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Full Length Article

Cloning and Identification of Salt Overly Sensitive (*SOS1*) Gene of Sugarcane

Nuttapong Kaewjiw[1], Chanakan Laksana[2] and Sontichai Chanprame[1,3,4*]
[1]*Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand and The Center of Excellence on Agricultural Biotechnology, (AG-BIO/PERDO-CHE), Thailand*
[2]*Faculty of Agricultural Technology, Burapha University Sakaeo Campus, Sakaeo 27160, Thailand*
[3]*Center for Advanced Studies for Agriculture and Food, Kasetsart University Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand (CASAF, NRU-KU, Thailand)*
[4]*Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen Campus, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[*]For correspondence: agrstc@ku.ac.th

Abstract

Salinity greatly affects gylcophyte plants such as sugarcane. It is one of the major factors that limit plant growth and consequently crop productivity. The aim of this study was to clone the full length of the *SOS1* gene, a plasma membrane Na^+/H^+ antiporter and to characterize the gene through *in silico* characterization and real time quantitative PCR. Sugarcane, wild sugarcane, commercial cultivar (KPS94-13) and interspecific hybrid (biotec2) plants at the age of 1.5 months old were subjected to 1/10 Hoagland nutrient solution supplemented with 0, 100 and 200 m*M* NaCI for 0–5 days. Total RNA from the leaves and roots of the plants were used as the template for first strand cDNA synthesis. The full length of *SoSOS1* was 3,390 bp. The phylogenetic analysis revealed that putative *SoSOS1* derived from KPS 94-13 was classified into the SOS1 group. The analysis of trans-membrane protein using TMPRED program showed 11 putative trans-membrane domains. The analysis of *SoSOS1* cDNA expression in the leaves and roots of the three sugarcane genotypes by real-time PCR revealed that the *SoSOS1* cDNA expression levels in roots were higher than in leaves during 1–3 days period and subsequently the leaves were at higher levels than in roots during 4–5 days period at 100 m*M* NaCI stress. While at 200 m*M* NaCI stress the *SoSOS1* cDNA expressions in leaves of the commercial cultivar and interspecific hybrid sugarcane were higher than in the roots.

Keywords: Hoagland solution; Real-time PCR; Na^+/H^+ antiporter; Saline soil; Gene expression

Introduction

Soil salinity is a major limiting factors to plant growth and productivity of many plant species such as chamomile (Razmjoo *et al*., 2008), lettuce (Al-Maskri *et al*., 2010) and cotton (Shaheen *et al*., 2012). High soil salinity induces ionic stress from Na^+ toxicity. Excess Na^+ and Cl^- can induce protein conformation changes and membrane depolarization which leads to ion toxicity. In addition, salinity also affects leaf expansion by reducing leaf area and affecting chlorophyll content which results in a decrease of photosystem II efficiency (Sengar *et al*., 2013). Severe salt stress also interrupts homeostasis in ion distribution and water potential leading to molecular damage, stunting and even death of the plant (Zhu, 2001). Plant adaptations to prevent the accumulation of Na^+ are divided into three mechanisms which consist of: localization of Na^+ influx; Na^+ compartmentalization in the vacuole and finally Na^+ exclusion (Chakraborty *et al*., 2012). *SOS1* (salt overly sensitive 1) which encodes for a plasma membrane Na^+/H^+ antiporter is one of the mechanism responsible for sodium efflux and controlling long-distance Na^+ transport from root to shoot (Yue *et al*., 2012). It is important for maintaining a low level of Na^+ at the cellular level, allowing plant survival under salt stress conditions. The *SOS* (salt overly sensitive) pathway consists of three domains, *SOS1*, *SOS2* and *SOS3*. An increase of Ca^+ from salt stress is sensed by SOS3 and then activates *SOS2* to become a *SOS2*/*SOS3* protein kinase complex. After that *SOS1* will be activated by the *SOS2*/*SOS3* protein kinase complex (Shang *et al*., 2012) and Na^+ is transported out of the cell. *SOS1* gene was first cloned from *Arabidopsis* (Shi *et al*., 2000). In addition, *SOS1* has also had been cloned from and characterized in several other plants for examining of salt tolerance in plants including *Arabidropsis* (Shi *et al*., 2003), wheat (Xu *et al*., 2008), reed plant (Takahashi *et al*., 2009), sweet potato (Shang *et al*., 2012) and *Brassica* (Chakraborty *et al*., 2012).

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Salt tolerance is a complicated trait in which many genes are involved. This makes a difficulty for success of a salt tolerance breeding program. One of the mechanisms that plants use to deal with salt stress is Na^+ exclusion from plant cell. The mechanism is powered by the operation of a plasma membrane H^+-ATPase (Yamagushi and Blumwald, 2005). Thus molecular analysis of genome at the DNA level is an additional tool for the breeders, and allows them to transfer and combine desirable genes with greater precision for greater benefits (Khan *et al*., 2001). Cloning and identifying genes which respond to salt stress is the first important step which must be accomplished for efficiency improvement of salt tolerance in sugarcane. Thus, the aim of this study was to clone the full length of the *SOS1* gene and to characterize the gene at molecular level and real time quantitative PCR.

Materials and Methods

Plant Materials and RNA Extraction

The 1.5 months old seedlings of wild sugarcane (*Saccharum spontaneum*), commercial cultivar (*S. officinarum* cv. KPS94-13, the drought tolerance cultivar) and interspecific hybrid (cv. Biotec2) were hydroponically cultured in 1/10 Hoagland salt solution (Hoagland and Arnon, 1950) supplemented with 0, 100 and 200 m*M* of NaCl. The total RNA from the young leaves and root tissues of each sample was extracted every day until the fifth day by using the method described by Laksana and Chanprame (2015). The total RNA was converted to first strand cDNA by reverse transcription PCR following manufacturer's protocol (Thermo Scientific).

Amplification of Full Length *SOS1* cDNA from Commercial Cultivar

The specific primers for the *SOS1* gene were designed using nucleotide sequence information from GenBank (www.ncbi.nlm.nih.gov). Primer sequences are shown in Table 1. The *SOS1* specific primers were used for amplification of the full length of *SOS1* by using the PCR technique. In 20 μL of the reaction consisted of 100 ng of first strand cDNA derived from the commercial cultivar (KPS 94-13) under 0 m*M* NaCl, 2 μL of 10x buffer (Fermentas), 1 μL of 1 m*M* dNTP, 2 μL of 25 m*M* $MgCl_2$, 0.25 μL of 10 μM of forward and reverse primer and 1U of high-fidelity DNA polymerase (5U/μL) (Thermo Scientific). The mixture was incubated at 95°C for 3 min for pre-denaturing. Then, the mixture was incubated in 3 steps for 30 cycles; denaturation at 94°C for 30 sec; annealing at 58°C for 30 sec; extension at 72°C 90 sec and a final extension at 72°C for 5 min. The PCR product was analysis on 0.8% (w/v) agarose gel electrophoresis at 100 V for 40 min.

Table 1: Primer sequences used to amplify *SOS1* gene

Primer name	Primer sequence (5'…3')	Remark
SOS1-F	ATGGGCGGCGAGGGTGAGCC	For full length *SOS1* cDNA
SOS1-R	CTACTGGTCCTGCGGCGG	
Partial SOS1F	GGAACAATGTTTGTGTTCTT	For real-time PCR
Partial SOS1R	TCTTCAAGCATTCCCCAGTA	
GAPDH-F	CACGGCCACTGGAAGCA	Reference gene
GAPDH-R	TCCTCAGGGTTCCTGATGCC	
eEF-1a-F	TTTCACACTTGGAGTGAAGCAGAT	Reference gene
eEF-1a-R	GACTTCCTTCACAATCTCATCATAA	

Cloning and Sequencing

The PCR products were extracted from 0.8% (w/v) agarose gel by using PCR cleanup and gel extraction kit (NucleoSpin® Gel and PCR clean-up) following the company's protocol step by step and were cloned into pGEM®-T Easy vector following the company's protocol (Promaga). Finally, they were sequenced by First Base Laboratory (Malaysia). The nucleotide sequences were compared with nucleotide sequences from the GenBank database using the BLAST program from www.ncbi.nlm.nih.gov/BLAST.

Expression Analysis of *SOS1*

The expression levels of the *SOS1* cDNA were evaluated by quantitative real-time PCR. The primer sequences are shown in the Table 1. The cDNA derived from the leave and root tissues of the plants was subjected to 100 and 200 m*M* NaCl for 0–5 days and used as the template for analysis. The amplification reactions consisted of 100 ng of first strand cDNA, 5 µL of 2x Sensi FAST SYBR No-ROX Mix buffer (Bioline Reagent Ltd.), 0.4 μL of 10 μM of forward and reverse of each primer, deionized water was added to make up the final volume of 10 μL. The amplification was performed by using the following condition: preliminary denaturation at 95°C for 2 min. Then, the mixture was incubated in 3 steps for 40 cycles; denaturation of double strand cDNA at 95°C for 15 sec; annealing at 58°C for 15 sec and extension at 68°C 20 sec. The amplification was conducted by using Mastercycle® epRealplex 4S (Eppendorf®). The levels of gene expression of each treatment were compared with the control (0 day) and normalized with two reference genes which were *GAPDH* and *eEF-1a.* Both of the reference genes are the constitutive gene expressions and widely used as reference genes in sugarcane (Ling *et al*., 2014). Three biological replicates of the real-time PCR analysis were performed in three technical replicates of each sample.

Statistical Analysis

Statistical analysis was performed by using SPSS20.0 software. Results were performed as mean ± SE (standard error of the mean; n=9). Differences in the data were

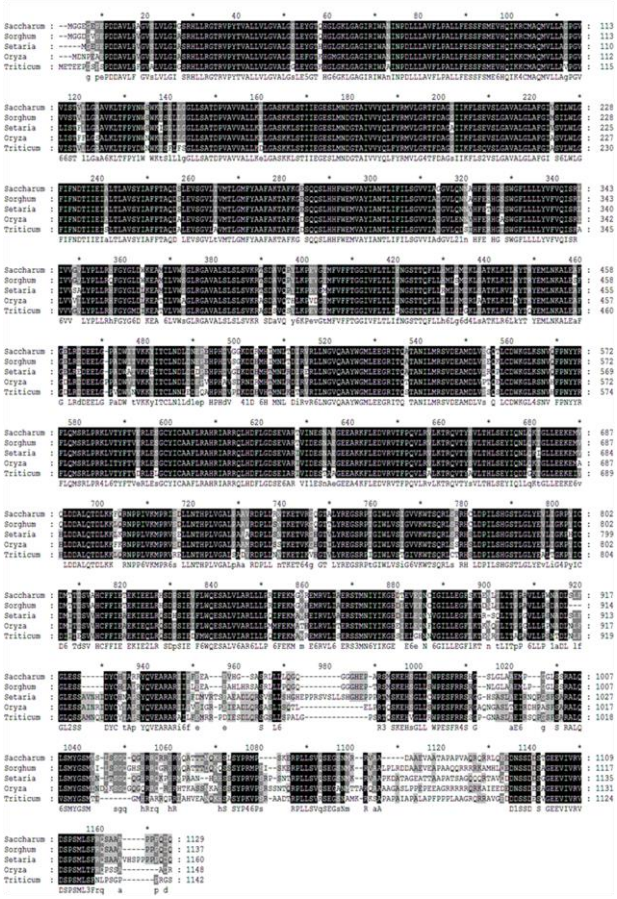

Fig. 1: The multiple alignment of deduced amino acid sequence from full length *SoSOS1* cDNA of KPS 94-13 (*S. officinarum*) to *SOS1* of other plant species by using the GeneDoc program

compared by ANOVA followed by post-hoc multiple comparisons by using Duncan's multiple range test. The differences were investigated significant at $P<0.05$.

Phylogeny Analysis

For classification, the group of protein derived from the full length of the *SOS1* cDNA in KPS94-13, the full and partial length of *SOS1*, *SOS2* and *SOS3* from many other plant species were obtained from the GenBank database and inverted into amino acid sequence. After that the phylogenetic tree was constructed based on amino acid sequence by using the ClustalW program.

Results

Cloning of *SOS1* Full Length cDNA from Commercial Cultivar

In this study, the *SOS1* full length of commercial cultivar sugarcane (KPS94-13) was cloned by using the PCR technique with specific primers to the *SOS1* gene. A PCR product size of 3,390 bp was obtained. After which, the obtained DNA was sequenced and blasted to the NCBI data base for identification. The results showed that this sequence was homologous to the *SOS1* cDNA of many plant species, including, *Sorghum bicolor* for 95% (XM_002443629.1), *Setaria italica* for 91% (XM_004963297.3), *Oryza sativa* for 86% (AY785147.1) and *Triticum aestivum* for 84% (FN356232.1) (Fig. 1). This is the first report of the identification of the full length of *SOS1* cDNA in sugarcane and named it as *SoSOS1*. In addition, the hydrophobicity plot analysis of deduced amino acid sequence showed that the N-terminal portion of SoSOS1 was highly hydrophobic and has 11 predicted trans-membrane domains (Fig. 2A). *SoSOS1* was predicted to encode a polypeptide of 1,129 amino acid residues with a theoretical molecular mass of 125.41kDa. The 11 predicted trans-membrane domains were underlined (Fig. 2B).

Phylogenetic Analysis

The nucleotide sequence of putative *SoSOS1* cDNA was translated into an amino acid sequence and analyzed for phylogenetic relationship with SOS1, SOS2, and SOS3 protein of certain plant species. The result showed that putative ScSOS1 protein of KPS94-13 was classified into the SOS1 group which is the same as SOS1 in other plant species such as sorghum (Sb), corn (Zm), *S. italica* (Si) and rice (Os) (Fig. 3).

The transcription levels of *SoSOS1* cDNA of wild sugarcane, interspecific hybrid (Biotec2) and commercial cultivar (KPS94-13) subjected to 100 and 200 m*M* NaCl stress condition for 0 (control), 1, 2, 3, 4 and 5 days were evaluated by real-time PCR.

The transcription levels of *SoSOS1* in the roots receiving 100 m*M* NaCl were highest on the first day in all genotypes and the relative expression of the gene in commercial cultivar (KPS 94-13) was the highest and significantly different from the rest of genotypes and the relative expression decreased shapely on the later days (Fig. 4A). In leaves, the relative expressions were highest at 5th day of salt stress and KPS 94-13 showed the highest expression with significantly different from others. The relative expressions of the gene in wild and interspecific hybrid were not significantly different and very low compared to KPS 94-13 (Fig. 4B). In 200 m*M* NaCl, the transcription level of *SoSOS1*in root of KPS 94-13 was the highest one on the first day of stress with significantly different from the others while wild sugarcane with highest on 5th day (Fig. 4C). In leaves, KPS 94-13 was the highest relative to expression among all genotypes tested. The expression levels reached the highest at day 5 in all genotypes and KPS 94-13 with the highest expression genotype with significantly different from others (Fig. 4D).

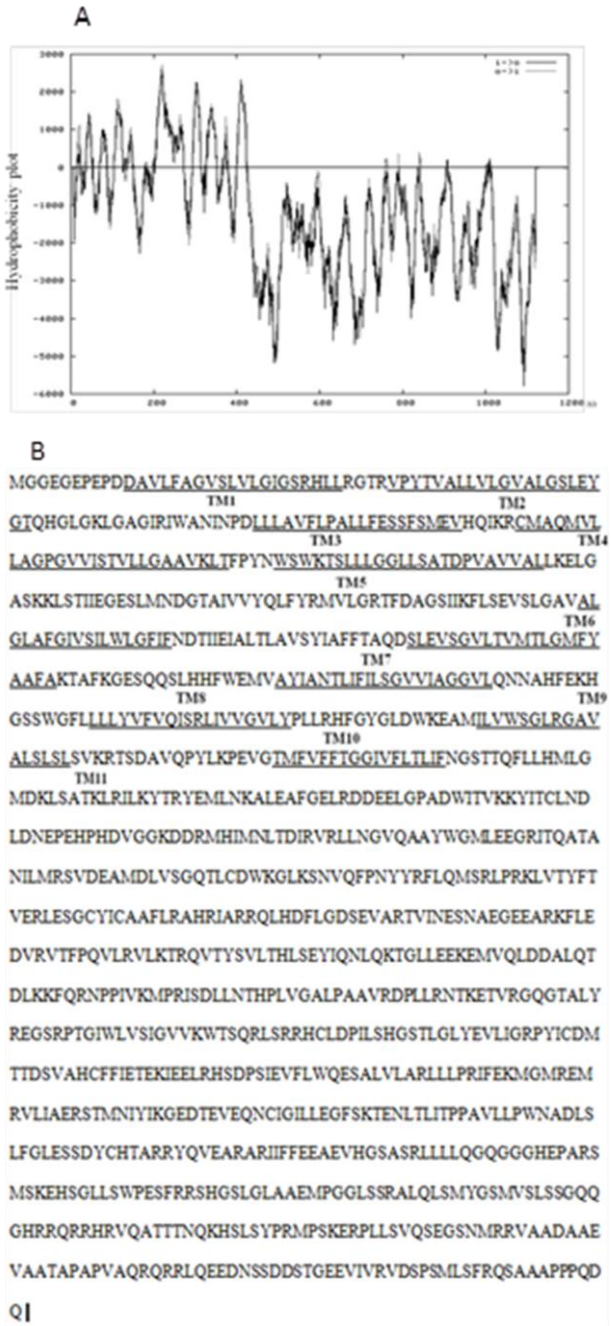

Fig. 2: *SoSOS1* is predicted to encode as a trans-membrane protein. (**A**) Hydrophobicity values were calculated by using the TMPRED program available at http://www.ch.embnet.org/cgi-bin/TMPRED_form_parser (**B**) The deduced amino acid sequence of *SoSOS1*. The 11 putative trans-membrane domains (TM) are underlined

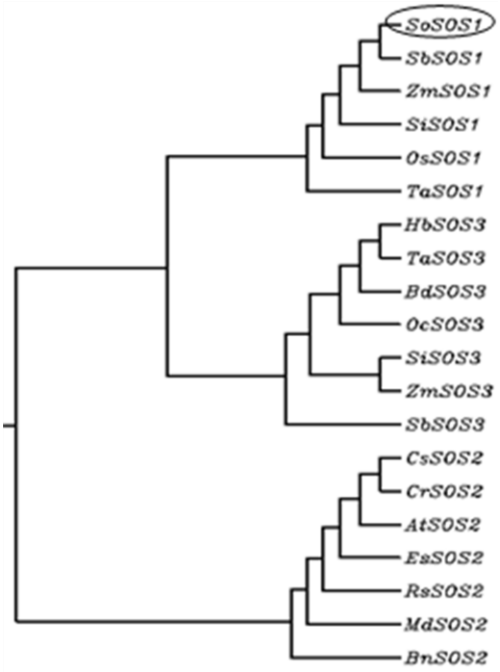

Fig. 3: The phylogenetic relationship between the amino acid sequence of putative *SoSOS1* and the amino acid sequence of *SOS1*, *SOS2* and *SOS3* of other plant species were analyzed by using the ClustalW program. The protein accession numbers: *SbSOS1* (XM_002443629.1), *ZmSOS1* (XM_008647519.1), *SiSOS1* (XM_004963297.3), *OsSOS1* (AY785147.1), *TaSOS1* (FN356232.1), *BnSOS2* (AY310413.1), *RsSOS2* (XM_018624146.1), *AtSOS2* (AY099621.1), *EsSOS2* (XM_006405485.1), *CsSOS2* (XM_010452074.2), *CrSOS2* (XM_006283668.1), *MdSOS2* (KT336311.1), *OcSOS3* (KP330206.1), *BdSOS3* (XM_010232814.2), *SiSOS3* (XM_004961342.3), *ZmSOS3* (BT069484.1), *HbSOS3* (JN107535.1), and *SbSOS3* (XM_002440090.1)

Discussion

The *SOS1* is the essential genes for Na^+ detoxification (Deinlein *et al*., 2014). *SOS1* encodes for Na^+/H^+ antiporter, embedded in the plasma membrane. It is responsible for exclusion of Na^+ when there is an excess of Na^+ inside the cell and also for controlling long-distance Na^+ transport from root to shoot (Yue *et al*., 2012). From this study, it was found that the full length of *SoSOS1* cDNA in sugarcane was 3,390 bp which was similar to the size of *SOS1* in many plants, including, sorghum (Sb; 3,413 bp), *S. italica* (Si; 3,483), rice (Os; 3,447) and wheat (Ta; 3,429 bp). It was predicted to encode a polypeptide of 1,129 amino acid residues with a theoretical molecular mass of 125.41kDa, which is similar to the molecular mass of *SOS1* in *Arabidopsis thaliana* (127 kDa) (Shi *et al*., 2000). Furthermore, the phylogenetic relationship analysis of the putative *SoSOS1* amino acid sequence and *SOS1*, *SOS2* and *SOS3* of certain plants species also indicated that the putative SoSOS1 of commercial cultivar sugarcane cv. KPS 94-13 was classified into the SOS1 group which was in the same *SOS1* group as other plant species. In addition, it was found that *SoSOS1* protein was very closely related to

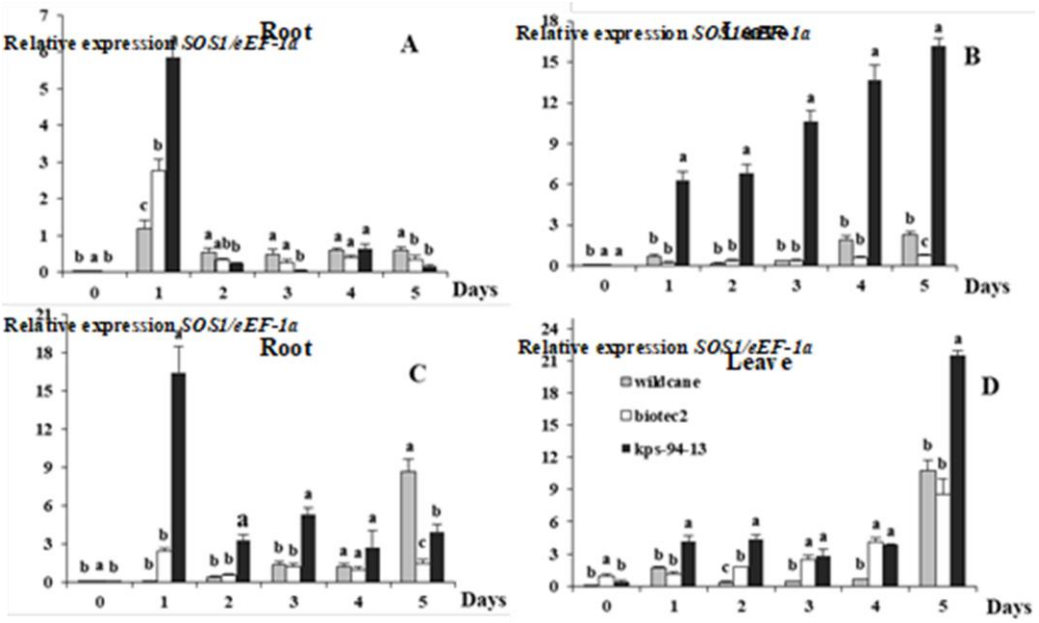

Fig. 4: The relative expression level of *SoSOS1* in the root (A, C) and leaves (B, D) of wild sugarcane, interspecific hybrid (Biotec 2) and commercial cultivar (KPS 94-13) subjected to 100 m*M* NaCl (A and B) and 200 m*M* NaCl (C and D) for 0-5 days. Data are means ± SE of the three biological replicates. Different letters on the bars indicate significant differences between species on the same day of stress at $P<0.05$ according to DMRT

sorghum more than to other species because sorghum belongs to the same family, Poaceae and the same tribe, Andropogoneae as sugarcane (Chittaranjan, 2007). Sorghum is also a member of the grass family and belongs to the saccharine sub-trib (Sorghum, Saccharum, Miscanthus) and shares a common ancestor from about 8–9 million years ago (Wang *et al.*, 2010). These results confirm that the putative *SoSOS1* is *SOS1* protein and may respond for Na^+ exclusion as the same as Na^+/H^+ antiporter of other plants.

In a part of determining the *SOS1* gene expression levels under salt solution were found that the *SoSOS1* expression patterns in the roots (Fig. 4A) and leaves (Fig. 4B) at 100 m*M* NaCl were similar to those at 200 m*M* NaCl (Fig. 4C and D). In roots at 100 and 200 m*M* NaCl both showed increasing *SoSOS1* expression levels in the early period and then decreased in the later period, while in the leaves the expression gradually increased from the early period and reached the highest expression in 5 days. When the plants are under salt stress condition, the root is the first organ in continuous contact with the solution and this causes the higher expression levels in the root rather than the leaves in the early period. Then Na^+ is transferred from root to shoot by *SOS1* activity, encodes for Na^+/H^+ antiporter responsible for exclusion of Na^+ and controls long-distance Na^+ transport from root to shoot (Yue *et al*., 2012), this causes higher expression levels in leaves than in roots in the later period. However, the roots of wild sugarcane receiving 200 m*M* NaCl showed a different expression pattern that gradually increased from the early day and reached the highest at the fifth day of stress. This might be because wild sugarcane is a wild species with readily adaptable to stress environments. The plant can with stand high salt stress with low expression of the concerned gene. If salt stress is prolonged, the expression of the gene will be up-regulated. When compared with the transcription levels of *SoSOS1* in roots and leaves are between 100 and 200 m*M* NaCl it was found that the transcription levels at 200 m*M* were higher than those at 100 m*M* NaCl. The concentrations of NaCl probably affect the *SoSOS1* expression levels as found in *Medicago truncatula* and *M. falcate* (Liu *et al*., 2015), *SOS1* can be up-regulated by NaCl stress. Considering the *SoSOS1* expression levels among the three genotypes it was found that KPS 94-13 showed the highest expression. KPS 94-13 is an open pollinated progeny of line no. 89-1-20. The *SoSOS1* gene in KPS 94-13 might come from the sugarcane clones planted surround the line 89-1-20 at that time. The difference of gene expression level among genotypes might be a consequence of the different amino acid sequence of each genotype. This may be important for developing salt tolerance plant through this gene in the future.

Conclusion

The full length of *SoSOS1* cDNA from commercial sugarcane cv. KPS 94-13 was 3,390 bp. Comparison and analysis of this cDNA with the NCBI database found that the *SoSOS1* cDNA showed high similarity to *SOS1* of *S. bicolor*. The analysis of trans-membrane protein of *SoSOS1* by using

the TMPRED program showed 11 putative trans-membrane domains. It indicates that this cDNA is *SOS1*, a plasma membrane Na^+/H^+ antiporter. Examination of *SoSOS1* expression levels in roots and leaves at 100 and 200 m*M* NaCl stress found that at 200 m*M* NaCl the expression levels were higher than at100 m*M* NaCl. In addition, the *SoSOS1* expression levels among three sugarcane genotypes found that KPS94-13was the highest expression. This information in concert with some physiological parameters that the sugarcane plants respond to salt stress will be of benefit for improving the salt tolerance of sugarcane in the future.

Acknowledgments

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