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PARTIAL CLONING AND EXPRESSION OF *ScBADH* AND *ScMIPS* GENE IN WILD AND CULTIVATED SUGARCANE UNDER MIMICKING SALINE SOIL CONDITIONS

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ABSTRACT

Partial sequences of *ScBADH* and *ScMIPS* were cloned and sequenced. Partial cDNA sequences of *ScBADH* and *ScMIPS* encoded for 138 and 116 amino acid residue proteins, respectively. The deducted amino acid sequence of ScBADH showed high similarly to BADH of many plant species. When the seedlings of wild and cultivated sugarcane, KPS 94-13, were subjected to nutrient solutions containing 1% NaCl for 0, 12, 24, 48 and 72 h, the expression of *ScBADH* in leaves was highest at 72 h, and after 12 h in roots. The accumulation of *ScMIPS* transcripts in the leaves of both sugarcane genotypes was highest at 48 h. Expression of *ScMIPS* gene in roots was highest at 24 h after receiving salt stress, and then decreased slowly. Results indicated that NaCl-stress could upregulate the expression of *ScMIPS* and *ScBADH*. Wild and cultivated sugarcane showed the same pattern of *ScMIP* and *ScBADH* transcript accumulation. However, wild sugarcane showed a higher transcript accumulation than the cultivated variety.

Key words: salinity**,** nutrient solution**,** qRT-PCR, phylogenetic tree, osmoprotectant

INTRODUCTION

Currently, areas used for growing plants are affected by abiotic stress factors, such as drought, salinity, flooding, and extreme temperatures that have long been known as major limitating factors for crop productivity (Boyor 1982). Salinity has been increasing annually. The increase in soil salinity is due to improper irrigation and the excessive use of fertilizers. Salinity appears to affect growth due to either the toxic effects of Na^+ or Cl^- accumulation or the low osmotic potential of the soil or solution (Pitman 1984), which can adversely crop plant productivity. Salt-tolerant plants are able to maintain an acceptably low concentration of NaCl in the cytosol by exclusion or sequestration within the cellular compartment, such as the vacuole and accumulation of K^+ and organic osmotica in the cytosol (Kramer 1984). Salt stress distorts homeostasis at both the cellular and whole plant, involving developmental, morphological, physiological, biochemical, and molecular mechanisms (Parida and Das 2005). Some of the most severe effects caused by salinity include cell membrane disruption, generation of reactive oxygen species, reduction in enzymatic and photosynthetic activities, and decreased nutrient acquisition (Hanumantha et al. 2016).

Accumulation of osmoprotectants is a common metabolic adaptation for salt-tolerant plants (Rathinasabapathi 2000, Suleiman et al. 2013). Many osmoprotectants, such as glycine betaine and *myo*-inositol, enhance the protection of plants from such stress. Betaine aldehyde dehydrogenase (BADH), which is encoded by *Betaine aldehyde dehydrogenase* (*BADH*) gene, is the key enzyme for betaine synthesis, favoring salinity tolerance (Tabuchi et al. 2005) and catalyzes the final step in the

synthesis of the osmoprotectant glycine betaine from choline. Choline is converted by choline monooxygenase (CMO) to betaine aldehyde, which is then converted to glycine betaine by betaine aldehyde dehydrogenase. *BADH*s genes have been cloned and characterized from many plant species, such as spinach (Rathinasabapathi 2000), sweet potato (Chen et al. 2014), and *Suaeda corniculata* (Wang et al. 2016). The *BADH* gene was cloned from *S. corniculata* and results suggested that *BADH* might be a positive regulator in plants during NaCl-stress response (Wang et al. 2016).

Myo-inositol-1-phosphate synthase (MIPS) catalyzes the conversion of D-glucose-6-P to D-*myo*-inositol-1-phosphate, followed by its specific dephosphorylation to free *myo*-inositol by the Mg^{++} dependent *L-Myo-inositol 1-phosphate phosphatase* (IMP) (Loewus and Murthy 2000). The *MIPS* gene has been isolated from several plant species such as *Arabidopsis thaliana* (Johnson 1994), *Nicotiana tabacum* (Hara et al. 2000), *Oryza sativa* (Yoshida et al. 1999), *Zea mays* (Larson and Raboy 1999), and *Ipomoea batatas* (Zhai and Liu 2009). Abreu and Aragão (2007) isolated the *MIPS* gene from yellow passion *PeMIPS* transcripts were expressed in the ovule, pollen grand, and leaves. The *MIPS* gene has also been shown to improve tolerance to abiotic stress in several plant species (Zhai et al. 2015).

Sugarcane is a naturally salt-sensitive plant. Saline soil is the primary problem causing low yield for sugarcane. A sugarcane breeding program aiming to improve salt tolerance is difficult to achieve due to trait complications. Understanding the response of sugarcane under salt stress at a molecular level is an important step in accelerating the realization of this program. This study sought to clone and identify the partial of *ScBADH* and *ScMIPS* sequences from wild (*Saccharum spontaneum* L.) and cultivated sugarcane (*S. officinarum* L.cv. KPS 94-13), and to investigate the expression of the genes under mimic saline soil condition.

MATERIALS AND METHODS

Plant samples and RNA extraction

Two-month old seedlings of wild sugarcane (*Saccharum spontaneum*) and cultivated sugarcane (*S. officinarum* cv. KPS 94-13) were grown in 1/10 Hoagland's nutrient solution containing 200 mM NaCl. Wild sugarcane is diploid with 80 chromosomes while the cultivated sugarcane is polyploid with 128 chromosomes. Leaf and root samples were collected at 0, 12, 24, 48 and 72 h after salt stress for RNA extraction. Total RNA was extracted from 0.1 g of leaves or root by the method described previously by Laksana and Chanprame (2015). RNA quality was determined through PCR using actin specific primers, while RNA quantity was determined using a nanodrop-spectrophotometer.

For first strand cDNA synthesis, the 12.5 uL reaction mixture consisted of 1 µg total RNA sample, 2 µM Oligo $(dT)_{18}$ primer, 0.8 mM dNTP, and RNase-free water were added and mixed gently. The reaction was incubated at 65 ºC for 5 min and then cooled at 4 ºC for a minimum of 2 min. After which, 1x reaction buffer, 0.5 U RiboRock RNAse inhibitor (Fermentas, Canada), 1 mM dNTP, and 1 µL Revert Aid M-MuLVRT (Fermentas) was added to the reaction tube, mixed gently and incubated at 42 ºC for 1 h. The reaction was thermally inactivated at 70 ºC for 10 min, then cooled at 4 ºC for at least 2 min. One-fourth U/ µL RNAseH was added for the degradation of any remaining total RNA.

Amplification of *ScBADH* and *ScMIPS* gene partial length from wild and cultivated sugarcane

The primers used in this study are listed in Table 1. The degenerated primers were designed based on the highly conserved region of the *BADH* and *MIPS* gene sequences of numerous plant species from the NCBI database, such as *Elaeis guineensis* (XP_010913436.1), *Oryza sativa* (ABI84118.1), *Hordeum vulgare* (ABO93605.1), *Triticum aestivum* (AAL05264.1), *Sorghum bicolor* (XP_002444357.1), and *Zea mays* (NP_001105781.1). PCR reaction was carried out in a total

volume of 20 µL containing 100 ng of the first strand cDNA template, 50 µM dNTPs, 1U of *Taq* polymerase (Fermentas), 5 mM $MgCl_2$, 1x buffer (Fermentas), 0.125 µM forward primer, and 0.125 µM reverse primer. The amplification was performed under the following conditions: initial denaturation at 95 °C for 3 min; then 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 1 min; then a final extension at 72 °C for 5 min. The amplification products were resolved on a 0.7% (w/v) agarose gel electrophoresis at 100 V for 40 min.

Table 1. Degenerate primers for amplifying partial of *BADH* and *MIPS* gene sequences.

Genes	Primers
BADH	Forward: 5'- ATGGGACATGGAYGATGT -3' Reverse: 5'- TTTTKCCACCAAGTTCCA -3'
MIPS	Forward: 5'- TGTTCATCGAGAGCTTCCG -3' Reverse: 5'- ATGGGMAGGAGGCTCTTGAA -3'

Note: Y = C/T, K= G/T, M= A/C

Sequencing and phylogenetic tree construction

The PCR products were eluted from the 0.7% (w/v) agarose gel using a PCR clean up and gel extraction kit (NucleoSpin® Extract II) following the manufacturer's protocol and were sequenced at First Base Laboratory (Malaysia). The sequences were compared with GenBank databases (Nucleotide BLAST) (www.ncbi.nlm.nih.gov/BLAST/) using default parameters, and then translated into amino acid sequences using Genetyx 5.0 (http://genetyx.software.informer.com/). The derived BADH and MIPS amino acid sequences were aligned to BADH and MIPS amino acid sequences of other plant species by using the Clustal X (http://www.clustal.org/clustal2/) and Genedoc 2.7 programs (http://genedoc.software.informer.com/2.7/). Clustal X was used for alignment. After which, alignment results were imported to GeneDoc 2.7 to generate a picture. A phylogenetic tree was constructed based on BADH and MIPS family members using the Neighbor joining method with 1,000 bootstrap replication using the ClustalW2 program (http://www.ebi.ac.uk/tools/msa/clustalw2).

Analysis of *ScBADH* and *ScMIPS* gene expression under mimic saline soil via quantitative PCR

The expression of *ScBADH* and *ScMIPS* in wild and cultivated sugarcane were investigated by real-time quantitative PCR. The accuracy of quantification was confirmed through normalization of S*cBADH* and *ScMIPS* expression to a reference transcript encoding for glyceraldehydes-3-phosphate dehydrogenase: *GAPDH* (GenBank accession no. CA254672). *GADPH* is identified as suitable reference gene for the normalization of gene expression under salinity/drought-treatment in sugarcane (Guo et al. 2014). Specific primers of the two genes for real-time PCR (Table 2) were designed from partial length *ScBADH* and *ScMIPS* cDNAs using Primer3 ver 0.4.0 (http://simgene.com/Primer3).

Table 2. Specific primers used for real-time quantitative PCR.

Genes	Primers
GADPH	Forward 5' CACGGCCACTGGAAGCA 3'
	Reverse 5' TCCTCAGGGTTCC TGATGCC 3'
BADH	Forward 5' TTGAACATTGTGACAGGATTAGG 3'
	Reverse 5'AGTTCCAGCGTAACAGGCTT 3'
MIPS	Forward 5' GCACAACACCTGTGAGGACT-3
	Reverse 5' TGAGGTAGCTCAGGATGGTG 3'

PCR reactions were performed in a total volume of 20 µL containing 500 ng of first strand cDNA template, 1x SensiFAST SYBR No-ROX mix buffer (Bioline Reagent Ltd.), 0.4 µM forward primer, and 0.4 µM reverse primer. The amplification was performed under the following conditions: initial denaturation at 95 °C for 30 sec; then 45 cycles of denaturation at 94 °C for 5 sec, annealing at 58 °C for 15 sec, and extension at 72 °C for 10 sec in a Mastercycler® ep realplex4 from Eppendorf®.

The expression of these genes was compared to control conditions (0 day) and the reference gene was *GADPH* gene for sugarcane. For each sample, the reactions were carried out in three biological replicates with three technical replicates each.

RESULTS AND DISCUSSION

The wild sugarcane (*Saccharum spontaneum*) is naturally more tolerant to abiotic stress compared to cultivated sugarcane. Cultivated sugarcane, KPS 94-13, is one of the most popular cultivars planted in the western region of Thailand and is more sensitive to abiotic stress than wild sugarcane. We expect that they may have some differences in the nucleotide sequences of their genes responsive to stress conditions. To prove this hypothesis we cloned the partial *BADH* and *MIPS* gene from both sugarcane genotypes and investigated the expression of the genes under salt stress conditions.

Cloning of partial *BADH* and *MIPS* gene

Saline soil is one of the major factors causing low yield in plants (Senger et al. 2013), affecting plant growth and reducing agricultural productivity worldwide. Sugarcane is one plant that shows high sensitvity to salinity. In this study, partial *BADH* and *MIPS* genes were first cloned from cDNAs from both wild and cultivated sugarcane cv. KPS 94-13. The PCR products were DNA fragments had a size of approximately 450 bp for *BADH* and 350 bp for *MIPS*, both corresponding to the predicted product size based on primer design results. The *BADH* amplicon had a 96% similarity with betaine-aldehyde 2, partial sequence from *Sorghum bicolor* (AGZ15752.1), while the *MIPS* amplicon had 99% similarity with myo-inositol-1-phosphate synthase sequence from *Saccharum spontaneum* (ALO50704.1). The sequences of these DNA fragments were translated to amino acid sequences of 138 and 116 amino acid residues for *BADH* and *MIPS*, respectively (Fig. 1).

Fig. 1. The deduced amino acid sequences of partial *BADH* (A) and *MIPS* (B) cloned from wild and cultivated sugarcane c.v. KPS 94-13.

The deduced amino acid sequences of BADH from both wild and cultivated sugarcane showed 99.3% identity, while sequences from both MIPS proteins showed 100% identity. These amino acid sequences were compared with sequences of other plant species from the GenBank database. Deduced amino acid sequence from both *BADH* cDNAs of wild and cultivated sugarcane cv. KPS 94-13 showed high similarity to *Sorghum bicolor* (96%, AGZ15752.1), *Zea mays* (96%, AQK52373.1), *Zoysia tenuifolia* (88%, BAD34956.1), *Oryza sativa japonica* (89%, BAT05491.1), and *Hordeum vulgare* (83%, BAB62846.1). We named it as *ScBADH*. Meanwhile, the amino acid sequence deduced from *MIPS* cDNA showed high similarity to *Zea mays* (99%, ACG33827.1) *Zoysia*

matrellam (97%, AIN39843.1) *Sorghum bicolor* (97%, KXG39974.1), *Triticum aestivum* (96%, EU371115.1), *Oryza sativa japonica* (96%, BAA25729.1), *Triticum aestivum* (96%, AGK06903.1), and *Nicotiana tabacum* (91%, NP_001311846.1). The start codon of the gene is included in this nucleotide sequence, we named it as *ScMIPS*. Partial of *ScBADH* and *ScMIPS* will be used for cloning the full length of *ScBADH* and *ScMIPS* in the future.

The *BADH* and *MIPS* gene in many plant species have been cloned and the gene expression level determined. Wang et al. (2016) cloned *BADH* gene from *Suaeda corniculata* and analyzed the expression profile of this gene. Meanwhile, *MIPS* gene of several plants have been cloned and studied, including rice (Yoshida et al. 1999), corn (Larson and Raboy 1999), and sweet potato (Zhai and Liu 2009). Abreu and Aragão (2007) isolated *PeMIPS* gene from *Passiflora edulis* and the expression of the gene was analyzed. The gene was expressed in the ovules, pollen grains, and leaves during stress conditions, and suggested that it is important for environmental stress response.

The amino acid sequences deduced from partial *ScBADH* and *ScMIPS* were used for phylogenetic tree construction. This revealed that *ScBADH* and *ScMIPS* from both wild and cultivated sugarcane are closely related to sorghum and corn (Fig. 2 and 3). There was segregation of these genes in monocotyledonous plants, especially sorghum, since sorghum and sugarcane are close relatives, and this evolutionary divergence is estimated as occurring as early as 5 million years ago (Dillon et al. 2007).

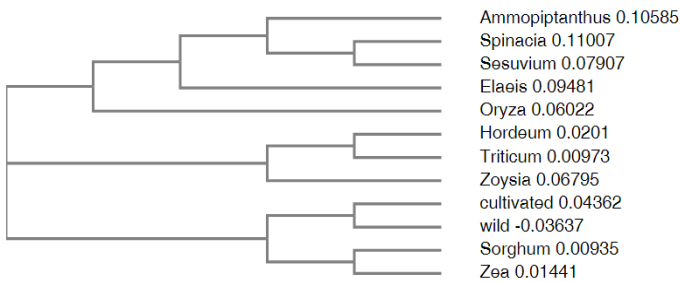

Fig. 2. Phylogenetic tree of *ScBADH* among different species was constructed based on deduced amino acid sequences by Neighbour-joining method with 1,000 bootstrap replication using ClustalW2 program .GenBank accession numbers of amino acid sequences used: *Ammopiptanthus nanus* (AIG52060.1), *Spinacia oleracea* (AAN52929.1), *Sesuvium portulacastrum* (AEK98521.1), *Elaeis guineensis* (XP_010913436.1), *O. sativa* (ABI84118.1), *Hordeum vulgare* (ABO93605.1), *T. aestivum* (AAL05264.1), *Zoysia tenuifolia* BAD34947.1), *S. bicolor* (XP_002444357.1) and *Z. mays* (NP_001105781.1).

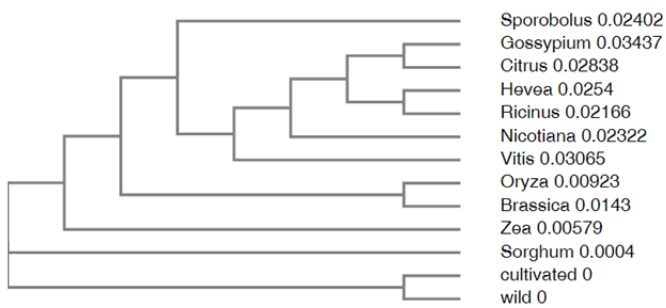

Fig. 3. Phylogenetic tree of *ScMIPS* among different species was constructed based on deduced amino acid sequences by Neighbour-joining method with 1,000 bootstrap replication using ClustalW2 program .GenBank accession numbers of amino acid sequences used : *Sporobolus*

alterniflorus (ADC33414.1), *Gossypium hirsutum* (ACJ11714.1), *Citrus sinensis* (XP_006464258.1), *Hevea brasiliensis* (AFD61599.1), *Ricinus communis* (NP_001310667.1), *Nicotiana tabacum* (NP_001311846.1),*Vitis vinifera* (XP_010652823.1), *Oryza brachyantha* (XP_006651124), *Brassica juncea* (ABY74556.1), *Z. mays* (ACG33827.1) and *S. bicolor* (KXG39974.1)

The *BADH* and *MIPS* genes regulate the production of osmoprotectants which plants use for protection from the harmful effects caused by abiotic stress, such as salinity (Santos et al. 2010). These molecules accumulate in cells and balance the osmotic difference between the cell's surroundings and the cytosol. In the present study, expression patterns of *ScBADH* and *ScMIPS* genes in different tissues (leaves and roots) exposed to salt stress (200 mM NaCl) at different lengths of time (0, 12, 24, 48 and 72 h) were analyzed through quantitative real-time PCR (qRT-PCR). Roots, in particular, were selected since this is the first organ affected by salt stress.

Results showed that the expression of *ScBADH* and *ScMIPS* genes in leaves and roots were up-regulated compared to the control (0 h). Accumulation of the *ScMIPS* transcript in leaves for both sugarcane species was highest after 48 h (Fig. 4A) whereas, the accumulation of *ScMIPS* transcript in roots of each sugarcane species after 24 and 48 h exhibited highest expression, with no-significant difference between the two time periods (Fig. 4B). Expression in leaves was higher than that in the roots, except after 24 h, where expression in the roots of wild sugarcane was a little bit higher than in leaves. This finding is in accordance with An-jun et al. (2016) who analyzed the expression of *MIPS* gene in pumpkin and suggested that the expression of this gene exhibited tissue specificity, with highest expression level in leaves when subjected to salt, abscisic acid (ABA), and drought stress. It is important to recognize that different tissues and cells in a plant are adapted for specific and often very different purposes. It is therefore not surprising that the expression levels of genes will differ from tissue to tissue and from cell to cell, depending on the tissue/cell's function (Roy et al. 2014). The accumulation of *ScBADH* transcript in leaves was highest after 72 h (Fig. 4C), whereas in roots, the expression was highest after 12 h (Fig. 4D).

The comparison of relative expression of *ScBADH* between roots and leaves revealed that its expression in roots was higher than in leaves. This result is similar to the expression of *IbBADH* in *Ipoea batatas*, which is strongly expressed in roots (Chen et al. 2014). These results indicate that the expression of both *ScMIPS* and *ScBADH* were induced by NaCl-stress. This agrees with Wang et al. (2016) who reported that *BADH* gene is a positive regulator in plants during its response to NaCl-stress. Wild and cultivated sugarcane showed the same pattern of expression for both genes. However, the relative expression of both genes in wild sugarcane was higher than in cultivated sugarcane. This phenomenon may correlate with observations that wild plants species are generally more tolerant to abiotic stress than cultivated species (Bolger et al. 2014, Li et al. 2014). Both genes regulate the production of osmoprotectants. The higher the gene expression, the more osmoprotectants produced and may result in higher salt-stress tolerance. Comparison between the relative expression of *ScBADH* and *ScMIPS* genes showed that *ScBADH* expression was lower than *ScMIPS* expression. This may be because MIPS enzyme is the only known enzyme to catalyze the conversion of glucose 6-phosphate to inositol phosphate (Lackey et al. 2003), which in turn is a precursor for many inositol-containing compounds that are implicated in various physiological and biochemical processes, including growth regulation, cell membrane biogenesis, hormonal regulation, stress signaling, and plant immunity (Kaur et al. 2013). However, BADH enzyme catalyzes the final step in the synthesis of the osmoprotectant glycine betaine from choline (Chen and Murata 2002). The results indicate that the expression pattern for both genes were similar when expression was up-regulated to the highest level, a short time after receiving salt stress, and then gradually decreased.

The higher expression of *ScMIPS* in sugarcane compared with *ScBADH* expression might indicate a better osmotic adjustment of inositol to salt stress than glycine betaine. The expression level

of both genes, especially *ScMIPS*, may be useful as a parameter to assist in the selection for salt tolerance in sugarcane breeding.

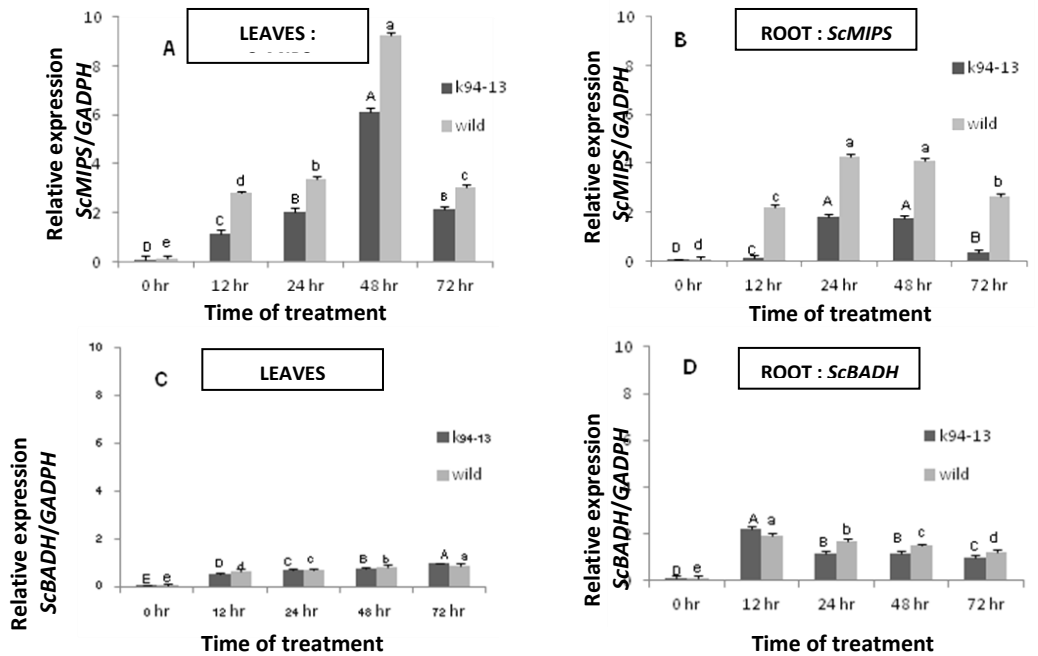

Fig. 4. The expression of *ScMIPS* and *ScBADH* gene in leaves (A: *ScMIPS*, C: *ScBADH*) and root (B: *ScMIPS*, D: *ScBADH*) of wild and cultivated sugarcane subjected to 200 mM NaCl for 0, 12, 24, 48 and 72 h. The different capital letters or small letters on the boxes indicate significant differences with $p<0.05$ while the error bars represent standard error. Each treatment composed of three biological and three technical replicates.

CONCLUSION

Partial cDNA sequences of *ScBADH* and *ScMIPS* were cloned from wild and cultivated sugarcane (KPS 94-13). The deduced amino acid sequence from *ScBADH* showed a high similarly to the BADH of other plant species, such as *Sorghum bicolor* (96%), *Zea mays* (96%) *Oryza sativa japonica* (89%), while ScMIPS deduced protein showed a high similarly to MIPS from *Zea mays* (99%), *Zoysia matrellam* (97%), *Sorghum bicolor* (97%), *Triticum aestivum* (96%), *Oryza sativa japonica* (96%), and *Nicotiana tabacum* (91%). Wild and cultivated sugarcane showed the same pattern of *ScMIP* and *ScBADH* transcript accumulation. Salt stress could up-regulate the expression of both genes, wherein the expression of *ScBADH* from the roots was higher than in leaves, while the expression of *ScMIPS* dominated in leaves. Investigations for gene expression correlation with physiological parameters responding to salt stress in sugarcane may be used as an index for salt tolerance selection in sugarcane breeding.

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