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Cloning and expression study of sugarcane (*Saccharum* sp.) sucrose transporter gene (*SoSUT4*)

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Abstract

Sugarcane (*Saccharum* sp.) is a vital commodity for global sugar production and biomass generation, with sucrose being the primary sugar accumulated predominantly in the stem. The sucrose transporter protein is essential in facilitating sucrose transport across cells and over long distances within plants, from source to sink tissues. This study focused on the cloning and expression analysis of the *SoSUT4* gene in the Bululawang sugarcane variety. A partial coding sequence of *SoSUT4*, comprising 802 nucleotides and encoding a 267-amino acid protein, was successfully cloned and sequenced. Sequence analysis revealed that the *So*SUT4 protein shares high similarity with other SUT4 proteins in monocotyledonous plants, particularly with *Saccharum spontaneum* and *Saccharum* hybrid. Bioinformatics predictions indicated that the *So*SUT4 protein is localized to the plasma membrane and contains six transmembrane helices. Gene expression analysis further demonstrated that *SoSUT4* expression was significantly higher in the middle internodes of the stem compared to the youngest midsection of the leaves. This expression pattern correlates with higher sucrose accumulation in the stem, as reflected by elevated Brix levels in the stem (19.61%) compared to the leaves (19.48%). This finding suggests that *SoSUT4* is essential for sucrose translocation to the stem, which serves as the primary storage site for sugar. The study provides valuable insights into the SoSUT gene family in sugarcane, particularly highlighting the role of SoSUT4 in sugar transport and accumulation. Future research should further investigate the underlying mechanisms of *SoSUT4* and related genes to enhance our understanding of their impact on sugarcane yield, with potential applications for genetic engineering aimed at improving crop productivity.

[Keywords: brix, relative expression, *SoSUT4*]

Introduction

The sugarcane plant (*Saccharum* sp.) is a significant commodity for sugar production and has the potential to produce a substantial amount of biomass. The primary sugar in sugarcane is sucrose, which accumulates in the stem. Sucrose is the main product of carbon dioxide fixation during photosynthesis and plays a crucial role in the plant's allocation of assimilated carbon (Sauer, 2007). According to Subiyakto et al. (2016), sugar is a major source of calories in the Indonesian diet, alongside other staple foods. Its importance is reflected in government policy, which classifies sugar—particularly crystallized sugar—as one of the nine essential foods. As a result, the policy mandates that sugar must be available in sufficient quantities, be of high quality, affordable, and easily accessible to consumers.

The Center for Agricultural Data and Information (2022) reported that global sugarcane productivity has not yet reached optimal levels. In 2022, Indonesia's sugarcane productivity was around 67 tons per hectare, placing it 36th in the world. However, domestic production remains insufficient to meet national demand, resulting in sugar imports from countries such as Thailand, India, Brazil, and Australia (Statistics Indonesia, 2023). That year, sugar imports increased to 6.01 million tons, valued at US$ 2.998 billion, surpassing the previous year's import levels. This reliance on imports highlights the need for agricultural innovations to improve sugarcane yield and ensure self-sufficiency.

One way to increase sugarcane productivity is through breeding programs that develop new, high-yielding varieties. A primary goal of these programs is to enhance the sucrose content in sugarcane stems. Sucrose accumulation is influenced by several factors, including photosynthesis, enzyme activity in sucrose metabolism, transport to storage organs, carbon allocation, and mobilization of sucrose for

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vegetative growth (Conradie, 2011). Effective sucrose accumulation requires efficient translocation from source organs (leaves) to sink organs (stems). This process is facilitated by sugar transporter proteins, which regulate, transport, compartmentalize, and store assimilates within the plant. Wang et al. (2017) classified sugar transporter genes into several categories, including *SWEET* (Sugars Will Eventually Be Exported Transporters), *SUT* (sucrose transporters), and monosaccharide transporters.

SUT proteins play a crucial role in sucrose translocation through the phloem, particularly in the uptake and accumulation of high levels of sucrose. These proteins are essential for building sucrose reserves in plants. Zhang et al. (2016) reported that most plant species contain at least four types of SUT proteins. SUT protein-encoding genes have been identified in various crops, including pineapple (Rae et al., 2002), rice (Aoki et al., 2003), potato (Leggewie et al., 2003), *Arabidopsis* (Sivitz et al., 2008), sorghum (Milne et al., 2013), and wheat (Deol et al., 2013). In sugarcane, Niu et al. (2019) successfully cloned five *SUT* genes (*SoSUT1* to *SoSUT5*) and analyzed their expression in various tissues, including buds, inflorescences, leaves, stems, and roots. The study found that *SoSUT4* expression was negatively correlated with sucrose concentration in the stem internodes, while *SoSUT1*, *SoSUT2*, *SoSUT3*, and *SoSUT5* were positively correlated. These findings suggest that different SUT genes may have varying roles during sucrose accumulation.

Enhancing sucrose concentration in sugarcane stems can be achieved by silencing *SUT* genes that negatively affect sucrose content, such as *SoSUT4*. Studies in other crops have shown that manipulating *SUT* expression can have significant impacts. For instance, overexpressing *SUT* genes has resulted in increased tuber yield in potatoes (Sun et al., 2011), higher sugar concentrations in mature tomato fruit (Wang et al., 2016), improved rice yield components (Putri et al., 2020), and enhanced sucrose accumulation in tobacco root cells (Kariya et al., 2017). Additionally, Chincinska et al. (2013) found that suppressing *SUT4* improved sucrose efflux from developing tubers and leaves in potatoes.

Despite these advances, *SoSUT4* remains underexplored in sugarcane, particularly in Indonesian varieties. This research aims to isolate, clone, and analyze *SoSUT4* gene expression in Indonesian sugarcane varieties. The findings are expected to contribute to the development of high-yielding sugarcane through genetic manipulation and breeding programs, ultimately reducing Indonesia's dependence on sugar imports and enhancing the country's agricultural sustainability.

Materials and Methods

This study was conducted in The Indonesian Center for Biodiversity and Biotechnology (ICBB) and the Indonesian Palm Oil Research Institute (IOPRI) in Bogor, Indonesia, from January 2021 to December 2023. The Bululawang sugarcane variety (5-month old) from the ICBB experimental garden was used in the study. The midsection of the topmost (youngest) leaf and the middle internodes (5-8) were selected for RNA isolation and *SoSUT4* gene expression analysis.

Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted using the plant total RNA extraction kit (Geneaid Biotech Ltd., Taiwan) from approximately 100 mg of leaf and stem tissue from the 5-month old Bululawang sugarcane variety. Agarose gel electrophoresis was done to assess the purity and integrity of the RNA. cDNA synthesis was conducted using the ReverTra kit (Toyobo, Japan).

Cloning of putative SoSUT4 gene

The putative *SoSUT4* gene was amplified in polymerase chain reaction (PCR). Each reaction mixture contained 200 ng of cDNA, 20 µM each primers and MyTaq HS Red Mix PCR. The primers SoSUT4_F (5'-GGCATCCCGCACGCCTTCGC-3') and SoSUT4_R (5'-GTACCTTTGGGTCTCC TGGG-3') were used to amplify the gene fragment. Amplification was performed in a thermal cycler (Takara Bio, Japan) following a standard protocol: pre-denaturation for 60 s at 95 °C and 35 cycles of denaturation (15 s at 95 °C), primer annealing (15 s at 60 °C), elongation (30 s at 72 °C), and final elongation (10 min at 72 °C). PCR products were confirmed using a UV transilluminator after electrophoresis on a 1% (w/v) agarose gel in 1× TAE buffer for 30 min at 100 volts.

The partial *SoSUT4* gene was cloned into pTA2 plasmid (Toyobo, Japan). The recombinant plasmid, pTA2:*SoSUT4*, was introduced into *E. coli* DH10B strain and the transformed colonies were confirmed by colony PCR. The pTA2:*SoSUT4* was extracted from the bacteria culture using ZR Plasmid Miniprep-Classic kit (Zymo Research, USA).

Bioinformatics analyses

Sequencing of the cloned partial *SoSUT4* gene was conducted using the Sanger sequencing technology. The resulting sequence was compared to similar sequences in the National Center for Biotechnology Information (NCBI) databases using BLASTn and BLASTx tools (https://www.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed using MEGA 11 with the maximum likelihood approach, based on the JTT matrix-based model (Jones et al.,

1992; Tamura et al., 2021). The nucleotide sequence of *SoSUT4* was translated into its corresponding amino acid sequence using Translate (https://web.expasy.org/translate/). The physicochemical properties, solubility, subcellular location, and transmembrane helices were predicted with, respectively, ProtParam (http://web.expasy.org/protparam), Protein-Sol (https://protein-sol.manchester.ac.uk/), DeepLoc 2.0 (https://services.healthtech.dtu.dk/services/DeepLoc-2.0/), and DeepTMHMM, a deep learning model for transmembrane topology prediction (https://dtu.biolib.com/DeepTMHMM).

SoSUT4 gene expression and Brix content analysis

The SensiFAST SYBR Hi-Rox kit (Bioline, USA) was used to measure *SoSUT4* gene expression in sugarcane leaves and stems through quantitative real-time PCR (qRT-PCR). Table 1 lists the primer sequences used for the experiment, which was run on StepOnePlus Real-Time PCR System (Applied Biosystems, USA), with the following setup: pre-denaturation at 95°C for 2 min; 40 cycles of denaturation (95°C for 5 s), annealing and extension (60°C for 15 s), and a melt curve stage with 15 s at 95°C, 1 min at 60°C, and another 15 s at 95°C. Gene expression was quantified using the cycle threshold (CT) values, following the method by Livak and Schmittgen (2001):

$$\Delta CT = CT_{SoSUT4} - CT_{actin}$$
$$\Delta\Delta CT = \Delta CT_{stem} - \Delta CT_{leaf}$$
$$\text{Relative expression} = 2^{-\Delta\Delta CT}$$

Brix content of leaves and stems was measured using a refractometer. Leaf and stem samples were ground with liquid nitrogen and extracted with 80% ethanol. After incubating at 80°C for 30 min, the samples were centrifuged at 13,000 g for 15 min. The supernatant was transferred to a new tube and re-extracted with 80% ethanol up to three times (Niu et al., 2019). Finally, the supernatant was analyzed using a Brix refractometer to determine the total sugar content.

Data analysis

The *SoSUT4* gene expression and sugarcane Brix contents were analyzed using ANOVA (analysis of variance) and LSD (least significant difference) T-test at a level of significance of 5% using Minitab software version 18.

Results and Discussion

The *SUT4* gene family plays a vital role in transporting sucrose from leaves to stems, influencing the final sugar content in sugarcane, where the stem serves as the primary storage organ. In this study, the partial coding sequence of *SoSUT4* was successfully cloned from the Bululawang sugarcane variety using cDNA derived from the stem. The amplified product was visualized through 1% agarose gel electrophoresis, revealing a DNA band of 802 base pairs (Figure 1A). The cloning of the partial gene into pTA2 vector plasmid was confirmed successful by gel electrophoresis showing a fragment of size 3.7 kb, approximately (Figure 1B).

Table 1. Primers used for quantitative real-time PCR analysis

Primer name	Sequence (5'-3')	Amplicon size (bp)
qSoACT forward	ATCACTTCCCTCGCACCAAG	154
qSoACT reverse	CCGGGCCAGACTCATCATAC	
qSoSUT4 forward	TGGCTATTCTGGGGCTTCCT	191
qSoSUT4 reverse	ACCCACAAGAGTTTCCACGC	

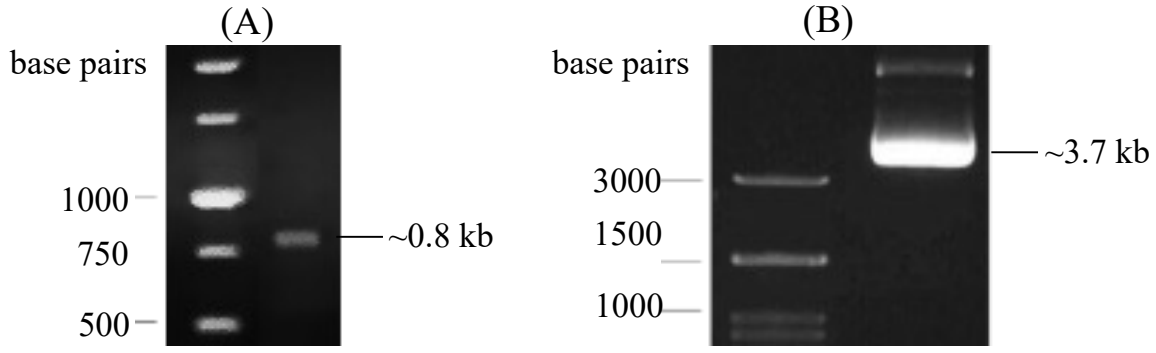

Figure 1. The size of (A) partial *SoSUT4* gene fragment and (B) plasmid pTA2 containing the partial gene (pTA2:*SoSUT4*)

The nucleotide sequence of the *SoSUT4* has been deposited in the GenBank under the accession PQ369497. This sequence encodes a 267-amino-acid protein (Figure 2) with a predicted molecular weight of 28.97 kDa and a theoretical isoelectric point (pI) of 4.76, suggesting the protein is relatively acidic. Amino acid composition analysis indicated that the *So*SUT4 protein is composed of 39.7% non-polar amino acids, 25.8% hydrophobic amino acids, 29.9% polar amino acids, and 14.6% aromatic amino acids, including cysteine residues. These features suggest a balanced distribution of hydrophobic and hydrophilic regions, indicating that the protein is likely membrane-associated.

Bioinformatics predictions revealed that *So*SUT4 protein contains six transmembrane helices, spanning specific amino acid positions (Figure 3). This topology aligns with its predicted role in transporting sucrose across the plasma membrane. Notably, this partial sequence contrasts with the full 1,509 bp *SoSUT4* open reading frame (ORF) identified by Niu et al. (2019), which likely resulted from differences in primer design during amplification. Such variations highlight the importance of targeted primer selection for characterizing specific gene regions.

Comparative sequence analysis using BLASTn and BLASTx revealed high similarity between the partial *So*SUT4 and known SUT4 proteins in related species. Specifically, the nucleotide sequence showed 98.38% identity with *S. hybrid* ROC22 (GQ485583.1), and the protein sequence exhibited 98.88% identity with *S. spontaneum* SUT4-h1 (ALS46598.1), suggesting evolutionary conservation of sucrose transport functions. Phylogenetic analysis supported these findings, clustering the Bululawang *So*SUT4 protein within the first family of SUT4 proteins, predominantly found in monocotyledonous plants (Figure 4). This grouping indicates functional similarity with high-yielding sugarcane varieties, such as *S. hybrid* and *S. spontaneum*, which are known for efficient sugar accumulation.

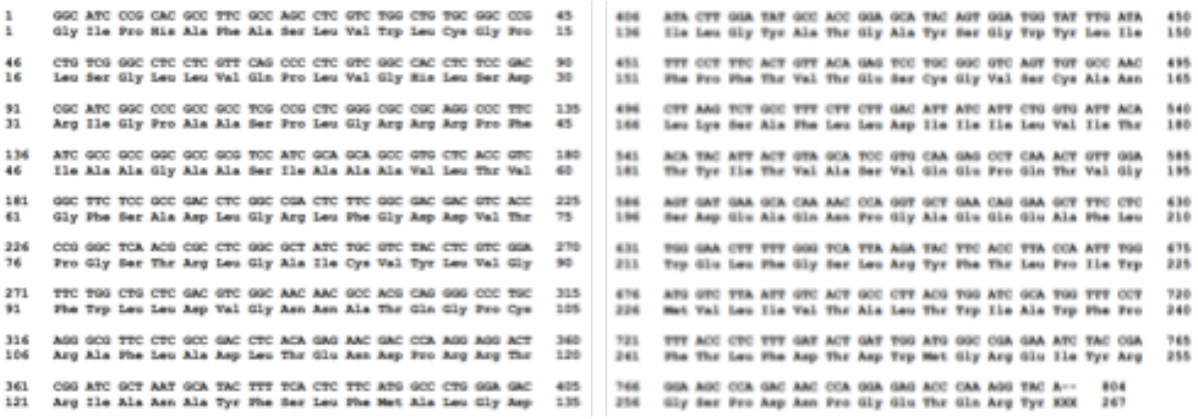

Figure 2. Partial nucleotide and protein sequence of *So*SUT4 of Bululawang variety

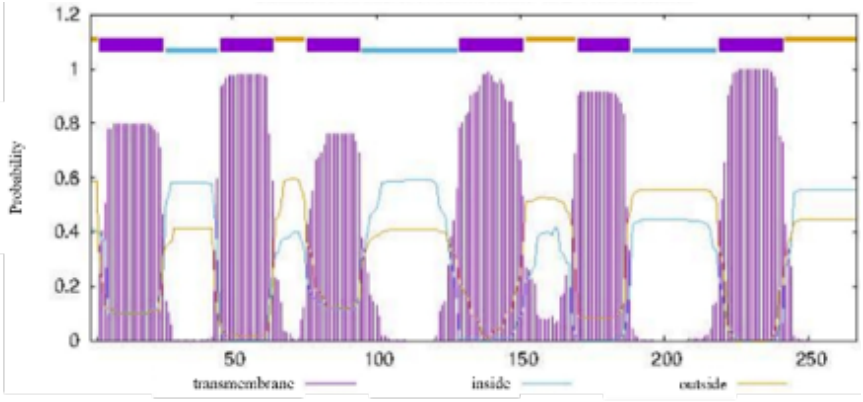

Figure 3. Predicted transmembrane structure of *So*SUT4 generated using TMHMM

The bioinformatics analysis also predicted that *So*SUT4 is most likely to be localized in the plasma membrane (Figure 5). These predictions align with previous studies that associate SUT4 proteins with sucrose transport across cellular membranes (Chincinska et al., 2013; Schulze et al., 2003). Moreover, the hydrophobic and hydrophilic distribution in *So*SUT4 protein suggests it forms a channel across the plasma membrane, facilitating sucrose symport, likely coupled with proton (H^+) movement (Donzella et al., 2023).

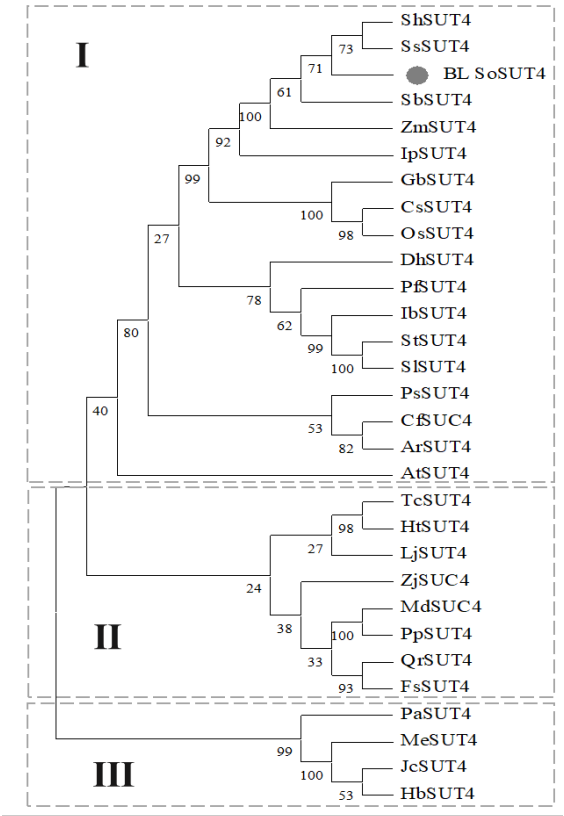

Figure 4. Phylogenetic tree of the SUT4 proteins from various plants, constructed with the maximum likelihood approach with a 100-fold bootstrap

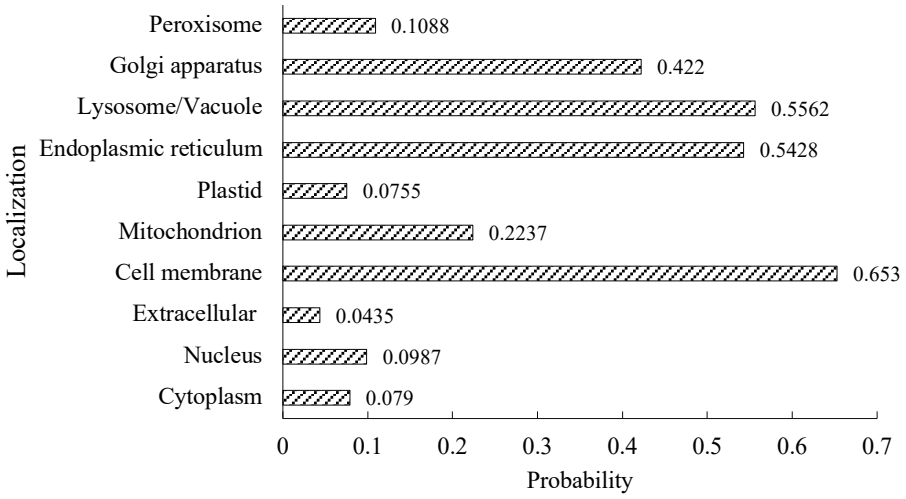

Figure 5. Prediction of *So*SUT4 protein subcellular localization generated using DeepLoc 2.0

Gene expression analysis revealed higher expression of *SoSUT4* in the stem tissue compared to leaves, with the stem showing 1.7 times greater expression (Figure 6A). This expression pattern correlates with increased sugar accumulation in the stem. Brix analysis of the Bululawang variety further supported this observation, with the sugar content in the stem reaching 19.61%, slightly higher than the 19.48% measured in the leaves (Figure 6B). Although the difference in Brix values was statistically nonsignificant, the higher expression of *SoSUT4* in the stem underscores the gene's role in facilitating sucrose transport to the storage tissues, contributing to sugar accumulation.

The findings in this study are consistent with earlier work by ElSayed et al. (2013), who reported that *SUT4* expression is elevated in sugarcane stems. However, differences in gene expression patterns compared to other studies (e.g., Zhang et al., 2016) suggest that environmental factors, developmental stages, and tissue-specific functions could influence sucrose transporter activity. The Bululawang variety's stem, as the primary storage site, aligns with previous research indicating that elite sugarcane varieties bred for high sugar yields exhibit elevated *SUT4* expression in the stem (Rae et al., 2005).

While previous studies have reported that SUT4 proteins typically contain 12 transmembrane domains, only six transmembrane helices were identified in this study, due to the partial isolation of the gene. Nonetheless, the identified helices and their predicted membrane-embedding properties provide insight into the *So*SUT4 protein's role in sucrose transport. These helices likely create a passage for sucrose across the plasma membrane, contributing to the plant's sucrose loading and unloading mechanisms during growth and development.

In conclusion, the successful cloning and characterization of the *SoSUT4*. Higher expression of the gene in stem tissue correlates with increased sugar accumulation, reinforcing the stem's role as the main sugar storage organ. These findings emphasize the evolutionary conservation of the *SoSUT4* across monocots and suggest that the gene may serve as a target for enhancing sugar yields in future breeding programs. Further research should focus on exploring regulatory networks controlling *SoSUT4* expression and validating the protein's functional role in sugarcane's sucrose metabolism to improve productivity.

Conclusion

The partial *SoSUT4* gene from Bululawang sugarcane variety was successfully cloned and its expression in leaf and stem was analyzed. The gene showed similar characteristics to *SUT4* genes observed in other plant species. *SoSUT4* was highly expressed in the stem which also had the highest Brix content. The recent finding of this study not only provides a valuable understanding of the *SUT* gene family in sugarcane but also facilitates further detailed investigation and examination.

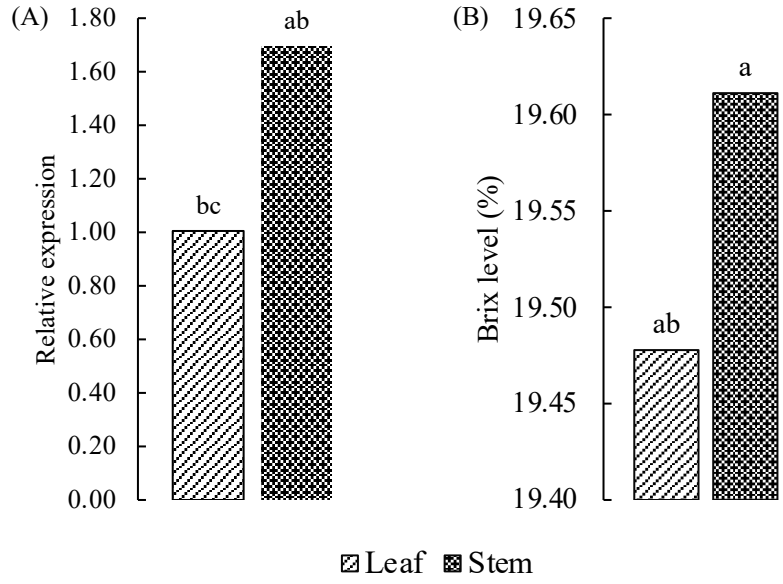

Figure 3. Relative expression of *SoSUT4* (A) and sugar contents (B) in leaf and stem of Bululawang sugarcane variety

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