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Molecular cloning and functional characterization of a novel cotton CBL-interacting protein kinase gene (*GhCIPK6*) reveals its involvement in multiple abiotic stress tolerance in transgenic plants

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ABSTRACT

Plant CIPKs were specific Ser/Thr protein kinases, which were activated through interaction with calcineurin B-like protein (CBL) containing four EF hands for Ca^{2+} binding. The CBL/CIPK complexes play an important role in signal transduction in biotic and abiotic stresses, as well as developmental processes. Here a Ser/Thr protein kinase gene (defined as *GhCIPK6*), which was isolated from RNA-Seq profile during cotton somatic embryogenesis in our previous research was characterized. The *GhCIPK6* gene contains an ORF of 1296 bp that putatively encodes a polypeptide of 431 amino acids with a predicted molecular mass of 48.46 kDa and isoelectric point of 9.12. Sequence alignment analysis confirmed that *Gh*CIPK6 has no intron, and it was homologous to *AtCIPK6*. Expression analysis of the *Gh*CIPK6 suggested that they might function in diverse tissues, including styles and anthers but not fibers. In addition, expression of the *Gh*CIPK6 gene was induced by salt, drought and ABA treatments. Overexpression of *GhCIPK6* significantly enhances the tolerance to salt, drought and ABA stresses in transgenic *Arabidopsis*, indicating that *GhCIPK6* acts as a positive regulator in response to salt and drought stress, and is supposed to be a potential candidate gene to improve stress tolerance by genetic manipulation in cotton and other crops.

1. Introduction

Plants need to meet the challenge from diverse environment changes during the whole growth and development process, including some abiotic stresses (drought, salinity, and cold). In order to respond and adapt to these stresses, plants can initiate a number of molecular, cellular, and physiological changes and evolve various survival mechanisms. Most abiotic stresses can elicit an increase of cytosolic free Ca^{2+} concentration in cells, and the change of Ca^{2+} concentration has been generally accepted as a secondary messenger to transduce the cellular responses to extracellular stimuli [17]. Cellular calcium signals are detected and transmitted by sensor molecules. There were three major classes of Ca^{2+} sensors identified in plants: calmodulin (CaM) and CaM-related proteins, calcium-dependent protein kinase (CDPK) and calcineurin B-like proteins (CBLs). CBLs represents a unique group of calcium sensor and plays a key role in decoding calcium transients by specifically interacting with and regulating a family of protein kinases (CIPKs) in higher plants [18]. The plant CIPK contains a specific Ser/Thr protein kinase domain that is activated through interaction with CBL containing four EF hands for Ca^{2+} binding [1]. Activated CIPKs can subsequently transduce calcium signals by phosphorylating downstream signaling components [7]. Genome sequence analysis of *Arabidopsis*, rice (*Oryza sativa*) and populus (*Populus trichocarpa*) revealed that there were 26 (*Arabidopsis*), 30 (rice) and 25 (populus) CIPK genes in their genome respectively [12,26,27].

The expression of CIPKs was regulated by many factors including abiotic stresses, plant hormones and nutrient deprivation. Some CIPKs were well illuminated in model plant Arabidopsis. It was initially identified in a genetic screen for a salt overly sensitive (SOS) phenotype [15]. *AtCIPK24* (SOS2) interacts with *AtCBL4* (SOS3) to transduce Ca^{2+} signal, thereby activates a plasma membrane-localized Na^+/H^+ antiporter (SOS1) and vacuolar H^+-ATPase to maintain ion homeostasis during salt stress [6,15]. *CIPK23*, which was regulated by the upstream regulators CBL1/9, are crucial for K^+ uptake under low-K^+ conditions, it directly interacts with and positively regulates the potassium transporter *AKT1* by phosphorylation. Homozygous cipk23 plants are hypersensitive to low K^+ concentrations [14,24]. Expression of *CIPK3* is enhanced during the early stages of seedling development, and the cipk3

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mutant shows abscisic acid (ABA) hypersensitivity during seed germination [10]. In contrast, expression of *CIPK20* rendered the transgenic plants hypersensitive to ABA, while RNAi lines showed insensitivity to ABA [5]. Similar interaction networks seem to exist in other plants, such as in rice which indicated by the presence of ten CBLs and 30 CIPKs and a conserved SOS pathway [16,22]. Transgenic plants with over-expressing *BnCIPK6* in *Arabidopsis* enhanced high salinity and low phosphate tolerance in transgenic plants, and activation of *BnCIPK6* (*Brassica napus*) confers *Arabidopsis* plants hypersensitive to ABA [2]. Ectopic expression of *a CaCIPK6* (*Cicer arietinum*) enhanced the salt tolerance in transgenic plants [21]. The expression of *AtCIPK6* was induced by ABA, osmotic and salt stress, and it was interacted with *AtCBL1/3/4* to confer the salt tolerant in chickpea [10].

Cotton (*Gossypium hirsutum* L.) is an important worldwide commercial crop grown as a source of natural fiber and edible oil. As a glycophytic plant, cotton shows higher drought and salt tolerance than other major crops [3,8]. However, the watering limitation and salinization of cotton cultivated areas remains an increasing threat to cotton production. And the salt and drought tolerance mechanism in cotton are far away from being clearly illuminated. Here, based on a cDNA fragment, zhu1_Ghi#S42301775 from RNA-Seq profile during cotton somatic embryogenesis (SE) [25], *GhCIPK6* were cloned and documented. Expression analysis of the *GhCIPK6* suggested that it may function in response to salt and drought stress. To investigate the functional role of the drought- and salt-induced *CIPK6* homologue from cotton, we expressed its constitutively active form in *Arabidopsis*. To our surprise, we observed that the transgenic seedlings displayed salt and drought tolerance in transgenic *Arabidopsis*. Gene expression analysis under drought-stress and normal growth conditions provided evidence of a dual role for *CIPK6* in stress responses. Our results provide evidence for involvement of the *GhCIPK6* in the drought-stress response, by regulating the expression of relative genes.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatments

The pre-germinated seeds of upland cotton (*G. hirsutum* L.) *cv.* YZ1 were planted in small pots with a depth of 1.0 cm. Three-week-old seedlings were used for gene expression analysis in response to ABA or various abiotic stresses at the stage of one fully expand euphylla and one bud. The leaves and roots were harvested at different time points after treatment (100 μmol L^{-1} ABA at 0.5 h, 1 h, 2 h, 4 h, and 8 h; 200 mmol L^{-1} NaCl and 15% PEG at 1 h, 2 h, 4 h, 8 h and 12 h), frozen in liquid nitrogen, and preserved at −70 °C for later RNA isolation. In parallel, three-week-old seedlings grown in natural conditions were used as control. For expression analysis, different stage samples during cotton SE [excised fresh hypocotyl (SE0 h), 24 h-cultured hypocotyl (SE24 h), 48 h-cultured hypocotyl (SE48 h), NEC, EC, somatic embryos (SEs)] were collected, various tissues (root, stem, leaf from three-week-old wild-type seedlings; style, anther, ovule and fiber from mature plants) were harvested respectively and immediately frozen in liquid nitrogen.

2.2. Cloning and sequences analysis of GhCIPK6

Full-length cDNA of *GhCIPK6* were obtained by 5′ and 3′ Rapid Amplification of cDNA Ends (RACE). The gene-specific primers (forward 5′ CCAAATATCCGCCTTGGCTCCGTC 3′, reverse 5′ GGTGAGG TTGCAGGGTCAAGAATGT 3′) of *GhCIPK6* were designed based on EST zhu1_Ghi#S42301775 (Accession number in GenBank ES816624), which differentially expressed during cotton somatic embryogenesis [25]. The RACE was performed using SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, USA) and Advantage™ 2 PCR Enzyme Kit (Clontech) in accordance with the manufacturer. The full-length cDNAs were amplified using gene-specific primers (*GhCIPK6*-full-F: 5′ AATGGCGGACAAAGCTAAAACC 3′, *GhCIPK6*-full-R: 5′ CTCACATAAATCAAGCCACAGTCG 3′). The genomic sequences of *GhCIPK6* gene was amplified using the genomic DNA as templates.

For phylogenetic analysis, the amino acid sequence of *GhCIPK6*, 26 *AtCIPKs*, *CaCIPK6* (ACC96114) and *BnCIPK6* (JF751063) were aligned using the ClustalX program [20] and then maximum parsimony analysis was performed by MEGA4 [19] using the neighbor-joining method with default.

2.3. qRT-PCR analysis

To determine the expression level of *GhCIPK6* in wild-type and transgenic plants, cotton RNA was isolated from the collected tissues using the method described in our previous report [20]. *Arabidopsis* total RNAs were extracted using Trizol reagent (Invitrogen, USA). Gene-specific primers (forward 5′ GGTGGGAAGTCA TTGTTTGATG 3′, reverse 5′ AAAGCAGCCCACTACCACAA 3′) were designed and synthesized and qRT-PCR was performed as previously described using three biological replicates and three technological replicates [25]. Relative quantitation of gene expression was calculated and normalized using *GhUBQ7* (GenBank accession number: DQ116441) as internal standard.

Table 1
Primer sequences used in RT-PCR.

Gene	Accesion number	Forward primer (5′–3′)	Reverse primer (5′–3′)
AtCIPK6	At4g30960	CATGGTTCTCACCTGAAGCTCG	TTGAACCAAGATGATTCCGTGA
GhCIPK6	KC465063	GGCGAGCAAGTCCAAGATTT	TTGGCTCCGTCGTACCCTTT
AtCBL1	At4g17615	ATCACTCAATGTTTTCCACCCC	TGGCAATCTCATCGACCTCC
AtCBL4	At5g24270	TCACGGTAGAAGAAGTGGAGGC	GATGAGCGATGGATTCAAGGAT
AtAKT2	At4g22200	TGACTAATTTGGTCGTGGAAGG	TTGGGAGCTGGTCAATAAGATG
AtKAT1	At5g46240	AGTCGGTGCCTTGTTTGCC	CCAGGTCTTTGCGGGGTT
AtSKOR1	At3g02850	GCTGGAGGTGACCCGAATAAG	ACAAGCAACGCCGCAACA
AtTPK1	At5g55630	TCCTCATCCGAGTAAAATCCC	CCGAGAAGACGAAGGCACA
AtDREB1A	At4g25480	CAGAGAATTCCGGATCCCAATGAACTCATTTTCTGCT	CCGCACTCGAGGTCGACCGTCGCATCACACATCTC
AtDREB2A	At5g5410	GATCCGAATTCATGGCAGTTTATGATCAGAGTGG	CAGCACTCGAGGTCGACGGATCCTCTGTTTTCAC
AtRD22	At5g25610	GGTTCGGAAGAAGCGGAGAT	GGAAACAGCCCTGACGTGATAT
AtRD29A	At5g52310	GGAAGACCTGGATACGGTGA	GTGCTCTGTTTTGGCTCCTC
AtABI1	At4g26080	ACCGATGCTCTGCGATGG	CGAAGATGTGAGACGGGAAAA
AtABI2	At5g57050	AGTCGCTGTTCCATTCAGACC	TCCGTCGCCAGAACAAGAA
AtACTIN2	At3g18780	TTCCTCATGCCATCCTCCGTCTT	CAGCGATACCTGAGAACATAGTGG

2.4. Plasmid construction and plant transformation

To construct the overexpression vector, full ORF of *GhCIPK6* was amplified using a pair of primers (CIPK6OE-F: 5′ GGGGACAAGTT TGTACAAAAAAGCAGGCTGTAATGGCGGACAAAGCTAAAACC 3′ and CIPK6OE-R: 5′ GGGGACCACTTTGTACAAGAAAGCTGGGTACTCACA-TAAATCAAGCCACAGTCG 3′) and then constructed to pK2GW7.0 vector by homologous recombination. The overexpression construct of *GhCIPK6* was used to transform into *Arabidopsis* wild type plants by a floral dipping method mediated by *Agrobacterium* strain GV3101. The seeds of positive transgenic plants carrying the *GhCIPK6* constructs were individually harvested. Homozygous transgenic lines were used for further investigation.

2.5. Stress tolerance assay in transgenic Arabidopsis plants

Homozygous transgenic lines were used to test tolerance to stress treatments. Seeds of wild-type and transgenic plants were surface-sterilized in 75% ethyl alcohol for 1 min and 95% ethyl alcohol for 3 min and then rinsed five times with sterile water. The seeds were sown on 1/2 MS solid medium containing different concentration of NaCl and ABA, vernalized at 4 °C for 2 days, and

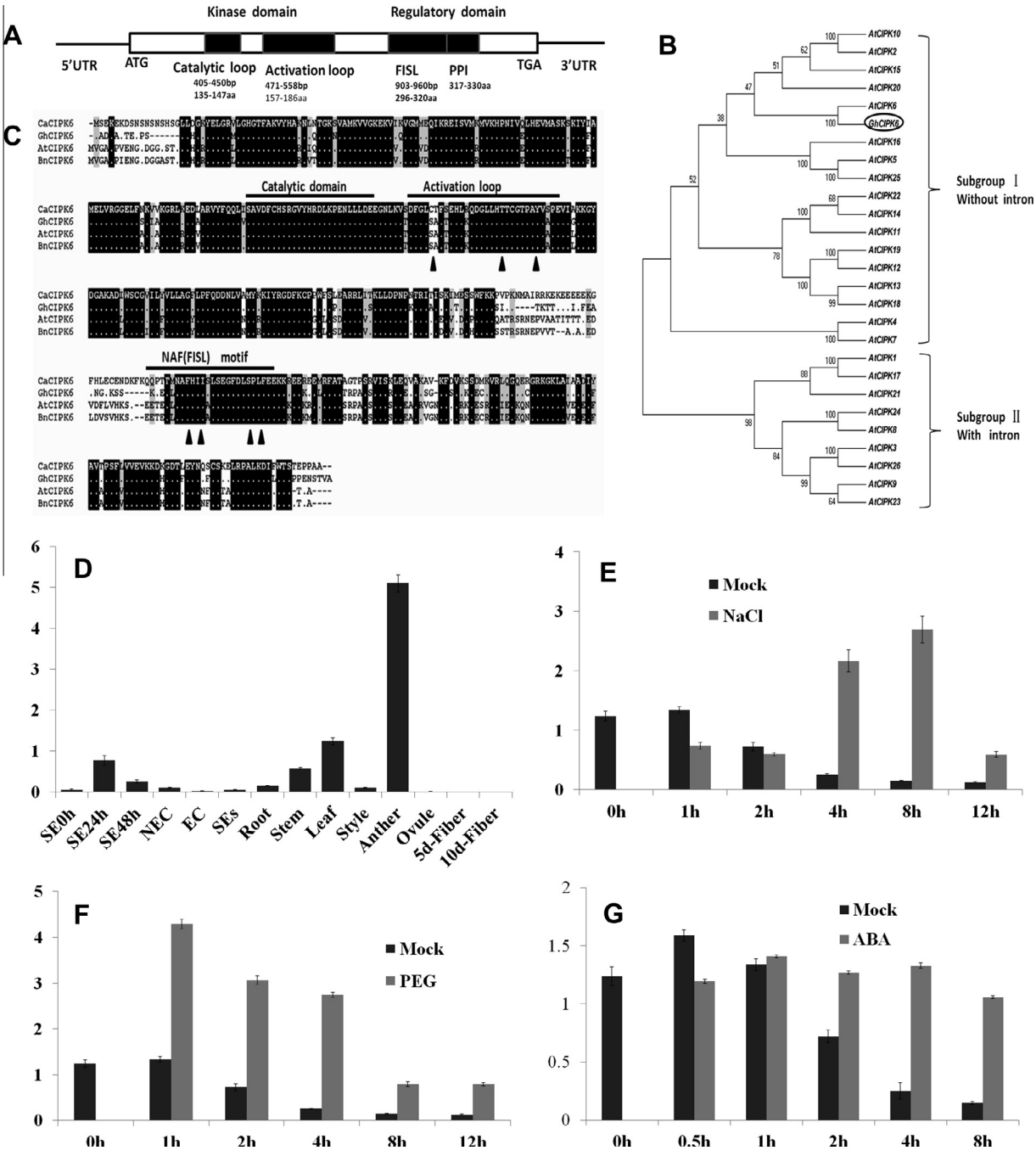

Fig. 1. Sequence and expression analysis of *GhCIPK6*. (A) Schematic diagram of the domain structure of *GhCIPK6*. Rectangles represent exon and lines represent untranslated regions of the gene. The overall structure of *GhCIPK6* comprises a N-terminal kinase domain and a regulatory C-terminal domain that are separated by a junction domain. (B) Phylogenic analysis of *GhCIPK6* (from cotton) with 26 *AtCIPK* proteins (from *Arabidopsis*). (C) Amino acid sequence comparison of *GhCIPK6* (KC465063), *CaCIPK6* (ACC96114), *BnCIPK6* (JF751063), and *AtCIPK6* (AF285106). Arrowheads below the sequences indicate the conserved amino acids. Solid and dashed above the sequences indicate subdomains of Activation loop and Ser/Thr protein kinases, respectively. (D) qRT-PCR analysis of *GhCIPK6* transcripts during cotton SE and other cotton tissues. (E–G) Expression patterns of *GhCIPK6* under 200 mM NaCl, 15% PEG, and 100 μM ABA treatments in *G. hirsutum* YZ1.

then transferred to 22 °C for growth. Germination rate was scored a few days after transferring. For seedling growth in NaCl and ABA, 3-day-old seedlings were transferred to 1/2 MS medium containing the given NaCl and ABA concentrations. Growth was monitored using survival rate and plant height assay.

To test the drought tolerance of transgenic plantlets in soil, self-rooted plantlets (WT, overexpression lines) were transferred to soil. After 4 weeks, plantlets grown in pots were deprived of water for 20 days. After re-watering, they were incubated at 22 °C for another 3 days. The normal irrigated pots were used as control. All experiments were conducted in triplicate.

2.6. Expression analysis of stress-related genes in transgenic Arabidopsis

To study the possible effects of GhCIPK6 overexpression in *Arabidopsis* on stress-related genes, *AtCBL1* (*At4g17615*), *AtCBL4* (*At5g24270*), *AtAKT2* (*At4g22200*), *AtKAT1* (*At5g46240*), *AtTPK1* (*At5g55630*), *AtSKOR1* (*At3g02850*), *DREB1A* (*At4g25480*), *DREB2A* (*At5g5410*), *RD22* (*At5g25610*), *RD29A* (*At5g52310*), *ABI1* (*At4g26080*), and *ABI2* (*At5g57050*) were used for RT-PCR analysis under the normal condition and drought stress. The primers used are list in Table 1.

3. Results

3.1. Cloning and bioinformatics analysis of GhCIPK6

Based on the sequence of the cDNA fragment, zhu1_Ghi#S42301775 from RNA-Seq profile during cotton somatic embryogenesis [25], we obtained the full length cDNA by rapid amplification of cDNA ends (RACE) and designated as *GhCIPK6* (GenBank accession number: KC465063), which contains an ORF of 1296 bp that putatively encodes a polypeptide of 431 amino acid residues with a predicted molecular mass of 48.46 kDa and an isoelectric point of 9.12, with 113 bp 5′ UTR and 272 bp 3′ UTR. Subsequently, the complete genome DNA sequence was isolated from *G. hirsutum*. It was found that the *GhCIPK6* gene contains no intron in its open reading frame in comparing with its cDNA sequence. *GhCIPK6* encodes a CBL-interacting protein kinase, exhibiting the same family signature. The mature protein contains an N-terminal SNF1-like kinase catalytic domain and a C-terminal regulatory domain with a CBL-interacting NAF/FISL module, including 4 conserved subdomains, a catalytic loop (135th–147th amino acid), a activation loop (157th–186th amino acid), a FISL domain (296th–320th amino acid) and a PPI domain (Fig. 1A).

For phylogenetic analysis, the amino acid sequence of *GhCIPK6* was first aligned with 26 *AtCIPKs*. *GhCIPK6* exhibited high similarity to *AtCIPK6* (Fig. 1B). In NCBI blast, the homology of amino acid sequence of *GhCIPK6* to *RcCIPK* (EEF28408), *PtCIPK9* (ABJ91216), *AtCIPK6* (AF285106) and *CaCIPK6* (ACC96114) was 88%, 85%, 82% and 78%. Further comparative phylogenetic aligning analysis of the *GhCIPK6*, *AtCIPK6*, *CaCIPK6* and *BnCIPK6* (JF751063) protein sequences indicated that the NAF/FISL module was conserved in these species, except a few mismatching amino acid (Fig. 1C).

3.2. GhCIPK6 response to abiotic stresses and ABA

qRT-PCR was performed to determine the expression levels of *GhCIPK6* in different tissues. Total RNA was isolated from different stage of cotton SE (SE0 h, SE24 h, SE48 h, NEC, EC, SEs), various tissues (root, stem, leaf, style, anther, ovule and fiber). The result

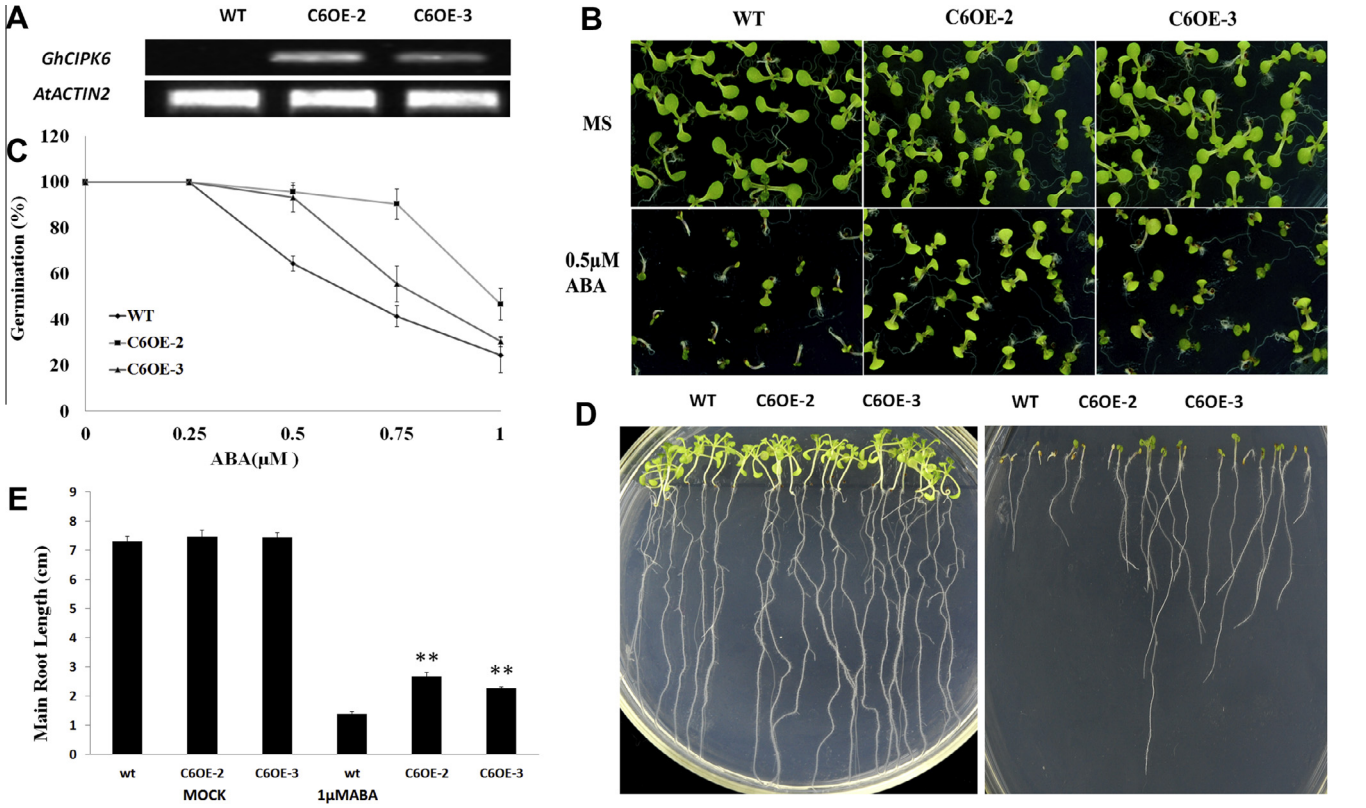

Fig. 2. Reduced ABA sensitivity in *GhCIPK6*-overexpressed plants. (A) RT-PCR analysis of *GhCIPK6* expression from transgenic *Arabidopsis* and wide type. The gene *AtACTIN2* was analyzed as a quantitative control. (B) The phenotype of *GhCIPK6*-overexpressing and wild-type *Arabidopsis* in 1/2 MS medium with or without ABA. (C) Germination rates of wild-type and transgenic seeds after 10rd on 1/2 MS medium containing different concentrations of ABA. (D) Representative images of wide type and transgenic lines roots grown on MS agar medium (left) or MS medium containing 1 μM ABA (right), the photograph was taken on day 14 after transferred to 22 °C. (E) Effects of ABA (1 μM) on root length of Col-0 and transgenic lines. Root lengths were measured 14 days after transferred to 22 °C.

showed that *GhCIPK6* transcripts were produced in all cotton tissues tested including callus, root, hypocotyl, leaf, style, anther, ovule and fiber. However, its expression levels varied in different tissues. Anther produced the highest level of *GhCIPK6* transcripts, while EC, ovule and fiber did the lowest. *GhCIPK6* transcripts were accumulated at 24 h hypocotyls but showed less in EC and somatic embryos during cotton SE. Moreover, *Gh*CIPK6 was strongly expressed in the leaves, but with almost no detectable expression in the root (Fig. 1D).

In addition, the expression of *GhCIPK6* was also analyzed in response to various abiotic stresses including salinity (200 mM NaCl); ABA (100 μm ABA) as well as PEG simulated drought (15% PEG) stresses. The results indicated that expression of *GhCIPK6* was positively induced by salinity, PEG and ABA. Especially, it could be induced dramatically by PEG and during early incubation and NaCl during late incubation (Fig. 1E–G). Therefore, it may come to a conclusion that *GhCIPK6* expression specifically responds to salt, drought and ABA in cotton.

3.3. Reduced ABA sensitivity in GhCIPK6-overexpressing Arabidopsis

To ascertain whether *GhCIPK6* plays any roles in ABA signaling, we ectopically overexpressed the *GhCIPK6* gene in *Arabidopsis* and examined its effects on the stress response in these plants. Several homozygous lines were obtained by selfing the 30 original T_0 transformants for two generations. Two homozygous lines (OE-2, OE-3) with *GhCIPK6* expressed were selected for further analysis (Fig. 2A). The seedling growth of two transgenic lines showed no obvious difference from that of corresponding control plants, when grown on medium without ABA. After treatment with 0.5 μM ABA, transgenic lines had significantly bigger shoots and longer roots than the control (Fig. 2B). Without treatment with ABA, the seed germination rate and seedling growth of transgenic plants was comparable to that of wild-type plants. However, following a lower dosage (0.5 μM) ABA treatment, the transgenic lines germinated and grew to a higher extent than did the wild-type seeds (Fig. 2B and C). After 10 days, about 75% of the transgenic seeds but less than 50% of the wild-type seeds germinated. A higher dosage (1 μM) of ABA severely retarded post-germination growth of both wild-type and transgenic seedlings, and this effect was more pronounced in wild-type seedlings (Fig. 2D and E). These results suggest that *GhCIPK6* positively regulates *Arabidopsis* respond to ABA-mediated signalling.

3.4. Improved salt and drought tolerance in GhCIPK6-overexpressing Arabidopsis

To determine whether the *GhCIPK6* plays a role in stress response pathways, the response of *GhCIPK6*-overexpressing *Arabidopsis* to salt stress was analyzed using the same transgenic lines as indicated above. We compared the germination rate on 1/2 MS medium containing different concentration NaCl with the same medium without NaCl (control), it was found the seed germination rate and seedling growth of *GhCIPK6*-overexpressing plants was comparable to that of wild-type plants in 1/2 MS medium without NaCl (Fig. 3A). However, the same assay on the medium containing high concentrations of NaCl ($\geqslant$150 mM) indicated that *GhCIPK6*-overexpressing seeds were more tolerant to NaCl treatments than were control seeds, the transgenic lines germinated and grew to a higher extent than did the wild-type seeds (Fig. 3B–E). For example, on medium containing 150 mM NaCl, two *GhCIPK6*-overexpressing transgenic lines showed 84% and 78% germination in

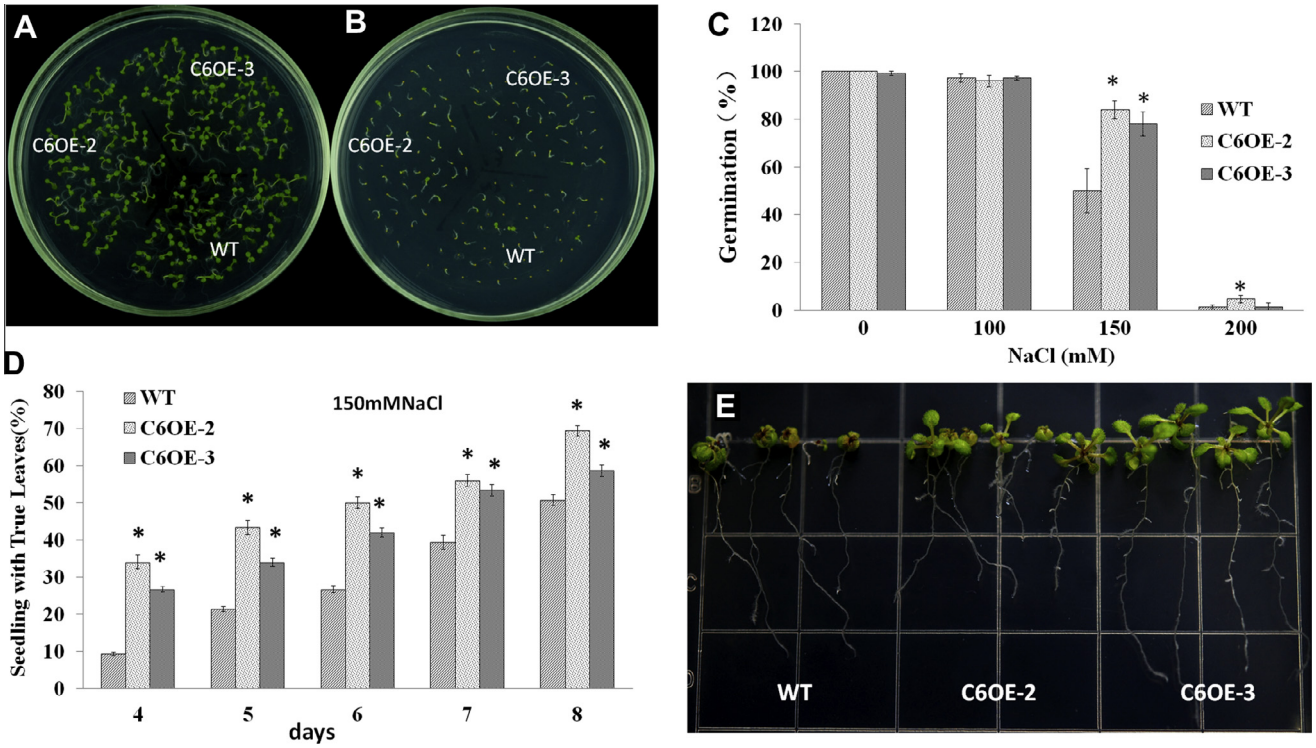

Fig. 3. Salt resistant assays of *GhCIPK6*-overexpressed *Arabidopsis*. (A) Germination assay of *GhCIPK6*-overexpressed and wild-type *Arabidopsis* seeds in 1/2 MS medium without NaCl in 3 days. (B) Germination asssy of *GhCIPK6*-overexpressed and wild-type *Arabidopsis* seeds in 1/2 MS medium containing 150 mM NaCl in 3 days. (C) Germination asssy of the wild-type and transgenic lines seeds at 3rd after being transferred to 1/2 MS medium at 22 °C under different concentrations of NaCl (0, 100, 150, 200 mM). Results are presented as average values and standard errors from three experiments. (D) Ratio of *GhCIPK6*-overexpressed and wild-type *Arabidopsis* seedlings with true leaves on MS medium supplemented with 150 mM NaCl at different growth days. (E) *GhCIPK6*-overexpressed *Arabidopsis* have more vigorous shoots and lateral roots than wild-type.

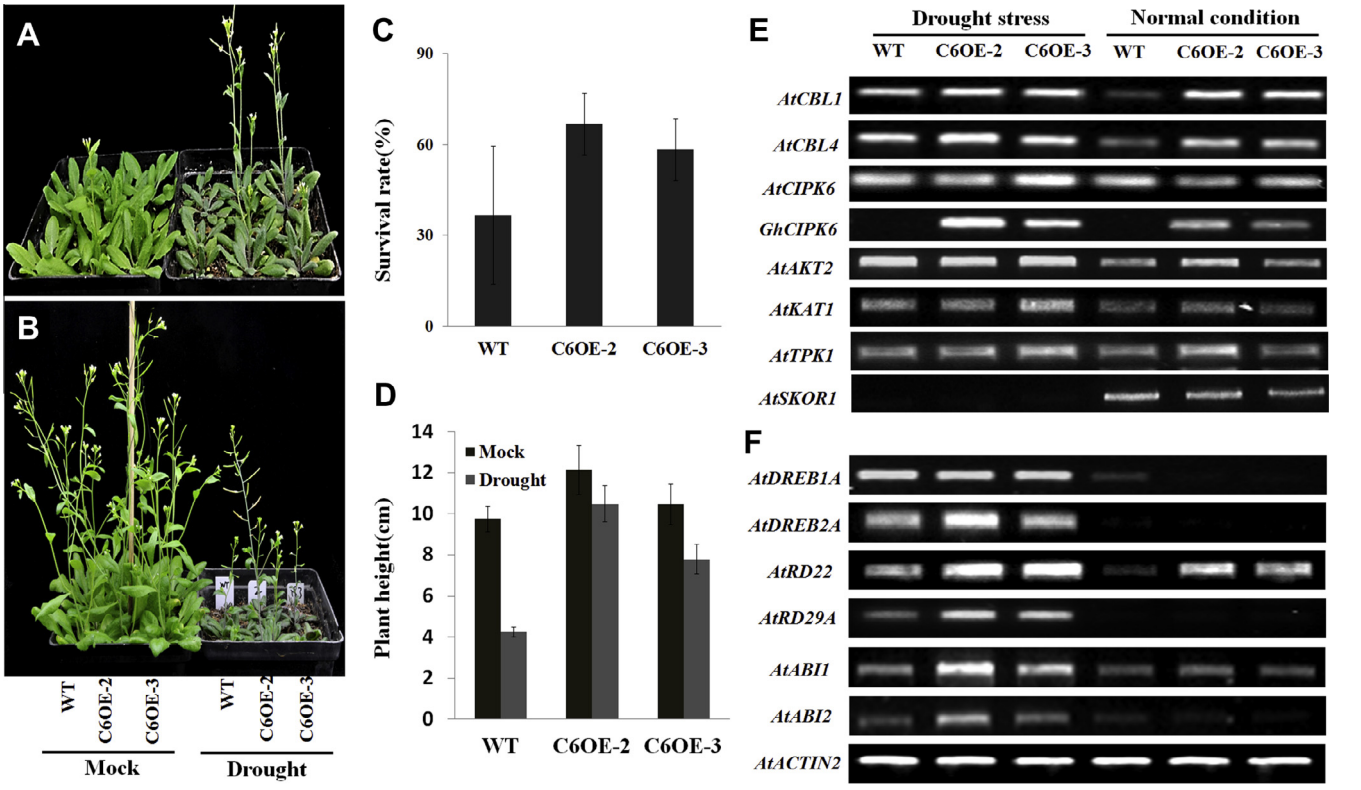

Fig. 4. Drought resistance assays of *GhCIPK6*-overexpressed *Arabidopsis*. The phenotype of transgenic and WT 4-week-old seedlings (A) after water-deficiency for 12 days, (B) after water-deficiency for 20 days, and (C) The survival rate of transgenic and WT seedlings at the 3rd day after rehydration. (D) The plant height of transgenic and WT seedlings under normal and water-deficit conditions. (E) RT-PCR analysis of genes related to *CIPK* mediated signalling pathways such as *AtCBL1* (*At4g17615*), *AtCBL4* (*At5g24270*), *AtCIPK6* (*At4g30960*), *AtAKT2* (*At4g22200*), *AtKAT1* (*At5g46240*), *AtTPK1* (*At5g55630*), *AtSKOR1* (*At3g02850*) and (F) abiotic stress-responsive genes: *AtRD22* (*At5g25610*), *AtRD29A* (*At5g52310*), *AtDREB1A* (*At4g25480*), *AtDREB2A* (*At5g5410*), *AtABI1* (*At4g26080*), *AtABI2* (*At5g57050*) in wild-type and the overexpression lines under normal and drought conditions. *AtACTIN2* was used as an internal control.

3 days, whereas about 50% of control seeds germinated (Fig. 3C). Moreover, the transgenic lines seedlings have more true leaves than wild-type on medium containing 150 mM NaCl during early culture days (Fig. 3D). Further analysis indicated that the shoot of *GhCIPK6*-overexpressing transgenic lines have more vigorous shoots and lateral roots than wild-type (Fig. 3E). These results indicate that *GhCIPK6* overexpression rendered plants more resistance to high salt during early development.

Drought tolerance assays were performed using the same transgenic lines. Water was withheld from the 4-week-old wild-type and homozygous transgenic plants grown in the same pot for 20 days. After a 12-day drought period, the leaves of all the plants become grayish green. The wild type plants grow slowly, while the transgenic plants grow fast and early-maturing (Fig. 4A). After a 20-day drought period, the leaf of all the plants began to shrivel and wilt. When they were re-watered after drought treatment, transgenic plants recovered faster than wild-type plants (Fig. 4B). Moreover, the survival rate of *GhCIPK6*-overexpressing plants was higher than that of wild-type under drought conditions (Fig. 4C). The plant height showed no obvious differences between transgenic lines and controls under normal conditions, however, the transgenic lines were higher than controls after drought treatment (Fig. 4D). It was indicated that overexpression of *GhCIPK6* conferred an increased drought tolerance in transgenic *Arabidopsis*.

3.5. Overexpression of GhCIPK6 activated CBL/CIPK mediated stress response signaling pathways

To determine whether *GhCIPK6* overexpression has any effect on stress response pathways in transgenic plants, we chose to monitor the expression patterns of several genes related to *CIPK* mediated signaling pathways such as *AtCBL1*, *AtCBL4*, *AtAKT2*, *AtKAT1*, *AtTPK1*, and *AtSKOR1*. The transcript of *AtCBL1* and *AtCBL4* was induced in *GhCIPK6*-overexpressing lines under normal conditions. They were further increased to a high level under drought stress, with the expression of the downstream voltage-gated inward rectifying K^+ channels *AtAKT2*, *AtKAT1*, *AtTPK1* increased, especially the *AtAKT2*, while the outward K^+ channels *AtSKOR1* decreased under drought stress (Fig. 4E). These results indicated that *GhCIPK6* might participate in relative signaling pathways.

Moreover, we chose to monitor the expression patterns of several ABA- or abiotic stress-responsive genes, including *DREB1A*, *DREB2A*, *RD22*, *RD29A*, *ABI1*, and *ABI2* in the control and *GhCIPK6*-overexpressing lines. As shown in Fig. 4F, the transcript of *DREB1A*, *DREB2A*, *RD22*, *RD29A*, *ABI1*, and *ABI2* was barely detectable in wild-type plants under normal conditions. However, all *GhCIPK6*-overexpressing plants produced high levels of mRNA for *RD22*, *RD29A*, and *ABI1*.

To examine this possibility further, we determined if *GhCIPK6* overexpression had altered the gene expression pattern under drought conditions (Fig. 4F). In *GhCIPK6*-overexpressing plants, most of these genes were active before the stress treatment, as indicated by a substantial level of mRNA accumulation. Drought stress induced the expression of all stress gene markers such as *RD22*, *RD29A*, *DREB1A*, *DREB2A*, *ABI1*, and *ABI2* in the control plants. However, in *GhCIPK6*-overexpressing plants, most of these genes, including already activated before the stress treatment genes, such as *RD22*, *RD29A* and *ABI1*, were further induced under drought stress and produced higher "total" levels of mRNA than those in the control plants.

4. Discussion

Plants are constantly confronted by multiple abiotic stresses and have evolved efficient ways to reallocate metabolic resources rapidly among different cellular and physiological signaling pathways in order to adapt to the changing environment. One of the earliest responses to stress signals is an increase in cellular calcium in plants [17]. Based on a RNA-Seq profile during cotton SE, we have isolated a large number of putative abiotic stress responsive genes from cotton [25]. In the present study, we characterized the cotton *GhCIPK6* gene, which might conduct calcium signals through CBL/CIPK complex under abiotic stresses. Our results suggest that overexpression of *GhCIPK6* enhances tolerance to salt and drought stresses and reduces ABA sensitivity in transgenic *Arabidopsis* plants.

Potassium (K^+) is an essential macronutrient and a major osmoticum for plant growth and development. Plants often adapt to low K^+ conditions by increasing their K^+ uptake capability. There are many types of K^+ transporter in plants, Such as the inward rectifying K^+ channels *AKTs*, *KATs*, *TPKs* and outward K^+ channels *SKOR* [4]. Earlier analyses in *Arabidopsis* revealed the important role of CBL/CIPK signal transduction system on controlling the K^+ channel under stress conditions. CBL1/CBL9-CIPK23/*CIPK6*/*CIPK16* activated a voltage-gated inward K^+ channel (*AKT1*) in a Ca^{2+}-dependent manner [13,14,24]. In this study, the expression of influx K^+ channels *AKT2*, *KAT1* and *TPK1* was accumulated, especially in the *GhCIPK6*-overexpressing plant under drought stress, while the expression of the efflux K^+ channel *SKOR1* decreased (Fig. 4E). These results indicated that the increase of K^+ influx and decrease of K^+ efflux in *GhCIPK6*-overexpressing plants happened under drought stress. The *GhCIPK6* might confer increased drought tolerance by regulating the K^+ channel, resulting in deep changes in the cell membrane equipment for K^+ transport, and probably affecting the membrane conductance for K^+, and the balance between transporter and channel activities (*AtAKT2* and *AtSKOR1*).

ABA regulates diverse cellular processes and transduces environmental signals to protect plants from abiotic stresses [23]. Plants have to adjust ABA levels constantly in response to changing physiological and environmental conditions. The mechanisms by which plants respond to stress include both ABA-dependent and ABA-independent processes [11]. Reverse genetics analyses have uncovered crucial functions of CBLs and CIPKs in the plant response to ABA. Previous studies reported that *a cipk3 loss-of-function mutant was* hypersensitive to ABA [9]. In this study, several lines of evidence suggest that *GhCIPK6* may be involved in the ABA signaling pathways directly or indirectly. Firstly, qRT-PCR analyses revealed that the expression of *GhCIPK6* was strongly induced by exogenous ABA (Fig. 1G). Secondly, the transcript levels of a number of ABA-regulated genes were enhanced in the transgenic lines compared to the wild-type plants (Fig. 4F). More direct evidence came from the functional analysis of ectopically expressed *GhCIPK6* in *Arabidopsis*, which showed that overexpression of *GhCIPK6* results in reduced ABA sensitivity during both seed germination and the vegetative stages (Fig. 2). More physiological, biochemical and molecular experiments are necessary to elucidate the complex signaling mechanism of *GhCIPK6*.

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