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Overexpression of an *Aeluropus littoralis* Parl. potassium transporter gene, *AlHAK1*, in cotton enhances potassium uptake and salt tolerance

J. F. Liu · S. L. Zhang · H. L. Tang · L. Z. Wu · L. J. Dong · L. D. Liu · W. L. Che

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Abstract Potassium (K) deficiency and salinity are major environmental stresses affecting the growth and agricultural production of cotton (*Gossypium hirsutum*) plants worldwide. In an effort to improve potassium uptake and tolerance to salt stress through genetic engineering, we inserted *AlHAK1* into cotton via *Agrobacterium*-mediated transformation, and then transgenic lines were obtained. To test their response to K-starvation and salt stress, we cultured seedlings from the wild type (WT) and three homozygous overexpression lines (T_3 generation) in plastic pots and treated them with a modified half-strength Hoagland's solution supplemented with different concentrations of potassium or sodium: NT (normal treatment with standard amounts of K and Na), KT (additional 0.05 mM KCl), ST (additional 150 mM NaCl), or KST (0.05 mM KCl plus 150 mM NaCl). After 15 days, all transgenic lines exhibited significantly larger values for shoot and root lengths and biomass (shoot dry weight or root dry weight) when compared with WT plants. Most root morphological parameters for the transgenics were also increased, e.g., total lengths, specific root lengths and surface areas. However, average root diameters were significantly lower than that of the WT ($P < 0.05$ or $P < 0.01$). Under salt-stress conditions, the ratios for K^+/Na^+ were higher in the leaves and roots of transgenic plants, and they also had less malondialdehyde and hydrogen peroxide (H_2O_2) than the WT tissues. Those responses paralleled greater activities by the antioxidant enzymes superoxide dismutase and peroxidase. We clearly demonstrated that cotton plants transformed with a high-affinity K^+ transporter gene have enhanced K^+ uptake and salt tolerance. These findings could serve as a promising step toward the development of new cotton cultivars with improved potassium uptake and tolerance to salt stress, and they have significant implications for increasing crop yields on high-salinity soils where potassium levels are low.

Keywords Cotton (*Gossypium hirsutum* L.) · *AlHAK1* · Transgenic plants · K^+ uptake · Salt tolerance

J. F. Liu and S. L. Zhang have contributed equally to this work.

J. F. Liu (✉) · S. L. Zhang · H. L. Tang · L. J. Dong
College of Life Sciences, Hebei University,
Baoding 071002, China
e-mail: jianfengliu@hbu.edu.cn

L. Z. Wu
North China Key Laboratory for Crop Germplasm Resources of Education Ministry, Agricultural University of Hebei, Baoding 071002, China

L. D. Liu
Institute of Cash Crops, Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang 050051, Hebei, China

W. L. Che
Academy of Agriculture and Forestry Sciences of Langfang, Langfang 065000, Hebei, China

Introduction

Potassium (K) is an essential nutrient that is the most abundant cation in plants, where it constitutes up to 10 % of the total dry matter (Leigh and Wyn Jones 1984). A deficiency in K^+ causes regional withering of old leaves and death of meristems, eventually leading to serious reductions in shoot growth and crop yields (Gierth and Mäser 2007). In addition to its importance in developmental and reproduction processes, K^+ acquisition is essential to defense mechanisms against salt stress that arises mainly because of excessive amounts of Na^+ (Wu et al. 1996). The high demand for K can be met through effective uptake from the soil solution by roots, followed by transport into the aerial parts of the plant. Membrane transport of K can be mediated either by channels that utilize membrane potential to facilitate K transport down along its electrochemical gradient or else by secondary transporters (Gierth and Mäser 2007). Many genes encoding K^+ channels and transporters have been identified (Horie et al. 2011; Xu et al. 2014; Nieves-Cordones et al. 2014; Hammou et al. 2014). These candidate genes have been confirmed in plant genomes as members of three major transporter families: the KT/HAK/KUP, TRK/HKT transporters; the cation proton antiporter family and K^+ channels.

Numerous genes encode KT/HAK/KUP transporters in evolutionarily diverse organisms ranging from green algae to angiosperms (Cabrera et al. 2012). Their ubiquity implies that these genes play important roles in nutrient acquisition and survival in K^+-poor environments (Grabov 2007). For example, *Arabidopsis thaliana* has 13 KT/HAK/KUP K^+ transporters (Rubio et al. 2000) while *Oryza sativa* has at least 17 transporters within that family (Bañuelos et al. 2002). The *HAK5* in *Arabidopsis* belongs to a *HAK-1* type transporter that works as a high-affinity K^+ transporter (Nieves-Cordones et al. 2010). Overexpression of those genes enhances potassium uptake and salt tolerance in transgenic plants (Horie et al. 2011; Xu et al. 2014; Mansour 2014). These results demonstrate that *HAK* genes are high-affinity K^+ uptake components that function predominantly under K^+-starvation.

Cotton (*Gossypium hirsutum* L.) is one of the most important cash crops in the world. Optimum yields depend upon the availability of potassium throughout the growing season (Pettigrew 2003). Compared with other field crops such as corn or soybean, cotton appears to be more dependent upon K-availability and shows symptoms of deficiency much earlier under limited soil-K (Cope 1981). The incidence of K-deficiencies in cotton cultivation is now increasing in many countries for three reasons: (1) a negative K-balance in the soil, (2) adoption of modern cultivars that feature accelerated fruit set and greater boll loads (Tian et al. 2008; Rengel and Damon 2008) and (3) the popularization of transgenic Bt (*Bacillus thuringiensis* Berliner)-cotton, which is more susceptible to K-deficiencies and salt stress (Zhang et al. 2007) Such deficiencies negatively influence photosynthesis (Wang et al. 2012), root development (Zhang et al. 2009a), overall plant growth, and premature senescence (Pettigrew 2003; Xia et al. 2011). These responses ultimately result in lower lint yields and poor fiber quality. Although these physiological phenomena have been studied extensively, no reports have been made about efforts to improve plant growth by expressing a high-affinity K^+ transporter gene, such as *AlHAK1* from *Aeluropus littoralis* Parl., to enhance potassium uptake.

Our study objective was to obtain a new cotton germplasm with better potassium uptake and salt tolerance. The goal was to increase crop productivity on potassium-deficient and high-salinity soils. To achieve this, we inserted *AlHAK1* into the cotton genome. Overexpression lines not only had greater potassium uptake when compared with the wild type (WT), but also showed significantly stronger salt tolerance and activities by antioxidant enzymes. We believe that this is the first report of work done to improve those traits in a crop plant through constitutive expression of a high-affinity K^+ transporter gene.

Materials and methods

Plant materials

Lumianyan28 is transgenic Bt (*Bacillus thuringiensis* Berliner)-cotton variety, and transgenic Bt (*Bacillus thuringiensis* Berliner)-cotton, which is more susceptible to K-deficiencies and salt stress, so we choose this varity as our transformation material. The procedures for seed germination, seedling growth, and isolation of shoot apices have been described previously (Gould and Magallanes-Cedeno 1998; Liu et al. 2011).

Isolation of *AlHAK1* cDNA

Total RNA was isolated from K^+-starved, 4-day-old *A. littoralis* plants by the acid guanidinium phenol chloroform method. As our PCR template, first-strand cDNA was synthesized from 1 μg of total RNA with a first-strand cDNA synthesis kit (Perkin Elmer, Branchberg, NJ, USA). The *AlHAK1* cDNA was amplified by PCR using a sense primer (5′-GCAAGCGGCCTCCGTATCACG-3′) and an antisense primer (Oligo dT Primer) for *AlHAK1*. The PCR products were cloned into the pUC118 vector with a TaKaRa BKL kit (Takara Bio Inc., Tokyo, Japan) and then sequenced. The putative ORF sequence of *AlHAK1* was identified in GenBank (Accession No. DQ645465). For amplifying the cDNA, a PCR primer (AF: 5′-CATCTAGAATGTCGCTCGAGGTCGAG-3′) and an antisense primer (AR: 5′-GCGAGCTCC-TATATTTCATATGTGATCCC-3′) were designed based on the ORF sequence information.

Bioinformatics analysis

The cDNA sequences were analyzed with DNAstar software and the BLAST program (http://ncbi.nlm.nih.gov). Open reading frames (ORFs) were identified with an ORF finder (NCBI), and the protein sequences were deduced. Preliminary properties of the encoded protein were predicted by ProtParam (Appel et al. 1994) while membrane-spanning structures were estimated by TMHMM (Sonnhammer et al.1998). Sub-cellular localization was predicted by Psort (Horton and Nakai 1997).

Cotton transformation using shoot apices

The ORF of *AlHAK1* was placed under the control of the CaMV 35S promoter in the pBI121 vector (Fig. 1). The resulting plasmid with an *AlHAK1* expression cassette was used to transform the apices from cotton shoots according to the method of Gould and Magallanes-Cedeno (1998) and Liu et al. (2011). This transformation required four major steps: (1) obtaining shoot apex explants and pre-culturing them for 2 d in an MS medium; (2) co-cultivation with an *Agrobacterium* suspension containing the pBI121-*AlHAK1* vector; (3) transferring the suspension to a co-cultivation medium, where it was held under darkness for 2 days; and (4) transfer to a shoot induction medium. The regenerated primary plants were designated as the T_0 generation. These were self-pollinated and, ultimately, T_3 transgenic lines were obtained for further analysis. Those seeds were germinated in a greenhouse at 28/20 °C (day/night) under a 16-h photoperiod.

Molecular characterization of putative transgenic plants

Southern blotting was performed according to the procedure described by Sambrook et al. (1989). Genomic DNA samples (70 μg) were digested with *Eco*R I enzymes, then separated in 1 % (w/v) agarose gels and transferred to membranes (Hybond-N^+ positively CHGD Nylon Transfer Membrane; Amersham Biosciences). For our probe a 500-bp fragment of the *AlHAK1* amplified from pBI121 with primers PF (5′-TGCTCTTCTCAGTCCAGCGTTTCG-3′, forward) and PR (5′-ACCTCTCCGCAGGAATCACA-3′, reverse) was labeled with DIG (DIG High Prime DNA Labeling and Detection Starter Kit I; Roche) according to the manufacturer's instructions.

Western blots were used to verify protein expression in leaf extracts. Anti-AlHAK1 antibody obtaining by the exogenous expression of whole protein was used as antigen, and this antigen could recognize the exogenous expression of AIHAK protein, and only a single band was observed in the vicinity of the 87Kd. Briefly, those proteins were resolved via SDS-PAGE and electrophoretically transferred to a PVDF membrane (Bio-Rad, USA). Each blot was first incubated with a rabbit anti-*AlHAK1* antibody (1:1,000 dilution) and then with an alkaline phosphatase-tagged secondary antibody.

Plant growth conditions

Seeds of WT and transgenic cotton plants were surface-sterilized with 10 % H_2O_2 for 30 min and then germinated in a sand culture at 26 °C. At the trefoil stage (15 days after germination), uniformly sized seedlings were transferred for hydroponic culturing in 16- × 13- × 16-cm plastic pots filled with modified half-strength Hoagland's solution. The following supplements were added to the solutions: NT (normal treatment with standard amounts of K and Na), KT (additional 0.05 mM KCl), ST (additional 150 mM NaCl), or KST (0.05 mM KCl plus 150 mM NaCl). For all treatment types, the Hoagland's

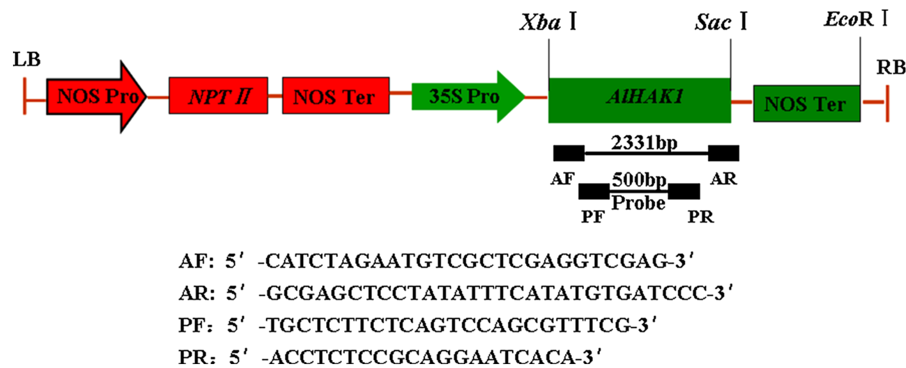

Fig. 1 Schematic representation of pBI121-*AlHAK1* plasmid construction. pBI121-*AlHAK1*, carrying *AlHAK1* and *NPT II*, was driven by 35S and NOS promoters, respectively. *RB* right border, *AlHAK1* high-affinity K^+ transporter gene, *NPT II* neomycin phosphotransferase. Locations for restriction maps and all primers are shown on construct

solution also contained 2.5 mM Ca $(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM $(NH_4)H_2PO_4$, 0.2 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.1 mM EDTA-Fe–Na, 20 μM H_3BO_3, 5 ρM $(NH_4)_6Mo_7O_{24}$, and 1 μM $MnSO_4$. Three seedlings were cultured in each box under a day/night temperature regime of 28/16° and a 12-h photoperiod provided by fluorescent light (240 $\mu Em^{-2}\ s^{-1}$). Deionized water was added daily to replace the water lost through evapotranspiration. The pH was maintained at 6.5 by adding concentrated solutions of NaOH, and air was bubbled into the nutrient solution to provide O_2 and achieve nutrient homogeneity.

Sampling method and assessment of physiological parameters

At 15 days after the stress experiments began, i.e., 25 days after germination, every fifth seedling was selected from each treatment. Entire root systems were scanned with an EPSON Transparency unit (Seiko Epson Corp., Tokyo, Japan) and then analyzed with WinRHIZO version 4.0b (Regent Instruments Inc., Quebec, Canada). The specific root length was calculated as total root length divided by root dry weight. The sampled plants were separated into their root and shoot portions. Tissues were oven-dried at 80 °C for 48 h and weighed on a digital balance.

Measurements of potassium and sodium levels

Leaf and root tissues were collected from each treatment type, then rinsed with distilled water, and oven-dried at 80 °C for 20 h. The dried samples (approximately 1.0 g each) were boiled in a mixture of nitric acid and perchloric acid (4:1, HNO_3:$HClO_4$) in the presence of glass beads for 5 h. The digested plant materials were filtered, diluted with distilled water, and analyzed for Na^+ and K^+ contents with an atomic flame photometer (FP6410).

Determination of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents, and assays for enzyme activity

Three transgenic lines (T_3) and wild-type plants (9 plant/each line) were cultured in plastic pots filld with MS solution. Each plastic pot contained three seedlings, held in place with polystyrene foam, and three replications were across plastic pots. 10-day-old WT plants and T_3 seedlings from *AlHAK1*-transgenic Lines OE2, OE5, and OE7 containing one insert locus were exposed to 150 mM NaCl for 72 h. Their leaves were harvested for biochemical analysis. Hydrogen peroxide was measured spectrophotometrically after reaction with KI (Alexieva et al. 2001). The reaction mixture consisted of 0.5 mL 0.1 % trichloroacetic acid leaf extract supernatant, 0.5 mL of 100 mM K-phosphate buffer and 2 mL reagent (1 M KI w/v in fresh double-distilled water H_2O). The reaction was developed for 1 h in darkness and absorbance measured at 390 nm. Then the amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Leaf of transgenic and wild plants were initially incubated with 1 % potassium iodide and 0.1 % butylated hydroxytoluene at 50 °C for 20 min, and then with

0.4 % TBA at 60 °C for 60 min. The MDA-TBA complex formed was extracted with isobutyl alcohol and measured by high-performance liquid chromatography with fluorescence detection Zhang et al. (2009b).

Leaves were homogenized at 4 °C in 100 mM K-phosphate buffer (pH 7.8), 10 mM $MgCl_2$, 0.2 mM EDTA. The homogenate was centrifuged at 17,000×g for 30 min to yield a crude enzyme extract. Peroxidase activity and superoxide dismutase activity (SOD) were monitored as described previously (Ranieri et al. 2000).

Statistical analysis

Data were examined by Origin software (Version 7.5) and one-way ANOVAs. Values were considered significantly different at $P < 0.05$ or $P < 0.01$ for all experiments. Treatment results were compared by tests for least significant differences.

Results

AlHAK1 cloning and bioinformatics analysis

The cDNA of a high-affinity K^+ transporter (*AlHAK1*) gene in an *A. littoralis* Parl was obtained according to the sequence of the GenBank uncleotide sequence database with the Accession Number DQ645465. By nucleic acid sequence analysis, *AlHAK1* sequence and public sequence of DQ645465 contain the same nucleic acid sequence identity. The full-length *AlHAK1* cDNA sequence comprises 2,844 bp, and its ORF encodes a protein of 777 amino acids with a calculated molecular mass of 87 kDa. The results of TMHMM predicted that the protein *Al*HAK1 is located mainly on plasma membrane with 12 transmembrane (Fig. 2a). Subsequently, the subcellular localization experiment showed that the transiently expressed *Al*HAK1–GFP fusion protein was clearly localized to the cytomembrane, whereas GFP occurred in both the nucleus and cytoplasm (Fig. 2b). Therefore, these results indicated that the *Al*HAK1 protein is indeed localized to the cytomembrane.

Generation of transgenic cotton

A total of 919 shoot apex explants, each five mm long taken from 5-day-old seedlings were cultured; of which, a total of 45 (5.0 %) plantlets survived on 75 mg/L kanamycin-containing medium. Plants expressing the *nptII* gene showed no symptoms, while the leaves of wild-type plants turned yellow and then mottled with brown spots (Fig. 3a). Subsequently, the kanamycin-resistant transformants were further confirmed by Southern blot analysis of *Eco*RI -digested genomic DNA. Examining their hybridization signals showed that OE2, OE5 and OE7 transformants each harbored one insert locus in the genome while OE6 had two. However, DNA isolated from the WT did not hybridize with the probe (Fig. 3b). The presence of *AlHAK1* in these four lines was also proven through western blot analysis. Immunoreactive bands were observed in all proteins extracted from leaves of the transgenics but none was detected in the WT samples (Fig. 3c). Thus, the results verified that *AlHAK1* was integrated into the transformants and expressed in these plants.

Overexpression *AlHAK1* gene promoted growth of transgenic plants

The different phenotypes of transgenic cotton and WT plants were also observed under different treatments. Under NT conditions, morphological or developmental phenotypes were similar between the WT and all OE plants (Fig. 4a). However, under stress conditions, the transgenics displayed better performance whereas growth of WT plants was significantly reduced (Fig. 4a). For KT, only the lower leaves from OE lines exhibited minor deficiency symptoms compared with almost all of the WT leaves, which showed typical symptoms of K-deficiency, including brown scorching and chlorosis between the veins. When plants were subjected to ST conditions, leaf tips from OE lines were somewhat etiolated while almost all WT leaves were discolored and had dropped. Transgenic plants also had better performance than the WT under KST conditions (Fig. 4a). For root phenotypes of transgenic cotton and WT plants, root phenotypes were similar between the WT and OE plants (Fig. 4b) under NT conditions, However, under stress conditions, the transgenics displayed robust performance whereas growth by WT plants was significantly reduced (Fig. 4b). Therefore, all of these results demonstrated that overexpression of *AlHAK1* enhanced the capacity for K^+ uptake and conferred salt tolerance in transformed cotton seedlings.

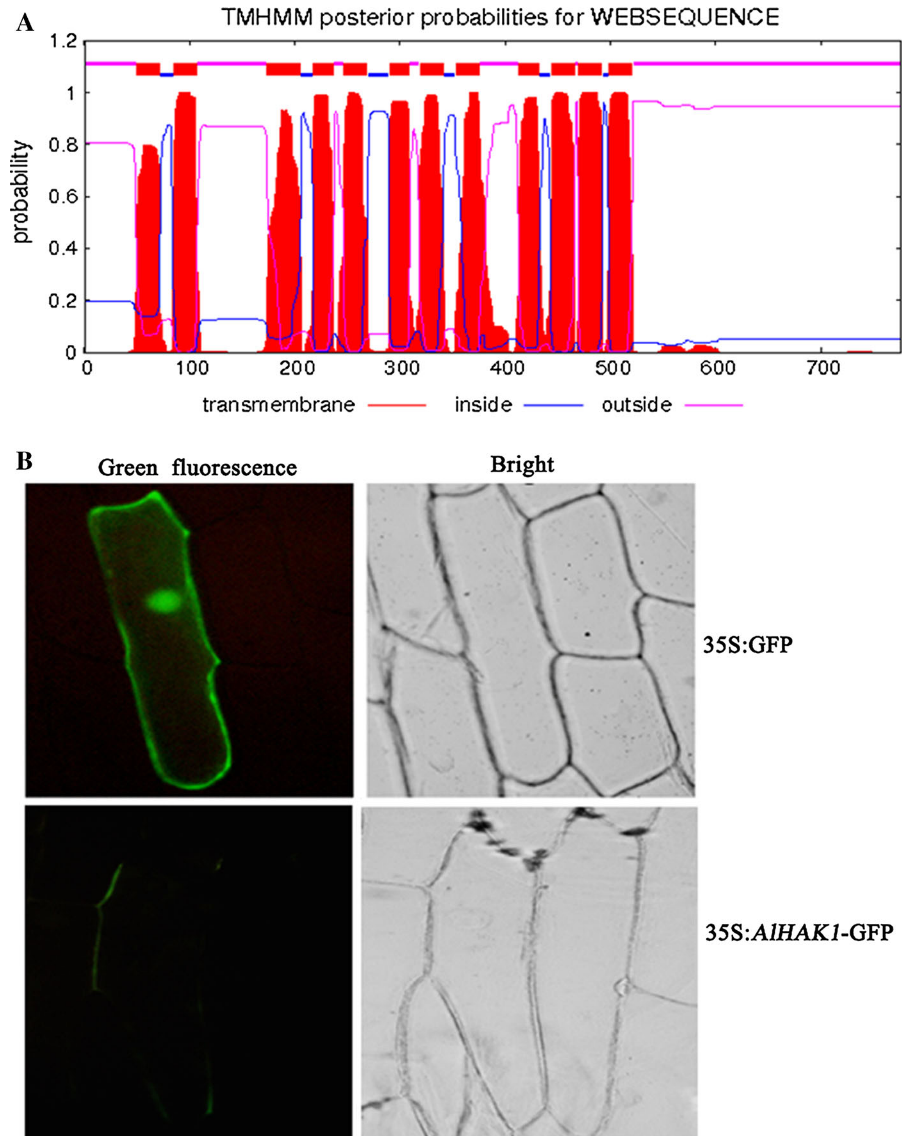

Fig. 2 Bioinformatics analysis and subcellular localization of *Al*HAK1 protein **a** Sub-cellular localization prediction of *AlHAK1* protein. **b** transmembrane analysis of *Al*HAK1 protein *AlHAK1*

Effects of *AlHAK1* overexpression on biomass production and shoot and root morphology parameters

15 days later, transgenic and WT seedlings performance significantly different in their amounts of biomass. For example, in response to KT, shoot dry weights were 35.6, 22.0 and 32.2 % higher in the three transgenic lines while their root dry weights were 32.1, 25 and 21.4 % greater than in the WT (Table 1). Similar trends were noted under ST and KST conditions. Shoot and root lengths also differed significantly between OE and WT plants (Table 2). Under KT, shoots were 19.8, 16.9 and 19.9 % longer in the transgenics while roots were 24.7, 16.7 and 23.3 % longer than in the WT.

Further, those parameters in the OE lines also improved with treatments of KT, ST and KST. Total root lengths were 32.6–80.5 % greater in the transgenics under KT conditions and were also significantly increased under ST and KST conditions (Fig. 5a). Values for specific root length and root surface area were also higher in OE lines under all stress treatments, but their average root diameters were smaller under

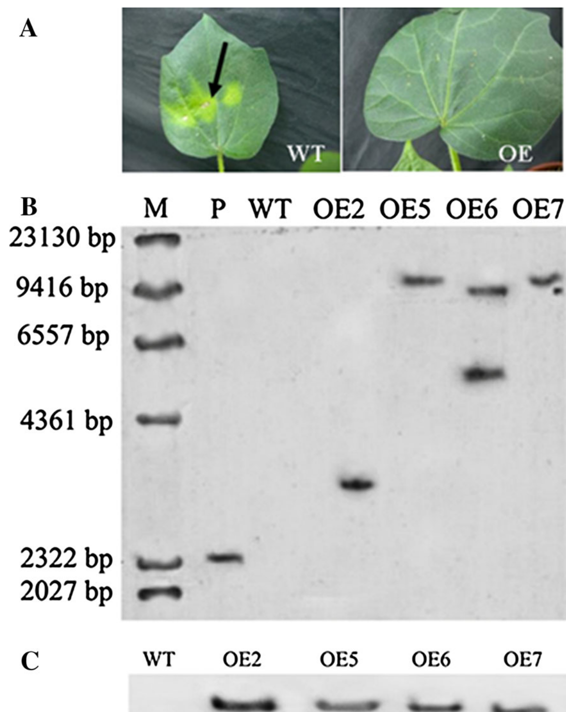

Fig. 3 Detection of *AlHAK1* in T_0 transgenic and WT cotton. **a** Kanamycin leaf-painting assay. The arrow indicates the specific sites of kanamycin application. **b** Southern blot analysis. Genomic DNA was digested with *Eco*R I and probed with 500 bp of *AlHAK1*. **c** Detection of *AlHAK1* protein in leaf extracts by Western blot analysis. *M* DNA Molecular Weight Marker II (Roche), *P* pBI121-*AlHAK1* plasmid, *WT* wild-type plants, OE2, OE5, OE6, and OE7, overexpression lines. The *arrow* indicates the specific sites of kanamycin application

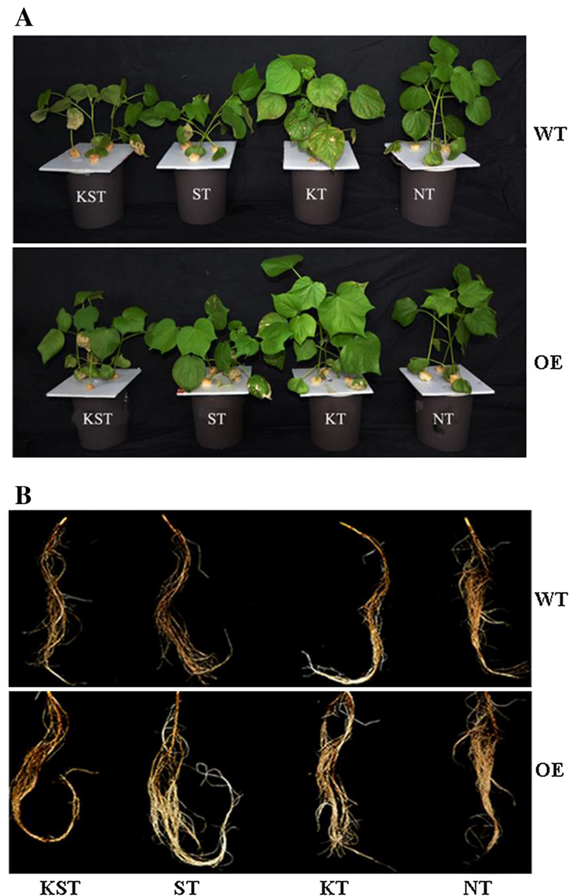

Fig. 4 Overexpression *AlHAK1* gene in transgenic and promoted growth performance of transgenic plants. **a** Growth performance of T_3 transgenic and WT plants after 15 days of treatment. **b** Root morphology of transgenic lines and WT plants. *WT* wild-type plants, *OE* overexpression lines, *NT* normal treatment, *KT* 0.05 mM KCl, *ST* 150 mM NaCl, *KST* 0.05 mM KCl plus 150 mM NaCl

such conditions (Fig. 5b–d). Again, those findings showed that overexpression of *AlHAK1* strengthened growth performance, enhanced the capacity to take up K^+ and other nutrients, and conferred salt tolerance.

Na^+ and K^+ status

The contents of Na^+ and K^+ in leaves and roots of transgenic OE lines and WT plants were measured after subjecting to non-salt and salt stresses (150 mM NaCl). Under non-salt stress, both transgenic cotton plants and WT plants showed similar levels of Na^+ in leaves and roots. Under salt stress, the Na^+ levels in leaves and roots increased in both WT and transgenic plants. However, in the leaves and roots, the increase in transgenic lines was higher than that of WT plants (Fig. 6a). K^+ levels in the leaves were more than twice the levels in roots under non-salt stress, and all plants showed similar levels under non-salt stress. Under salt stress, the K^+ level in WT decreased to about 50 % in both leaves and roots, while transgenic plants appeared to maintain a level similar to those under non-salt stress for both leaves and roots (Fig. 6b). We further found that K^+/Na^+ ratio was similar between leaves and roots in all genotypes under non-salt stress. However, in the presence of 150 mM NaCl, that ratio was up to 3.22-fold and 3.35-fold higher in both the leaves and roots of transgenic plants when compared with WT ratios (Fig. 6c). This suggested that the OE plants had possibly invoked a mechanism for salt tolerance by which they could maintain a high K^+/Na^+ ionic balance in a saline environment by regulating Na^+ efflux and K^+ influx.

Table 1 Effects of stress treatments on dry weights of shoots and roots from transgenic and wild-type cotton plants

Treatment	Shoot dry weight (g)				Root dry weight (g)			
	WT	OE2	OE5	OE7	WT	OE2	OE5	OE7
NT	0.71 ± 0.07	0.82 ± 0.07	0.76 ± 0.08	0.81 ± 0.08	0.42 ± 0.02	0.45 ± 0.02	0.43 ± 0.02	0.40 ± 0.07
KT	0.59 ± 0.06	0.80 ± 0.11*	0.72 ± 0.09*	0.78 ± 0.07*	0.28 ± 0.01	0.37 ± 0.07*	0.35 ± 0.09*	0.34 ± 0.05*
ST	0.63 ± 0.09	0.72 ± 0.11*	0.73 ± 0.10**	0.72 ± 0.01*	0.25 ± 0.02	0.35 ± 0.01**	0.34 ± 0.02*	0.30 ± 0.06*
KST	0.49 ± 0.10	0.64 ± 0.11*	0.65 ± 0.07*	0.64 ± 0.07*	0.20 ± 0.01	0.29 ± 0.03*	0.26 ± 0.06	0.27 ± 0.07

Data are mean ± SD (n = 5)

NT Normal treatment with standard Hoagland's solution containing 2.5 mM K^+, *KT* standard Hoagland's plus 0.05 Mm KCl, *ST* standard Hoagland's plus 150 mM NaCl, *KST* standard Hoagland's plus 0.05 mM KCl and 150 mM NaCl

* $P < 0.05$; ** $P < 0.01$

Accumulations of H_2O_2 and MDA and activity by antioxidant enzymes under salt stress

To test whether contrasts in salt tolerance between OE and WT plants are related to oxidative stress, we measured their levels of H_2O_2 and MDA. Under non-salt stress, no differences were found among the genotypes (Fig. 7a). However, in response to excess NaCl, more H_2O_2 and MDA was accumulated in the WT. For example, OE plants had up to 34.6 % less in H_2O_2 and 39.2 % in MDA when compared with the WT (Fig. 7a). This indicated that overexpression of *AlHAK1* conferred greater tolerance to oxidative stress that arose from excess salt.

To investigate the possible underlying cause for lower H_2O_2 accumulations in the transgenic lines, we monitored the activity of major antioxidant enzymes. Under non-salt stress, SOD and POD activities did not differ significantly among genotypes (Fig. 7b), but they were much higher (up to 59.0 % increases for both) in the OE plants than in the WT when excess NaCl was added to the Hoagland's solution (Fig. 7b). These data indicated that overexpression of *AlHAK1* was associated with a more efficient antioxidant system that counteracted the negative effects of oxidative and salt stresses.

Discussion

The HAK gene family plays critical roles in responses to potassium-starvation and salt stress (Gierth and Mäser 2007; Yang et al. 2009; Tomoaki et al. 2011). Moreover, the cotton remains difficult to genetic engineer primarily because of its resistance to *Agrobacterium* infection and its recalcitrance to in vitro regeneration. Therefore, an alternative approach using shoot apices as explants was developed for free of genotype-dependence, with time-saving in regeneration of cotton plantlet in vitro. So, in this study, this method was used to transfer the high-affinity K^+ transporter gene (*AlHAK1*) into cotton to develop potassium efficient-uptake and salt-tolerant cotton cultivar.

K^+ uptake in the high-affinity range of concentrations and its components have been widely studied. In previous study, In common ice plant, transcript levels of *McHAK1* and *McHAK4* is increased by K^+ starvation and salt stress of 400 mM NaCl in leaves

Table 2 Effects of stress treatments on shoot and root lengths from transgenic and wild-type cotton plants

Treatment	Shoot length (cm)				Root length (taproot length) (cm)			
	WT	OE2	OE5	OE7	WT	OE2	OE5	OE7
NT	31.4 ± 3.3	31.6 ± 2.7	32.8 ± 3.7	31.7 ± 3.4	31.2 ± 2.5	26.1 ± 2.6	29.9 ± 3.5	28.4 ± 4.0
KT	23.7 ± 1.2	28.4 ± 2.3*	27.7 ± 3.0*	28.4 ± 3.0*	30.0 ± 3.6	37.4 ± 1.5*	35.0 ± 4.0*	37.0 ± 4.2*
ST	16.0 ± 1.3	19.3 ± 1.8*	20.9 ± 2.2*	18.5 ± 1.3*	28.9 ± 5.2	36.3 ± 3.6**	35.8 ± 5.7*	34.2 ± 3.5*
KST	12.1 ± 2.7	17.2 ± 1.7**	15.4 ± 0.6*	15.2 ± 0.9*	25.4 ± 3.9	33 ± 3.8*	29.5 ± 4.4	32.2 ± 7.5*

Data are mean ± SD (n = 5)

NT Normal treatment with standard Hoagland's solution containing 2.5 mM K^+, *KT* standard Hoagland's plus 0.05 mM KCl, *ST* standard Hoagland's plus 150 mM NaCl, *KST* standard Hoagland's plus 0.05 mM KCl and 150 mM NaCl

* $P < 0.05$; ** $P < 0.01$

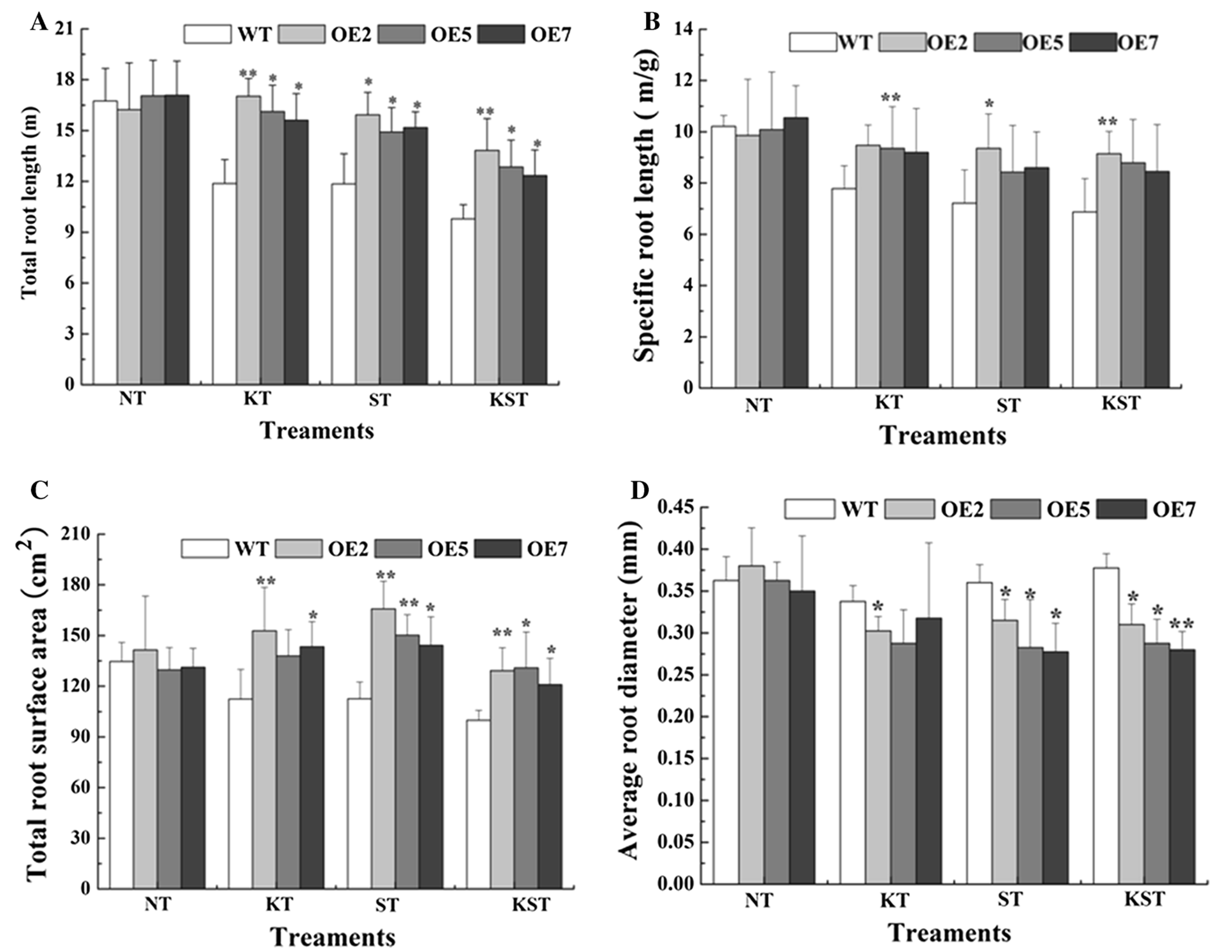

Fig. 5 Effects of treatments on root morphology parameters of transgenic lines and WT plants. **a** Total root lengths. **b** Specific root lengths. **c** Root surface areas and **d** Average root diameters. *WT* wild-type plants, *OE* overexpression lines, *NT* normal treatment, *KT* 0.05 mM KCl, *ST* 150 mM NaCl, *KST* 0.05 mM KCl plus 150 mM NaCl. Data are presented as mean ± SD (n = 5). (*$P < 0.05$; **$P < 0.01$)

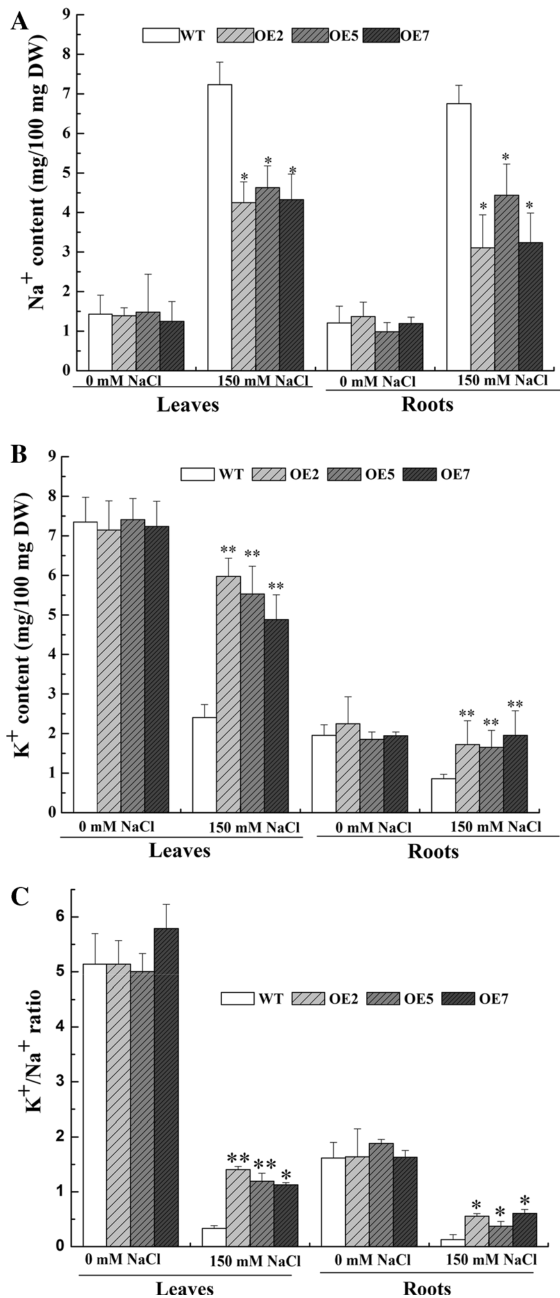

Fig. 6 Comparison of Na^+, K^+ contents and K^+/Na^+ ratio between OE transgenic lines and WT plants after 15 days of 150 mM NaCl treatment. **a** Content of Na^+ in leaves and roots of plants. **b** Content of K^+ in leaves and roots of plants. **c** K^+/Na^+ ratios in leaves and roots of plants. Data are mean ± SD of 3 independent measurements (*$P < 0.05$; **$P < 0.01$)

and roots (Su et al. 2002). Also, accumulation of barley *HvHAK1* transcripts is enhanced by K^+ deprivation and exposure to high salt concentrations (Fulgenzi et al. 2008). In addition, *AtHAK5* gene of *Arabidposis thaliana* was observed that it is required for K^+ absorption necessary to sustain plant growth at low K^+ in the absence or in the presence of salinity (Nieves-Cordones et al. 2010). In this study, the *AlHAK1* gene was first cloned and functionally expressed in *Saccharomyces cerevisiae*, it is believed that this gene enables plants to maintain a high cytosolic K^+/Na^+ ratio under increased salinity (Su et al. 2007). Therefore, we also study the role of this gene in cotton under two important stress conditions: low K^+ supply or the presence of salinity. Notability, the growth was significantly improved in transgenic plants along with their uptake of ions and activities of antioxidant enzymes.

Plant members of the KT-HAK-KUP family of transport proteins were first identified in *Arabidopsis* and barley (*Hordeum vulgare*), and were classified into four major clusters (Santa-María et al. 1997). Transporters within Cluster I have key roles in K^+ capture from diluted K^+ solutions (Gierth et al. 2005). They are also likely involved in K^+ homeostasis during acclimation to NaCl stress. One gene in that cluster is *AlHAK1*, which was first cloned and functionally expressed in *S. cerevisiae* (Su et al. 2007). Therefore, it is believed that this gene enables plants to maintain a high cytosolic K^+/Na^+ ratio under increased salinity. We found that *AlHAK1* was successfully incorporated into the cotton genome and over-expressed in transgenic lines. Those transformed plants exhibited much greater tolerance to K-deficiency and salt stress when compared with the WT. Our findings support previous reports that expression of K^+-transporters by other plant, such as *OsHAK5* and *OSHKT2* in rice, improves K^+ uptake and confers salt tolerance (Yao et al. 2010; Tomoaki et al. 2011). Taken together, these observations unambiguously demonstrate that engineering of the HAK transporter is a useful tool for increasing K^+ uptake and salt tolerance, thereby enhancing overall plant performance in K-starvation and saline environments.

Cotton requires high levels of potassium but has limited capacity for its utilization (Dong et al. 2010; Jiang et al. 2011). However, K-resources are very scarce in China and have long been supplemented through the application of fertilizers that are lower in K than in either N or P (Cassman et al. 1989). Therefore, introduction of a transporter gene into cotton would be an efficient measure for increasing the

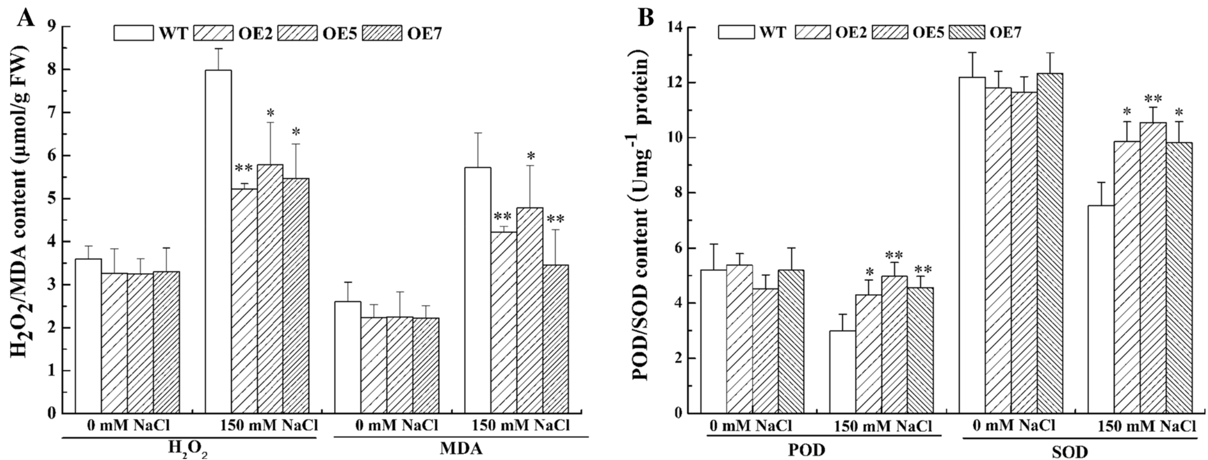

Fig. 7 Levels of oxidants and antioxidant enzymes in transgenic lines and WT plants after 48 h of treatment with either 0 mM NaCl or 150 mM NaCl. **a** H_2O_2 and MDA. **b** SOD and POD. Data are mean ± SD of 3 independent measurements (*$P < 0.05$; **$P < 0.01$)

level of potassium nutrition in that crop. Indeed, technologies involving genetic modifications have already greatly contributed to better plant growth, such as seen with Bt-cotton, which is resistance to insect attack. However, those transgenics have also proven to be more sensitive to K-deficiencies and high salt than their untransformed parents (Zhang et al. 2007; Yang et al. 2011). Those deficiencies can inhibit root elongation and lateral root formation, thereby influencing the uptake of nutrients from the soil because of significant reductions in total lengths, surface areas, and volumes (Shin and Schachtman 2004; Armengaud et al. 2004). Brouder and Cassman (1994) have shown that a larger or more efficient root system plays a decisive role in determining cotton tolerance to K^+-deficiency. In this study, we found that, under high salinity, OE lines had well-developed root systems and more fine roots, which meant that the interface between root and solution was larger than that for WT plants. We are currently analyzing the molecular mechanism by which overexpression of *AlHAK1* promotes root development.

Plants can generally survive better under saline conditions if they remain able to regulate their ionic homeostasis. Maintaining a high K^+/Na^+ ratio in the cells is a crucial aspect of salt adaptation. The extent to which a plant is salt-tolerant depends upon its ability to export Na^+ from the shoots and maintain an elevated K^+/Na^+ ratio in the cells (Martinez-Atienza et al. 2007). In an environment with excess NaCl, ion transporters such as members of the KT-HAK-KUP family, the low-affinity cation transporter LCT1, and the Na^+/H^+ antiporter NHA must be activated (Zhu 2001). Among these, the HAK1 transporters demonstrate a stronger tendency to move K^+ over Na^+ (Santa-María et al. 1997). We noted here that transgenic plants had higher K^+/Na^+ ratios in both leaves and roots, which implied that overexpression of the transporter gene changed the metabolism of potassium so that the cells could maintain a higher K^+ content. Saline conditions often lead to the accumulation of ROS, which can damage cellular membranes and mediate lipid peroxidation, causing oxidative stress (Xiong et al. 2002). Malondialdehyde content is widely recognized as an index of this peroxidation (Mittler 2002). We found that, under salt stress, levels of both H_2O_2 and MDA were markedly lower in OE plants than in the WT. This response may have been correlated with increased activities by ROS-scavenging SOD and POD. In addition, the greater amounts of *AlHAK1* transcript in OE plants induced a rise in enzyme activities, thereby conferring greater salt tolerance and enhancing the stability of proteins and cell membranes within our experimental saline environment (150 mM NaCl). These improvements in the capacity for K^+ uptake and salt tolerance by cotton are a promising step toward the development of new cultivars for agricultural production in soils where potassium supplies are deficient.

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