low  $\text{Na}^+/\text{K}^+$  ratio may maintain cell metabolic activities as well as salt tolerance (Blumwald, 2000). Although more  $\text{Na}^+$  and  $\text{K}^+$  were accumulated in the *A. thaliana* plants overexpressing *GhCLCg-1*, the  $\text{Na}^+/\text{K}^+$  ratio was still lower than that of the WT plants under 100 mM NaCl (Figure 6). By contrary, the stem and leaf accumulated more  $\text{Na}^+$  in the *TRV:GhCLCg-1* plants, leading to a higher  $\text{Na}^+/\text{K}^+$  ratios than that in the *TRV:00* plants after treatment with 200 mM NaCl (Figure 8). Considered together, these observations reveal that the *GhCLCg-1* play a vital role in salt tolerance of upland cotton by regulating ion accumulation.

## CONCLUSION

In conclusion, we revealed upland cotton plants accumulated  $\text{Cl}^-$  and were damaged by chloride salt stress. Gene expression profiles suggested that the expression of *GhCLCg-1*, which encodes a tonoplast-localized chloride channel, is up-regulated in response to chloride salt stress. The overexpression of *GhCLCg-1* in *A. thaliana* altered the accumulation of ions with a decrease of the  $\text{Na}^+/\text{K}^+$  ratios, and enhanced salt tolerance. Moreover, cotton plants, in which *GhCLCg-1* was silenced, accumulated more  $\text{Cl}^-$  and increased the  $\text{Na}^+/\text{K}^+$  ratios, leading to greater sensitivity to chloride salt. These results indicate that upland cotton plants accumulate  $\text{Cl}^-$  under salt conditions and

*GhCLCg-1* alleviates damages resulting from excessive amounts of chloride salt.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

WL and ZM conceived and designed the research. WL, JF, and WM performed the experiments and analyzed the data. ZM provided the materials. WL and JF prepared the figures and wrote the manuscript. ZM and YZ revised the manuscript. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.765173/full#supplementary-material>

**Supplementary Figure 1** | Cation contents in upland cotton plants.  $\text{Na}^+$  contents (mg/g DW) of upland cotton plants (roots, stems, and leaves) treated with no chloride (Mock) (A) or 200 mM NaCl (B) for 10 days.  $\text{K}^+$  contents (mg/g DW) of upland cotton plants (roots, stems, and leaves) treated with no chloride (Mock) (C) or 200 mM KCl (D) for 10 days. (E) Heatmap of the cation contents of upland cotton plants treated with no chloride (Mock), 200 mM NaCl, or 200 mM KCl. The cation concentrations are indicated by different colors and the colors are consistent in panels (A–E).

**Supplementary Figure 2** | Sequence alignment of the GhCLCg-1A, GhCLCg-1D, and AtCLCg. Conserved residues GxxGIPE, GKxGPxxH, and PxxGxLF are indicated by the red, blue, and green boxes, respectively.

**Supplementary Figure 3** | *GhCLCg-1* expression patterns in the stems of plants treated for 3 h with different NaCl concentrations (0, 50, 100, 150, and 200 mM). The  $2^{-\Delta\text{CT}}$  method was used to calculate relative expression levels. Error bars indicate the standard deviation (SD) of three biological replicates (\*\* $p < 0.01$ ;  $t$ -test).

**Supplementary Table 1** | Primers used in this study.

**Supplementary Table 2** | Genes used in this study.

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