

Co-overexpression of *AtCBL9*, *AtCIPK23* and *AtAKT1* enhances K^+ uptake of sugarcane under low- K^+ stress

Qiwei Li^{1*}, Lina Fan¹, Qingwen Luo¹, Huiyi He¹, Jiachang Zhang², Qiaoying Zeng¹, Yu Li¹, Wenling Zhou¹, Zhenrui Huang¹, Haihua Deng¹, Yongwen Qi^{1*}

¹Guangdong Key Laboratory of Sugarcane Improvement and Biorefinery, Guangzhou Sugarcane Industry Research Institute, Guangzhou 510316, China

²State Key Laboratory of Plant Physiology and Biochemistry, College of Agronomy and Biotechnology, China Agricultural University, Beijing 100094, China

*Corresponding author: liqiwei66@163.com; yongwen2001@163.com

Abstract

Low potassium (K^+) availability is a major constraint to sugarcane growth and production. Previous studies have identified a calcium-signaling pathway that includes two calcineurin B-like calcium sensors (CBL1/CBL9) and a target interacting protein kinase (CIPK23) that activates the inward shaker-like potassium channel (AKT1), responsible for K^+ uptake in plant roots. In this study, three components of the signaling pathway, *AtCBL9*, *AtCIPK23* and *AtAKT1* from *Arabidopsis thaliana* were co-overexpressed in sugarcane (*Saccharum* spp.) through particle bombardment. The results showed about 31% and 35% increase of K^+ content in transgenic plants in low- K^+ stress assay of tissue culture and hydroponic culture, respectively, compared to non-transgenic line. Under low K^+ supply condition, transgenic lines have longer roots, higher plant height, heavier dry weight than non-transgenic line, indicating the transgenic lines grow better than non-transgenic lines. This study indicated that co-overexpression of *AtCBL9*, *AtCIPK23* and *AtAKT1* could significantly increase sugarcane K^+ uptake ability and tolerance to low- K^+ stress. These findings would have significant implications for improving stress tolerance of sugarcane in areas where K^+ supply is low.

Keywords: Sugarcane, potassium (K^+), genetic engineering, low- K^+ tolerance.

Abbreviations: AKT1_inward shaker-like potassium channel AKT1; CBL_Calcineurin B-like protein; CIPK_CBL-interacting protein kinase; Hyg_Hygromycin B phosphotransferase; MS_Murashige and Skoog; MSI_MS induction medium; MSS_MS selection medium; MSSR_MS shoot-regeneration medium; PCR_Polymerase chain reaction; RT-PCR_reverse transcription-polymerase chain reaction.

Introduction

Sugarcane (*Saccharum* spp.) has been one of most important sugar crop in the world for hundreds of years. It is cultivated on 24 million hectares which correspond to 0.5% of global agricultural area and produces two-thirds of the world's sugar (Lembke et al., 2009). Sugarcane is also an important energy crop. Ethanol derived from the fermentation of sucrose is an increasingly important product that can be used as fuel, either directly or mixed with refined petroleum (Menossi et al., 2008). It has been estimated that ethanol from sugarcane may replace up to 10% of the world's refined petroleum consumption in the next 15 to 20 years (Goldemberg, 2007).

Being a long-duration crop with C4 metabolism, sugarcane produces very high biomass, which demands large amounts of moisture, nutrients and sunlight for optimum productivity. The cultivation of cane can rapidly deplete nutrients in soil, particularly potassium (K^+). It has been estimated that sugarcane removes 0.56–1.20 kg N, 0.38–0.82 kg P_2O_5 , 1.00–2.50 kg K_2O and other nutrients for a ton of cane (Zende, 1990; Gopalasundaram et al., 2012). K^+ is the most abundant cation that accumulates in cell sap of sugarcane plant. This nutrient is fundamental for sucrose synthesis and translocation of sucrose from leaves to stalks by acting as an enzyme activator in plant metabolism. Furthermore, it plays a significant role in controlling hydration and osmotic

concentrations within guard cells of stomata (Hartt, 1970; Schroeder, 1994). In absence of adequate K^+ supply, the number, height and sucrose content of millable stalks at harvest may be impaired (Ashraf et al., 2012). Generally, a healthy sugarcane crop contains more than 200 kg ha^{-1} K_2O in its aerial parts (Coale et al., 1993). Under irrigation, cane may remove 790 kg ha^{-1} K_2O from soil a year (Wood, 1990). Because excessive uptake of K^+ by sugarcane depresses the accumulation of sucrose during milling, K^+ fertilization must be kept to the minimum amount required for obtaining optimum yield and for helping regulate maturation.

Enhancement of K^+ uptake capability under low- K^+ conditions can reduce the potential damage on cane production caused by K^+ deficiency. Uptake of K^+ from external environment into living plant cells is accomplished through K^+ -transport proteins which located in the plasma membrane of root cells. The K^+ transporters are divided into six gene families in plants according to their responses to different external K^+ concentration, including three channel families (Shaker, TPK, and Kir-like families) and three transporter families (KUP/HAK/KT, HKT, and CPA families) (Buschmann et al., 2000; Ve'ry and Sentenac, 2003; Ashley et al., 2006; Wang and Wu, 2010). The AKT1, a shaker-type K^+ channel, has been confirmed as the most important

inward-rectifying K⁺ channel for K⁺ acquisition by plants (Hirsch et al., 1998). In *Arabidopsis thaliana*, AKT1 is primarily expressed in the epidermis of roots, where it participates in K⁺ transport from soil into root cells (Sentenac et al., 1992; Lagarde et al., 1996). Genetic analysis and electrophysiological studies showed that mediation of K⁺ uptake at low-K⁺ conditions via AKT1 is regulated by the calcium-dependent signaling pathway involving two calcineurin B-like calcium sensors (CBL1/CBL9) and a target kinase CIPK23. Under low-K⁺ condition, CBL1/CBL9 interacts with their common target kinase (CIPK23) and recruit it to the plasma membrane, where they jointly activate the K⁺ channel AKT1, which thereby, increases the K⁺ uptake capacity (Xu et al., 2006; Lee et al., 2007).

The knowledge about regulation of K⁺ uptake enables improvement of low K⁺ tolerance in crops by genetic engineering. In this study, we transformed *AtCBL9*, *AtCIPK23*, and *AtAKT1* from *Arabidopsis thaliana* that regulate K⁺ uptake under low-K⁺ stress into sugarcane, and found that co-overexpression of the three genes could substantially increase the K⁺ uptake capability of transgenic sugarcane plants. To the best of our knowledge, this is first report on improvement of crop low K⁺ tolerance by engineering multiple genes. This study would have significant implications for improving crop production on low-K⁺ soils, which is a serious agronomic limitation worldwide.

Results

Generation of transgenic sugarcane lines

A vector containing *AtCIPK23*, *AtCBL9* and *AtAKT1* (Fig. 1) was transformed into sugarcane using PDS 1000/He biolistic system. Transformants were selected on a medium containing 50 mg L⁻¹ Hygromycin B. Plantlets with well growth root system under selection pressure of 30 mg L⁻¹ Hygromycin B were regarded as transgenic candidates (Fig. 2A). The PCR analysis showed that all candidate plants contained the 398 bp positive amplification band from Hygromycin B phosphotransferase gene (*Hyg*), suggesting that there were no escapes in this selection system (Fig. 2B). A total of 67 independent events were generated in this study. Among the 67 events, there was 36 events contained all three genes of *AtCBL9*, *AtCIPK23* and *AtAKT1*, while one or two of the three genes were lost in the other 31 events. A 472bp fragment from *AtCBL9* and its super promoter (Fig. 1) was used as probe for southern blotting, which demonstrated that 8–15 copies of the transgenic insertions were detected in the tested plants (Fig. 3A).

The expression of *AtCBL9*, *AtCIPK23* and *AtAKT1* in transgenic lines

Specific primers of *AtCBL9*, *AtCIPK23* and *AtAKT1* were designed for detecting the expression of three genes in transgenic sugarcane. Obviously, specific bands of three genes were detected in transgenic sugarcane, while no bands were found in non-transgenic plants (Fig. 3B). The results suggested that *AtCBL9*, *AtCIPK23* and *AtAKT1* could express normally in roots of transgenic lines.

Transgenic plants showed enhanced K⁺ uptake ability under low-K⁺ stress

To examine whether or not overexpressing *AtCBL9*, *AtCIPK23* and *AtAKT1* could improve sugarcane tolerance to low-K⁺

stress, uniform plantlets of three independent transgenic lines (T1, T2, and T3) and one non-transgenic lines (CK) were transferred to high-K⁺ medium containing 20 mM k⁺ (MS) and low-K⁺ medium containing 100 μM K⁺. After 25 days, the growth of transgenic plants on MS medium was indistinguishable from that of non-transgenic plants (Fig. 4A). The dry weight of non-transgenic lines was higher than that of T1 and T3 and lower than that of T2 (Fig. 5A). The K⁺ content in non-transgenic lines was 44.92 mg g⁻¹ DW, and that of the transgenic lines was 44.35, 48.3, and 44.3 mg g⁻¹ DW, respectively. There was no obvious difference in K⁺ content between transgenic and non-transgenic lines (Fig. 5B). In low-K⁺ medium, the growth of transgenic and non-transgenic lines were restricted when they were compared with plants grown in MS medium, but the reduction in non-transgenic line was more severe. Transgenic lines possessed higher plant height and longer roots than non-transgenic lines, such as that showed in Fig. 4B. Moreover, the K⁺ content of the three transgenic lines (T1, T2 and T3) was 28%, 33%, and 31% greater than that of non-transgenic plants, respectively, which indicated that transgenic plants had significantly enhanced K⁺ uptake ability relative to non-transgenic control.

The hydroponic assay also showed that the transgenic plants had higher tolerance to low-K⁺ conditions than non-transgenic ones and the growth of non-transgenic plants was more seriously inhibited than the three transgenic lines under low-K⁺ stress. No obvious difference on morphological performance between non-transgenic and transgenic plants was found at 1 mM K⁺ solution, (Table 1). At 100 μM K⁺ solution, non-transgenic plants showed most slowly growth with lowest plant height and thinnest diameter of stalk. Then, the K⁺ content in roots and shoots of non-transgenic and transgenic plants were investigated (Fig. 6). At 1 mM K⁺ solution, the K⁺ content in shoots of transgenic plants varied from 37.7–42.4 mg g⁻¹ DW with an average of 40.2 mg g⁻¹ DW, which was approximately 3% higher than that of non-transgenic line (39.2 mg g⁻¹ DW). The K⁺ content in roots of transgenic plants was 17.1 mg g⁻¹ DW, 12.1 mg g⁻¹ DW, and 10.6 mg g⁻¹ DW, respectively, with an average of 13.2 mg g⁻¹ DW. The K⁺ content in transgenic plants roots was 8% higher than that of the non-transgenic line. At 100 μM K⁺ solution, both the K⁺ content of transgenic plants and non-transgenic plants were lower than that in 1 mM K⁺ solution (Fig. 6). The transgenic lines exhibited a much higher K⁺ content than non-transgenic plants with an average 35% and 14% increase in shoots and roots, respectively. The results indicated that transgenic plants had greater K⁺ uptake ability than non-transgenic plants, especially in lower K⁺ condition.

Discussion

Previous studies have demonstrated that the calcium-signaling pathway, including CBL9/CBL1, CIPK23 and AKT1 plays a key role in regulating plant K⁺ uptake under low-K⁺ stress (Xu et al., 2006; Lee et al., 2007; Zhang et al., 2010). In this study, we demonstrated that overexpression of *AtCBL9*, *AtAKT1*, and *AtCIPK23* in sugarcane could significantly improve sugarcane tolerance to low-K⁺ stress. Under low-K⁺ conditions, we found transgenic lines had faster root elongation and more root hairs than the non-transgenic lines, such as T2 in Fig. 4B. The average root surface area of transgenic lines (247 cm², 359 cm², and 259 cm², respectively) was all significantly larger than that of non-transgenic line (188 cm²).

Table 1. The plant height (PH), diameter of stalk (DS), length of leaf (LL), and width of leaf (WL) of non-transgenic line (CK) and transgenic lines (T4, T5, T6) in high and low K⁺ solutions.

Treatment (K ⁺)	Trait	CK	T4	T5	T6
1 mM	PH	18.6±0.71(b)	21.2±0.50 (a)	18.2±0.91 (b)	19.8±0.53 (a)
	DS	1.00±0.62(ab)	1.02±0.19(b)	0.98±0.23(ab)	1.12±0.16(a)
	LL	49.2±0.74(b)	48.1±0.98(b)	52.6±0.72(a)	56.4±0.96(a)
	WL	1.06±0.09(ab)	0.92±0.15(b)	0.88±0.11(c)	1.17±0.10(a)
100 μM	PH	13.2±0.76(c)	16.3±1.51(b)	15.7±0.53(b)	18.6±0.95(a)
	DS	0.59±0.22(c)	0.65±0.31(b)	0.68±0.21(b)	0.80±0.16(a)
	LL	36.0±0.79(c)	39.1±0.67(c)	44.6±0.93(b)	58.6±0.64(a)
	WL	0.83±0.09(a)	0.80±0.08(a)	0.80±0.11(a)	0.84±0.10(a)

Data are means ± SE; Letters denote different groups obtained from Tukey's test (P≤0.05).

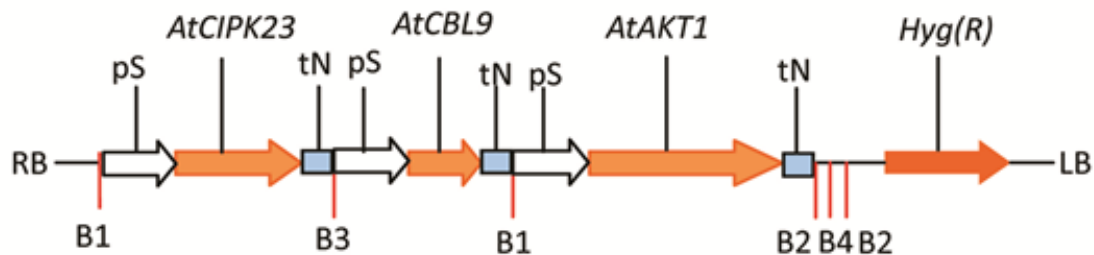


Fig 1. Schematic representation of the T-DNA region of the binary vector pMDC99. RB, right border; LB, left border; pS, Super promoter; tN, nopaline terminator; B1/2/3/4, attB1/2/3/4.

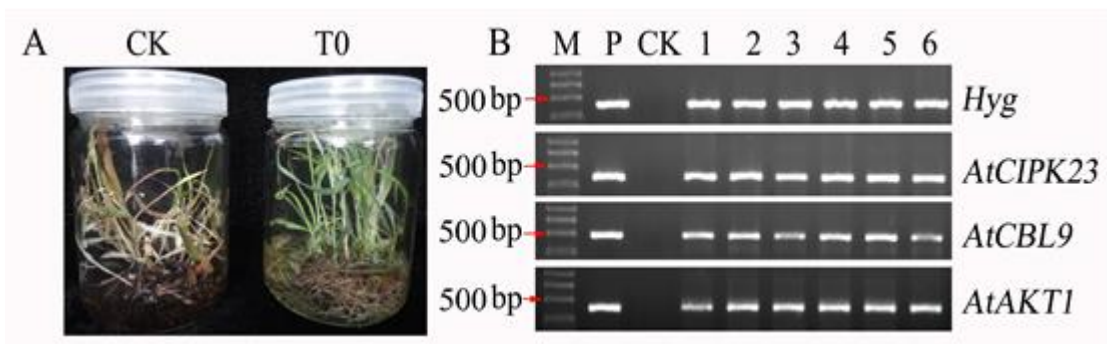


Fig 2. Identification of positive transgenic lines. (A) rooting of non-transgenic plants (CK) and candidate transgenic lines (T0) in medium containing 30 μg/L Hygromycin B. (B) PCR detection of fragments from *Hyg*, *AtCIPK23*, *AtCBL9*, and *AtAKT1* in candidate transgenic lines. M, DL 2000 DNA marker; P, plasmid DNA (positive control); CK, non-transgenic plant; 1–6, candidate transgenic plants.

Generally, the larger root surface area of plants could facilitate to increase contacting area of roots with external K⁺ and contribute plants to uptake K⁺. The greater K⁺ accumulation would help transgenic plants to sustain plant growth under low-K⁺ conditions. The smaller differences in growth and K⁺ content between transgenic and non-transgenic plants in the higher-K⁺ medium might be due to the fact that all lines could absorb adequate K⁺ for healthy growth.

There have been several reports on improving tolerance of crops to low-K⁺ stress by overexpression of genes examined in this study. Shi et al. (2002) reported that overexpression of *AtAKT1* could greatly increase rice K⁺ uptake ability. In K⁺-depletion assay, K⁺ accumulation in transgenic plants was 25.4–79.1% higher than that in controls. The study of Wang et al. (2011) showed that overexpression of *AtCIPK23* could much enhance potato tolerance to low-K⁺ stress. In low-K⁺ conditions, the non-transgenic line displayed reduction in

stature while transgenic lines sustained growth. Considering the complexity in plants for regulating K⁺ uptake (Lee et al., 2007; Wang and Wu, 2010), it might be more feasible to enhance low-K⁺ tolerance by engineering multiple genes responsible for K⁺-uptake. Thus, *AtCIPK23*, *AtCBL9* and *AtAKT1* involved the calcium-signaling pathway regulating K⁺ uptake response to low-K⁺ stress in plant were co-transformed into sugarcane, which indicated that co-overexpressing the three gene much enhance sugarcane K⁺ uptake ability and tolerance in low-K⁺ condition. While, no transgenic sugarcane with single gene of *AtCIPK23*, *AtAKT1*, and *AtCBL9* was available, we were not able to demonstrate function of single gene in enhancing sugarcane K⁺ uptake. In addition, previous studies have showed that there were considerable variations in nutrient uptake and use efficiency in sugarcane (Rakkiyappan et al., 2007; Chen et al., 2013). The total K⁺ needed for producing of one tone cane

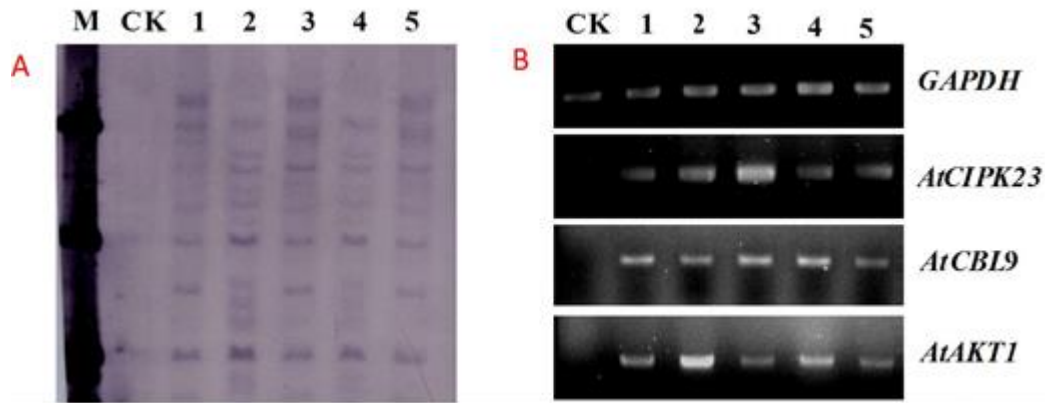


Fig 3. Southern blotting and reverse transcription PCR analysis of the transgenic plants. (A) Southern-blot analysis of transgenic lines using fragments of the pSuper-AtCBL9 sequence as the probe. DNA was digested by *EcoRI*. (B) Detection of gene expression in root by reverse transcription PCR analysis. M, Marker; CK, non-transgenic plant; 1–5, independent transgenic plants.

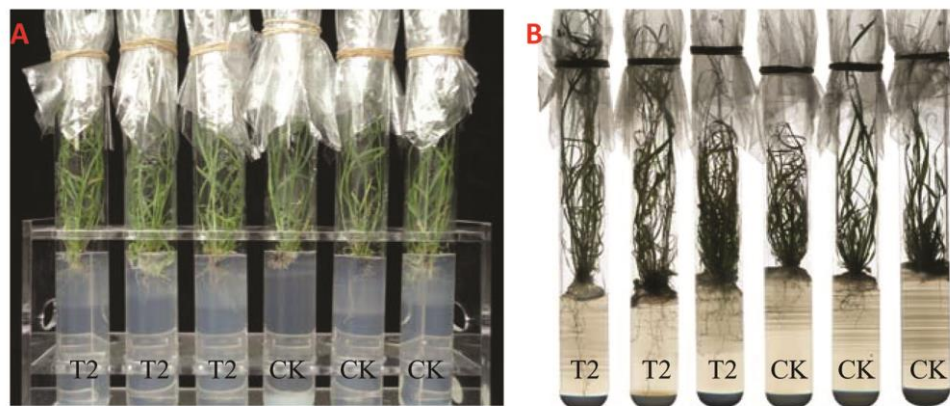


Fig 4. Phenotype of a transgenic line (T2) and non-transgenic line (CK) growing in high K^+ medium and low- K^+ medium. (A) high K^+ medium; (B) low- K^+ medium.

depends to variety and in some varieties could be more than twice or more. Because only one variety was investigated in this study, the effect of *AtCIPK23*, *AtCBL9* and *AtAKT1* on enhancing K^+ uptake in other varieties needs to be further researched.

The sugarcane genome size ranges from 760 to 926 Mbp, which is twice the size of rice genome and similar to that of sorghum (D'Hont and Glasznan, 2001). The size and complexity of sugarcane genome has inhibited efforts and investment in development of biotechnology and genetic tools for this crop. Fewer genes derived from sugarcane have been proven to be ideal for improving agronomic performance such as yield, sugar content, and tolerance to drought or low nutrition (N, P, K, etc.). The reports on transgenic sugarcane plants are mainly related to herbicide-, herbivory-, and virus-resistance (Menossi et al., 2008; Joyce et al., 2010). This study suggested that knowledge of plant responses to low- K^+ in other species is helpful for improving sugarcane to low- K^+ stress. Using the knowledge of other species in tolerance to environmental stress might be a feasible way in sugarcane improvement.

Materials and Methods

Plant materials

The most popular sugarcane cultivar in China, ROC22, was used in this study.

Gene constructs

The pMDC99-derived binary vector containing *AtCIPK23*, *AtCBL9*, and *AtAKT1* as shown in Fig. 1 was kindly provided by Prof. Weihua Wu, China Agricultural University. The three genes have the same Super promoter and tNos terminator. *Hyg* gene was used as the plant selection marker driven by CaMV35S promoter and a polyA terminator.

Generation of transgenic sugarcane

Transverse sections (1–2 mm) of immature leaf whorls above the apical meristem of shoot top were cultured on MS-based

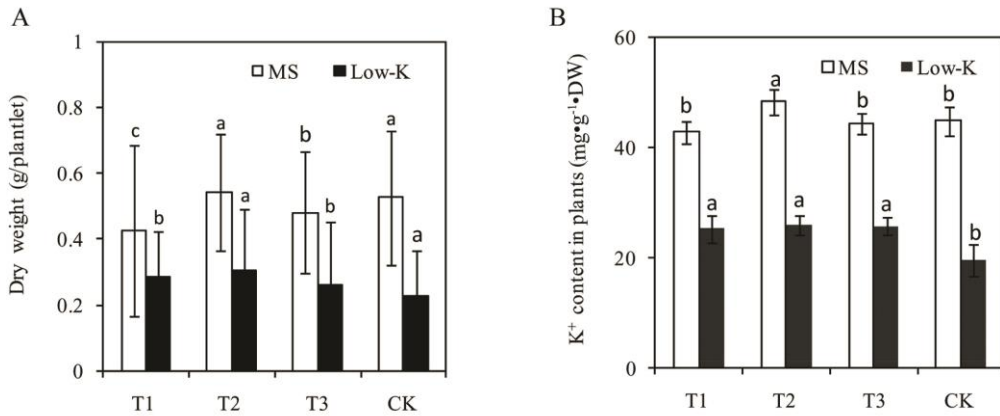


Fig 5. Dry weight and K⁺ content of transgenic plants (T1, T2, T3) and non-transgenic plants (CK) in high-K⁺ medium and low-K⁺ medium. Data are means ± SE. Letters denote different groups obtained from Tukey's test (P ≤ 0.05).

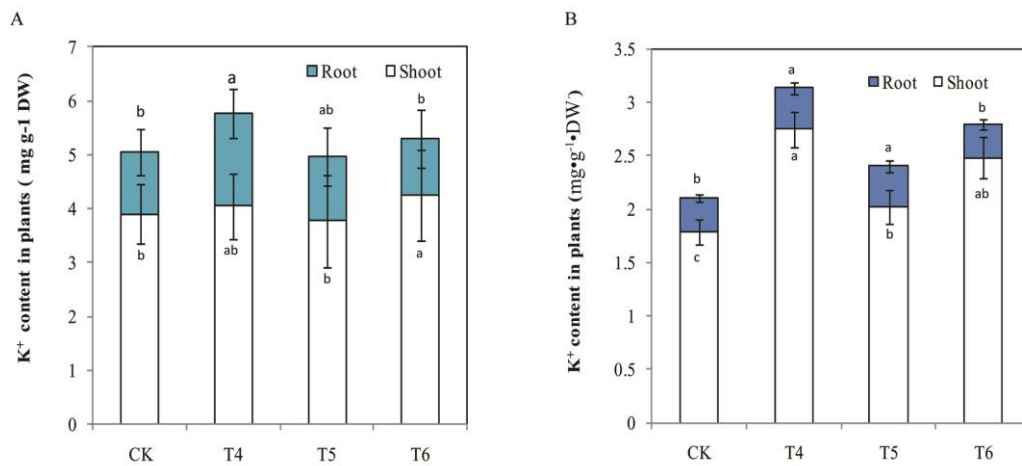


Fig 6. K⁺ content in shoots and roots of non-transgenic (CK) and three transgenic lines (T4, T5, T6) grown in solutions with K⁺ concentration of 1 mM (A) and 100 μM (B). Data are means ± SE. Letters denote different groups obtained from Tukey's test (P ≤ 0.05).

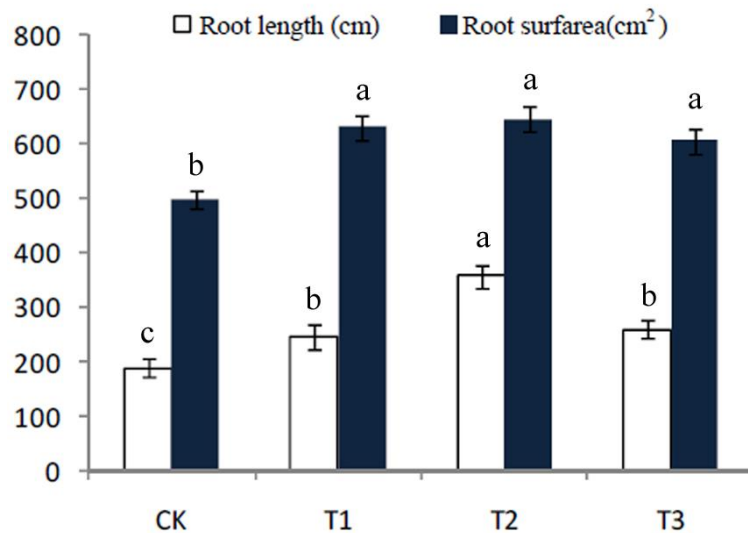


Fig 7. Root length and root surface area of non-transgenic (CK) and three transgenic lines (T1, T2, T3) grown in 100 μM K⁺ medium. Data are means ± SE. Letters denote different groups obtained from Tukey's test (P ≤ 0.05).

(Murashige and Skoog, 1962) embryogenic callus induction medium containing 3 mg L⁻¹ 2,4-dichlorophenoxy acetic acid, 15 g L⁻¹ sucrose and 8 g L⁻¹ agar (MSI) in the dark for 2 week. The initial induced calli were then subcultured onto fresh MSI medium every 2–3 week. The compact, cream-colored nodular embryogenic callus (Joyce et al., 2010) was selected for transformation.

Particle bombardment was carried out with pMDC99 carrying *AtCIPK23*, *AtCBL9*, and *AtAKT1* using PDS 1000/He biolistic system (Bio-Rad, Hercules, CA) at a pressure of 1,100 psi of helium. Prior to bombardment, the calli were placed on osmotic medium (MS + 0.2 Mm annitol + 0.2 Mm sorbitol + 15 g L⁻¹ sucrose + 8 g L⁻¹ agar) for 24 h. After bombardment, calli were incubated in the dark at 25 °C for 16 h and then were transferred to selection medium (MSS; MSI +50 mg L⁻¹ Hygromycin B) in continuous dark. After two rounds of selection (2 week per round) in MSS medium under continuous darkness, the remaining calli were moved to light (16-h photoperiod) at 27 °C on shoot-regeneration medium (MS + 2.0 mg L⁻¹ 6-Benzylaminopurine + 15 g L⁻¹ sucrose + 8 g L⁻¹ agar + 50 mg L⁻¹ Hygromycin B) for 3-4 weeks. Then, the shoots were cultured on rooting medium (MS+ 30 g L⁻¹ sucrose + 8 g L⁻¹ agar + 30 mg L⁻¹ Hygromycin B) for 4–6 weeks. Plantlets with well-developed root systems were transferred to pots containing a mixture of sterilized sand, soil, and farmyard manure (1:1:1). After 2–3 weeks, the acclimatized plantlets were transferred to a greenhouse.

PCR analysis

DNA was extracted from leaf tissues following a modified CTAB procedure (Hoisington, 1992). Polymerase chain reaction (PCR) was carried out in the first-generation transgenic lines. The primer sequences were as follow:
AtCIPK23, 5'-TTTCGGCGTGTAGGACATGGCAACC-3' and 5'-GTGTCCTACCACAGAGAACTACC-3';
AtCBL9, 5'-TTTCGGCGTGTAGGACATGGCAACC-3' and 5'-CTTCCTTGTTTATCAAGCCATCGTC-3';
AtAKT1, 5'-ATCGGTGATAGATAGTGGTG-3' and 5'-TGAATAGATACGCTGACACG-3';
Hyg, 5'-GAGAGCCTGACCTATTGCATCTC-3' and 5'-AGTACTTCTACACAGCCATCGGTC-3'.

The PCR program was as follow: 95 °C for 5 min; 32 cycles of 95 °C for 15sec; 55 °C for 30 sec; and 72 °C for 30 sec; then 72 °C for 10 min. The sizes of PCR product were 387 bp for *AtCIPK23*, 476 bp for *AtCBL9*, 356 bp for *AtAKT1* and 398 bp for *Hyg*. The products of PCR were separated by 1% agaros gel and the DL2000 marker (Takara, Japan) was used.

Southern bolt analysis

A 50 µg of genomic DNA from transformed plants and a non-transformed plant were digested with restriction enzyme *EcoRI* at 37°C for 16 h. Digested DNA was electrophoresed at 25 V for 20 h on a 0.8% (w/v) agarose gel, and then transferred to a positively charged nylon membrane (Osmonics Magnacharge nylon transfer membrane, Amersham Pharmacia Biotec, Little Chalfont, UK), according to the manufacturer's recommendations. The 476 bp fragment of *AtCBL9* gene and its promoter amplified from plasmid pMDC99 with the specific primers (5'-TTTCGGCGTGTAGGACATGGCAACC-3' and 5'-CTTCCTTGTTTATCAAGCCATCGTC-3') by DIG DNA Labeling Kit (Roche) as the probe. The membrane was hybridized with the DIG-labeled probe at 42 °C for 16 h. The hybridized membrane was washed and detected according to the protocol of DIG Nucleic Acid Detection Kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche).

Gene expression analysis

Total RNA was isolated from young leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). DNaseI was used to remove any contaminating genomic DNA from the RNA samples. First-strand cDNA was then synthesized using MMLV reverse transcriptase (Promega, USA) from 1 µg of total RNA with oligo(T)18 primers in a 20 µL reaction volume, and 1 µL of the reaction mixture was subjected to subsequent PCR in a 50 µL reaction volume. The primers for reverse transcription PCR (RT-PCR) analysis of *AtCIPK23*, *AtCBL9*, and *AtAKT1* were as follows:

RT-AKT1, 5' TTCTGCCTTCATCTACCG-3' and 5' AACCCAACATCCGTCCTG 3';
RT-CBL9, 5'-GTCGCAATCTCGTCCACC -3' and 5'-CAGACTTTACCTTTAGGCTTT-3';
RT-CIPK23, 5'-GCAACTGCCAACTGACCC-3' and 5'-CAGCCTTGATGATGTTGATG-3'.

GAPDH was used as a reference in reverse PCR analysis with primers 5'-CACGGCCACTGGAAGCA -3' and 5'-TCCTCAGGGTTCCTGATGCC-3'. The program used in RT-PCR was as follow: 35 cycles of 95 °C for 15sec; 55 °C for 30 sec; and 72 °C for 30 sec; then 72 °C for 10 min. The 1.5% gel and the DL2000 marker (Takara) were used for detecting products of RT-PCR.

Low-K⁺ stress assay

Transgenic plants derived from individual transformation events and non-transgenic plants were used for low-K⁺ stress assays in tissue culture and hydroponic culture. In tissue culture assay, a low-K⁺ medium was prepared following the method described by Xu et al. (2006). First, a K⁺-free MS medium was prepared and then supplemented with KCl to obtain a K⁺ concentration of 100 µM. The MS medium containing K⁺ content of 20 mM were designed as high-K⁺ medium. Big tubes with diameter of 2.0 cm were used for tissue culture assay and each tube contained 50 ml medium. Similar segments of micropropagated plantlets of three transgenic lines and the non-transgenic control were transferred to the low-K⁺ medium and high-K⁺ medium. Six replicates of each line were treated for low and high K⁺ condition. After treatment for 25 days, the phenotypes were observed and plant dry weight was measured after drying in oven at 80 °C for 48 h. For measurement of plant K⁺ content, the plantlets were ground to power and treated following the H₂SO₄-H₂O₂ ashing procedure. The K⁺ concentrations of the samples were determined using a flame photometer (Jenway PFP7, UK).

The hydroponic culture was carried out in greenhouse. Uniform plants (approximately 8 cm tall) of the three transgenic lines and the non-transgenic control were transplanted in the 10 L plastic basins. In each basin six seedlings were transplanted. The seedlings were treated in modified Hoagland's nutrient solutions with K⁺ content of 100 µM and 1 mM. The solution was changed every one week. After 45 days treatment, plant height, stalk diameter, leaf length and width were measured, and K⁺ content in roots and shoots was measured.

Statistical analysis

Statistical analyses were conducted using Microsoft Excel and SPSS (Chicago, IL, USA). An analysis of variance was employed to compare statistical differences on the basis of Tuket's t test.

Conclusion

In this study, we firstly reported improvement of sugarcane tolerance to low K⁺ stress through genetic engineering. By over-expression of *AtCBL9*, *AtAKT1* and *AtCIPK23*, sugarcane K⁺ uptake ability and tolerance to low K⁺ stress were greatly enhanced. Under low K⁺ conditions, K⁺ content in transgenic plants was up to 30% higher than that of non-transgenic plants, and the transgenic plants showed a less growth inhibition than non-transgenic plants. Because low K⁺ availability is an important constraint to sugarcane growth and production, these findings would have significant implications for sugarcane improvement.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30800700), the Earmarked Fund for China Agriculture Research System (CARS-20-1-4 and CARS-20-3-1), and the Science and Technology Planning Project of Guangdong Province, China (2012B020301008).

References

- Ashley MK, Grant M, Grabov A (2006) Plant responses to potassium deficiencies: a role for potassium transport proteins. *J Exp Bot.* 57: 425–436.
- Ashraf M, Afzal M, Ahmad R, Maqsood MA, Shahzad SM, Tahir MA, Akhtar N, Aziz A (2012) Growth response of the salt-sensitive and the salt-tolerant sugarcane genotypes to potassium nutrition under salt stress. *Arch Agron Soil Sci.* 58: 385–398.
- Buschmann PH, Vaidyanathan R, Gassmann W, Schroeder JI (2000) Enhancement of Na⁺ uptake currents, time-dependent inward-rectifying K⁺ channel currents, and K_v channel transcripts by K⁺ starvation in wheat root cells. *Plant Physiol.* 122: 1387–1398.
- Chen DW, Lu YL, Jiang Y, Chen ZH, Liu R, Ao JH, Huang Y, Zhou WL, Huang ZR, Li QW (2013) Study on nutritional features of potassium in different genotypes of tissue culture sugarcane seedling. *Chin Agric Sci Bull.* 29: 96–102
- Coale FJ, Sanchez CA, Izuno FT, Bottcher AB (1993) Nutrient accumulation and removal by sugarcane grown on Everglades Histosols. *Agron J.* 85: 310–315.
- D'Hont A, Glasznan JC (2001) Sugarcane genome analysis with molecular markers: a first decade of research. *Proc Int Soc SugarCane Technol.* 24: 556–559.
- Joyce P, Kuwahata M, Turner N, Lakshmanan P (2010) Selection system and co-cultivation medium are important determinants of Agrobacterium-mediated transformation of sugarcane. *Plant Cell Rep.* 29: 173–183
- Lembke CG, Nishiyama Jr MY, Sato PM, de Andrade RF, Souza GM (2012) Identification of sense and antisense transcripts regulated by drought in sugarcane. *Plant Mol Biol.* 79: 461–477.
- Goldemberg J (2007) Ethanol for a sustainable energy future. *Science.* 315:808–810.
- Gopalasundaram P, Bhaskaran A, Rakkiyappan P (2012) Integrated nutrient management in sugarcane. *Sugar Tech.* 14: 3–20.
- Hart CE (1970) Effect of potassium deficiency upon translocation of ¹⁴C in detached blades of sugarcane. *Plant Physiol.* 45: 183–187.
- Hirsch RE, Lewis BD, Spalding EP, Sussman MR (1998) A role for the AKT1 potassium channel in plant nutrition. *Science.* 280: 918–921.
- Hoisington D (1992) Laboratory protocols. CIMMYT applied molecular genetics laboratory, CIMMYT, Mexico, D.F
- Lagarde D, Basset M, Lepetit M, Conejero G, Gaymard F, Astruc S, Grignon C (1996) Tissue-specific expression of Arabidopsis AKT1 gene is consistent with a role in K⁺ nutrition. *Plant J.* 9: 195–203.
- Lee SC, Lan WZ, Kim BG, Li L, Cheong YH, Pandey GK, Lu GH, Buchanan BB, Luan S (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. *Proc Natl Acad Sci USA.* 104: 15959–15964.
- Menossi M, Silva-Filho MC, Vincenz M, Souza GM (2008) Sugarcane functional genomics: gene discovery for agronomic trait development. *Int J Plant Genom.* 2008: 458732.
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plantarum.* 15: 473–497.
- Rakkiyappan P, Thangavelu S, Bhagyalakshmi KV, Radhamani R (2007) Uptake of nitrogen, phosphorus and potassium by some promising mid late maturing sugarcane clones. *Sugar Tech* 1: 23–27
- Schroeder JI, Ward JM, Gassmann W (1994) Perspectives on the physiology and structure of inward-rectifying K channels in higher plants: biophysical implications for K uptake. *Annu Rev Biophys Biomol Struct.* 23: 441–471.
- Shi WM, Wang XC, Yan WD, Tang L, An ZZ, He SJ, Tang WZ, Cao ZH (2002) Over-expressing of potassium channel genes in rice plants and its effect on K uptake and accumulation. *Acta Agron Sin.* 28: 374–378.
- Sentenac H, Bonneaud N, Minet M, Lacroute F, Salmon JM, Gaymard F, Grignon C (1992) Cloning and expression in yeast of a plant potassium ion transport system. *Science.* 256: 663–665.
- Véry AA, Sentenac H (2003) Molecular mechanisms and regulation of K⁺ transport in higher plants. *Annu Rev Plant Biol.* 54: 575–603.
- Wang Y, Wu WH (2010) Plant sensing and signaling in response to K⁺-deficiency. *Mol Plant.* 3: 280–287.
- Wang X, Li J, Zou X, Lu L, Li L, Ni S, Liu F (2011) Ectopic expression of *AtCIPK23* enhances tolerance against low-K⁺ Stress in transgenic potato. *Am J Pot Res.* 88: 153–159.
- Wood RA (1990) The roles of nitrogen, phosphorus and potassium in the production of sugarcane in South Africa. *Fertilizer Res.* 26: 89–98.
- Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, Wu WH (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K⁺ transporter AKT1 in Arabidopsis. *Cell.* 125: 1347–1360.
- Zende GK (1990) Soil fertility management for higher sugar and sugarcane production. In: Rao PN (ed) Recent advances in sugarcane, pp 99–200. KCP Ltd, Vuyyuru, Andhra Pradesh.
- Zhang HC, Yin WL, Xia XL (2010) Shaker-like potassium channels in *Populus*, regulated by the CBL–CIPK signal transduction pathway, increase tolerance to low-K⁺ stress. *Plant Cell Rep.* 29: 1007–1012.