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Functional characterization of *GhAKT1*, a novel Shaker-like K^+ channel gene involved in K^+ uptake from cotton (*Gossypium hirsutum*)

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ABSTRACT

Shaker-like potassium (K^+) channels in plants play an important role in K^+ absorption and transport. In this study, we characterized a Shaker-like K^+ channel gene *GhAKT1* from the roots of *Gossypium hirsutum* cv. Liaomian17. Phylogenetic analysis showed that the GhAKT1 belongs to the AKT1-subfamily in the Shaker-like K^+ channel family. Confocal imaging of a GhAKT1-green fluorescent fusion protein (GFP) in transgenic *Arabidopsis* plants indicated that GhAKT1 is localized in the plasma membrane. Transcript analysis located *GhAKT1* predominantly in cotton leaves with low abundance in roots, stem and shoot apex. Similarly, β-glucuronidase (GUS) activity was detected in both leaves and roots of *PGhAKT1::GUS* transgenic *Arabidopsis* plants. In roots, the GUS signals appeared in the epidermis, cortex and endodermis and root hairs, suggesting the contribution of GhAKT1 to K^+ uptake. In leaves, *GhAKT1* was expressed in differentiated leaf primordial as well as mesophyll cells and veins of expanded leaves, pointing to its involvement in cell elongation and K^+ transport and distribution in leaves. Severe K^+ deficiency did not affect the expression of *GhAKT1* gene. *GhAKT1*-overexpression in either the *Arabidopsis* wild-type or *akt1* mutant enhanced the growth of transgenic seedlings under low K^+ deficiency and raised the net K^+ influx in roots at 100 μM external K^+ concentration, within the range of operation of the high-affinity K^+ uptake system. The application of 2 mM $BaCl_2$ resulted in net K^+ efflux in roots, and eliminated the differences between *GhAKT1*-overexpression lines and their acceptors indicating that the K^+ uptake mediated by GhAKT1 is also as Ba^{2+}-sensitive as AtAKT1.

1. Introduction

Potassium (K) is the most important and abundant cation in living plant cells and plays crucial roles in many physiological and biochemical processes, including enzyme activation, membrane transport, anion neutralization, co-transport of sugars, and osmoregulation (Clarkson and Hanson, 1980). Potassium ion (K^+) concentrations in soil usually range from 0.04 to 3%, but the worldwide distribution of K^+ is inconsistent. In the tropics and subtropics, one-quarter of the soil experienced a deficiency of K^+ (Munson, 1985). Moreover, the release of exchangeable K^+ is often slower than the rate of K^+ acquisition by plants and, consequently, K^+ content in some soils is very low (Johnston, 2005). Plant K status may further deteriorate in the presence of high levels of other monovalent cations such as Na^+ and NH_4^+ that interfere with K^+ uptake (Qi and Spalding, 2004; Rus et al., 2004; Spalding et al., 1999).

Cotton (*Gossypium hirsutum* L.) is more sensitive to low K^+ availability than most other major field crops, and often shows signs of K deficiency on soils not considered deficient in K^+ (Cassman et al., 1989). Widespread K deficiency in cotton has occurred in many countries (Oosterhuis, 1994; Tian et al., 2008), because of the negative K^+ balance in the soil, adoption of modern cultivars characterized by faster fruit set and greater boll load (Oosterhuis, 1994), and popularization of transgenic Bt (*Bacillus thuringiensis* Berliner) cotton (Tian et al., 2008), which is more susceptible to K deficiency (Yang et al., 2011; Zhang et al., 2007).

To ensure an adequate supply, plants have a number of redundant mechanisms for K^+ acquisition and translocation (Kochian and Lucas, 1988; Maser et al., 2001; Véry and Sentenac, 2003). In the past twenty years, a large number of genes encoding plant K^+ transporters and channels, such as the KT/KUP/HAK family, the HKT family, and the Shaker-like K^+ channel family, particularly for *Arabidopsis* (*Arabidopsis thaliana*), have been characterized (Fu and Luan, 1998; Kim et al., 1998; Quintero and Blatt, 1997; Santa-María et al., 1997). These K^+

Abbreviations: bp, base pair; CBL, calcineurin B-like proteins; cDNA, complementary DNA; CIPK, CBL-interacting protein kinase; cNMP, cyclic nucleotide binding domain; EST, expressed sequence tag; GFP, green fluorescent protein; GUS, β-glucoronidase; MS, Murashige–Skoog media; NCBI, National Center for Biotechnology Information; NMT, non-invasive ion flux measuring technique; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RNA, ribonucleic acid; RT-PCR, reverse transcription PCR; WT, wild type; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

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transporters vary in K^+ affinity, kinetics, transcriptional modulation, and regulatory mechanism, etc. (Gambale and Uozumi, 2006; Gierth and Mäser, 2007; Lebaudy et al., 2007), and compose a complex system for plant K^+ uptake and translocation. Among these K^+ transporters and channels, the KT/KUP/HAK family of K^+ transporters is involved in high-affinity K^+ uptake into the roots, and the Shaker-like K^+ channel family has been shown to code for voltage-gated highly K^+-selective channels active at the plasma membrane, which provide major pathways for wholesale K^+ uptake or secretion in most tissues and cell types (Gambale and Uozumi, 2006; Véry and Sentenac, 2003). In 1992, the *Arabidopsis* Shaker-like K^+ channels *AKT1* were isolated (Sentenac et al., 1992). AtAKT1 channel has been shown to mediate K^+ uptake within the K^+ concentrations that correspond to the high- and the low-affinity K^+ uptake systems described by Epstein in 1963 (Epstein et al., 1963; Hirsch et al., 1998; Ivashikina et al., 2001; Lagarde et al., 1996; Spalding et al., 1999).

AtAKT1 expression was preferentially localized in the peripheral cell layers of the root mature regions, which was consistent with a role of AtAKT1 in root K^+ uptake (Lagarde et al., 1996). After the cloning of *AtAKT1*, several cDNAs encoding K^+ channels with homology to AKT1 were obtained from other species, such as SKT1 from potato (*Solanum tuberosum*, Zimmermann et al., 1998), LKT1 from tomato (*Solanum lycopersicum*, Hartje et al., 2000), TaAKT1 from wheat (*Triticum aestivum*, Buschmann et al., 2000), OsAKT1 from rice (*Oryza sativa,* Golldack et al., 2003), ZMK1 from maize (*Zea mays*, Philippar et al., 1999), DKT1 from carrot (*Daucus carota*, Formentin et al., 2004), CaAKT1 from pepper (*Capsicum annuum*, Martinez-Cordero et al., 2005), NKT1 from tobacco (*Nicotiana tabacum*, Sano et al., 2007), and VvK1.1 from grapevine (*Vitis vinifera*, Cuéllar et al., 2010). Up to now, however, little is known about the function of K^+ channels in cotton.

In this study, we identified and characterized *GhAKT1*, a novel member of Shaker-like family, from the root of cotton cv. Liaomian17. The results would be beneficial for elucidating how cotton acquires K^+ and developing K^+ efficient cotton genotypes by using biotechnological approaches.

2. Materials and methods

2.1. Plant materials, growth conditions and treatments

Cotton (*G. hirsutum* L.), cv. Liaomian17, developed and provided by Cash Crops Research Institute, Liaoning Academy of Agricultural Sciences, China, was used in this study to isolate *GhAKT1*. Seeds were surface-sterilized with 9% H_2O_2 for 30 min, then germinated in sand and cultured in nutrient solutions with 12 h light/12 h dark at $30 \pm 2/22 \pm 2$ °C as described in Wang et al. (2012).

The *Arabidopsis* (*A. thaliana*) WT of the ecotype, Columbia was also used. The T-DNA insertion line *akt1* (SALK_071803) was ordered from the *Arabidopsis* Biological Resource Center (http://www.arabidopsis.org/abrc/). Low K^+ (50–100 μM) MS medium was prepared by modification of the normal MS medium containing 20 mM K^+ (KH_2PO_4 replaced by $NH_4H_2PO_4$ and partial KNO_3 replaced by NH_4NO_3) as described in Xu et al. (2006). For seed harvest, *Arabidopsis* plants were grown in potting soil mixture (rich soil: vermiculite = 2:1, v/v) and kept in growth chambers at 22 °C with illumination at 120 μmol m^{-2} s^{-1} for a 16 h light period. The relative humidity was ~70% (±5%).

2.2. Cloning and sequence analysis of GhAKT1 gene

To identify the cotton homologue of the *Arabidopsis* Shaker-like K^+ channel, AKT1, the total RNA was isolated from roots of Liaomian17 which were grown hydroponically in 2.5 mM K^+ or 30 μM K^+, as described in Wang et al. (2012). The amino acid sequences of *AtAKT1* were used as probes to screen the cotton (*G. hirsutum*) EST database in the GenBank. The candidate ESTs' sequences were subjected to contig analysis with the SeqMan program. The full-length sequence of *GhAKT1* gene was obtained through the 5′-and 3′-rapid amplification of cDNA ends (RACE) following the user manual of SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) by using the cDNA of Liaomian17 as the template. The gene-specific primers were as follows: QA-L (5′-ATGTTTCGAGGGTCAGTACTAT-3′) and ZA-R (5′-TTAAGGGTTTTGGGTGTCATTA-3′). The PCR product was cloned into the pGEM-T easy vector (Promega) and then sequenced. Phylogenetic analysis was performed with clustalX version 1.83 (Thompson et al., 1997) and MEGA4 (Tamura et al., 2007) by the neighbor-joining method. The amino acid sequence was analyzed with the SMART program (Schultz et al., 1998). Putative transmembrane spans were predicted by the TMPRED server (http://www.ch.embnet.org/software/TMPRED_form.htmL).

2.3. Subcellular localization of the GhAKT1 protein

The open reading frame (ORF) excluding a stop codon of *GhAKT1* was amplified by using primers with *Sac1* and *Xba*I restriction sites; the sequences of these primers were as follows: AYL (5′-CGAGCTCATGTTTCGAGGGTCAGTACTAT-3′) and AYR (5′-GCTCTAGAAGGGTTTTGGGTGTCATTA-3′). The constructs, including 35S-GFP (control) and 35S-GhAKT1-GFP were transformed into *Arabidopsis*. For GFP localization in cells, the roots of seven-day-old transgenic *Arabidopsis* plants were transferred onto glass slides, covered with slips, and observed under a confocal laser microscope (FV1000, Olympus, Japan). The tissue samples were soaked in 500 mM mannitol on glass slides for 10 min at room temperature to plasmolyze cells, and then observed for GFP signal under the same confocal laser microscope as above.

2.4. Isolation of the GhAKT1 promoter and promoter::GUS assay

Genomic DNA was extracted from cotton roots by the CTAB method. To isolate the *GhAKT1* promoter, an adaptor-ligated genomic library was constructed by ligating digested genomic DNA with adaptors from the Universal Genome Walker Kit (Clontech, Mountain View, CA, USA) according to manufacturer protocol. Primers designed to amplify putative promoter sequence were corresponding to the 5′-untranslated region (UTR) and upstream sequences of *GhAKT1* gene. Two gene-specific primers, AGSP1 (5′-CTGCTGCTCTTTTGGAAATGCTCTCTT-3′) and AGSP2 (5′-AGAGAGAGAGGAACCAAAGGCTTTACC-3′) were derived from the mRNA sequence and used for PCR-based DNA walking. After obtaining the putative promoter fragment (2187 bp), it was amplified by using the common downstream primer PAL (5′-TAATTTCTTTCTCACCCCACATTGT-3′) and PAR (5′-GCCTATGTTTACTGCTTCTCTTTTG-3′). The *PGhAKT1::GUS* construct was generated by fusing the promoter of *GhAKT1* in the front of the β-glucuronidase (GUS) coding sequence in pBGWFS7,0 vector via the Gateway system. The PLACE database (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) were used for promoter nucleotide sequence analysis.

For GUS staining, the transgenic plants were incubated overnight at 37 °C in 1 mg mL^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 5 mM potassium ferrocyanide, 0.03% Triton X-100, and 100 mM sodium phosphate buffer (pH 7.0). The tissues were cleaned with 70% ethanol (Lagarde et al., 1996), and then observed and photographed with a stereoscope (SZ-16, Olympus, Japan). For examination of the detailed GUS staining, the tissues were observed and photographed with a bright-field microscope.

2.5. Construction of vectors and transformation of Arabidopsis

The 35S::GhAKT1 construct was generated by cloning the coding sequence of *GhAKT1* into the binary vector, pBI121 under the control of the CaMV 35S promoter. The SUPER::GhAKT1 construct was generated by cloning the coding sequence of *GhAKT1* into pBIB vector under control of the SUPER promoter (Li et al., 2001). To generate *GhAKT1*-overexpression lines, *Arabidopsis* WT plants were transformed with the 35S::GhAKT1 construct, and *akt1* mutant plants with a