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DECIPHERING THE MYB TRANSCRIPTION FACTOR FAMILY IN SUGARCANE (*SACCHARUM OFFICINARUM*): IMPLICATION FOR ENHANCED ABIOTIC STRESS TOLERANCE

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ABSTRACT

Plants rely on a class of proteins known as transcription factors (TFs) to regulate their physiological and biochemical functions. Among the many different types of TFs, MYB family proteins are the largest and play a crucial role in regulating plant growth, development, and stress responses. MYB TFs have been extensively studied in numerous dicot and monocot plant species, but very little is known about them in the complex polyploidy genome of sugarcane. In order to better understand MYB genes in sugarcane, this study utilized biocomputational analysis to compare closely related Sorghum bicolor plants. The MYB genes in *Saccharum officinarum* Co86032 were then identified using the Primer blast tool and primers were designed specifically for these genes. The primers were used in PCR to amplify the MYB genes from both the leaf and stem tissues of the sugarcane plant. The resulting amplicons were sequenced to verify the presence of MYB genes. The open reading frame (ORF) of the sequenced amplicons was then checked using an ORF finder, and the resulting ORFs were analyzed for the presence of MYB gene domains using ScanProsite. The MYB protein sequences were further analyzed to identify nuclear export signals (NES) using Loc NES. The MYB transcription factor super family plays a critical role in plant growth and defense mechanisms. Identifying and analyzing the MYB genes in sugarcane is therefore of great importance for genetic crop improvement, particularly regarding abiotic stress. By better understanding the MYB domains in the upstream region, it may be possible to induce the over-expression of MYB genes, thereby enhancing sugarcane's resistance to abiotic stress. This study provides valuable insights into the MYB family of proteins in sugarcane, which could have far-reaching implications for the development of more resilient crops. By identifying and understanding the genes responsible for regulating plant growth and stress responses, we can help to ensure that crops are better equipped to handle the challenges posed by the environment.

KEYWORDS

MYB, NES (Nuclear export signal), FP, RP (forward and reverse primer), ScanProsite and WRKY.

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INTRODUCTON

Commercial sugarcane is a good source of bioelectricity and bioethanol because of its high sugar buildup. Abiotic and biotic stress both have an impact on this crop. This crop has higher water needs and is water sensitive, therefore abiotic stressors such as a water shortage affect plant growth and crop yield[1]. Water shortage is the most

prevalent abiotic stress, hence drought-tolerant breeds are essential for all nations with significant sugar cane production. Extensive study has revealed certain stress-responsive transcription factors from the MYB, WRKY, etc., families that mediate plant response and tolerance to abiotic stress. One of the biggest families of transcription factors, the MYB transcription factor is involved in a variety of physical and biochemical processes, including secondary metabolism, organ development, plant development, and cell cycle regulation. Additionally, they participate in biotic and abiotic stress responses. Findings imply that transcriptional regulation of stress-responsive genes is necessary to understand the mechanisms underlying plant stress responses and tolerance to abiotic stress and that these transcription factors may be crucial targets for the creation of crops with enhanced abiotic stress resistance[2]. Crops are better able to withstand the effects of drought stress because the MYB transcription factor controls how plants react to stressors and activate genes that are relevant to drought. Research suggests that MYB proteins are crucial in regulating how plants react to abiotic stress[3-6]. Being a tropical crop, drought and salt are the most frequent abiotic stresses that sugarcane encounters[1]. This study aims at the identification of the MYB gene of *Saccharum officinarum* Co86032 by comparing the closely related plant species *Sorghum bicolor* using Biocomputational methods. Initially, 4 primers were designed for MYB gene identification using Primer Blast. The polymerase chain reaction is likely affected most significantly by selecting the right primers (PCR)[7]. Two phases are normally included in the process of developing certain primers. Primers surrounding areas of interest are first created manually or with the use of digital tools, and then they are checked against a database of nucleotide sequences to see if there are any suitable targets. The BLAST software is frequently used to find primer targets. Primer-BLAST also allows exon/intron placement and excludes single nucleotide polymorphism (SNP) sites from primer placement. Adjusting the specificity threshold and other primer parameters is flexible using Primer-BLAST[7]. Primer-BLAST is an intuitive and easy-to-utilize online resource that is seamlessly integrated with the NCBI database. This tool offers a comprehensive and up-to-date solution for molecular biology research, leveraging its seamless integration with both GenBank and RefSeq. Primer-BLAST facilitates the design of polymerase chain reaction (PCR) primers by providing insightful information regarding the specificity and feasibility of the selected primers, including their melting temperature, propensity for cross-reactivity with existing sequences and likelihood of primer-dimer formation. By considering these parameters, researchers can make informed decisions regarding the optimal choice of primers for their experiments[8]. Designed primer for MYB genes allowed for PCR amplification with isolated *Saccharum officinarum* Co86032 (leaf and Stem) DNA[9]. Resulting in amplicons that were sequenced and checked for the presence of MYB genes *Saccharum officinarum* Co86032. ScanProsite enables protein searches for matches against both user-defined patterns and the PROSITE library of motifs. ScanProsite is a brand-new iteration of the web-based tool for finding PROSITE signature matches in protein sequences. The ability of function prediction based on profiles is strengthened by the identification of such traits[10]. Searches against signature databases, also known as secondary databases, are used to predict protein function, assign family identity, or find distant homologues[11]. Open reading frame finder searches for open reading frames (ORFs) in the DNA sequence you enter. The program returns the range of each ORF, along with its protein translation. Use ORF finder to search newly sequenced DNA for potential protein encoding segments[12]. To find protein matches against signatures from the PROSITE database, ScanProsite offers a web interface[13]. At every stage of the cell cycle, with the exception of mitosis, the nuclear envelope (NE) within eukaryotic cells acts as a physical barrier to keep the contents of the nucleus and cytoplasm apart geographically. Transcription and translation, two important cellular functions, are restricted to the nuclear and cytoplasmic compartments, respectively, by the NE. Prokaryotic and eukaryotic

organisms can be distinguished by a range of criteria, including intricate regulation of gene expression[14,15]. Nuclear proteins like transcription factors and histones must be imported into the nucleus from the cytoplasm in order for newly generated mRNA to be exported from the nucleus to be translated. Dynamic spatial and temporal control of proteins and other macromolecules between the cytoplasmic and nuclear compartments is necessary for complex biological processes like signal transduction to take place, which eventually necessitates transit over the NE in one or more directions[16]. The short cognate peptides known as "classical nuclear export signals" (NESs) are used to drive proteins out of the nucleus through the CRM1-mediated export pathway[17].

MATERIAL AND METHODS

Sett samples and leaves of *Saccharum officinarum* Co86032 were collected from ICAR-Sugarcane Breeding Institute, Veerakeralam, Tamil Nadu 641007.

Methodology

DNA Isolation

DNA was extracted from the sample using an MN plant kit - (Macherey-Nagel GmbH and Co. KG, Düren, Germany), hereafter called MN plant kit[9,15] following the manufacturer's instructions. ~500mg of Samples were taken and ground well into a fine powder with liquid Nitrogen using a sterile mortar and pestle. The powdered sample was transferred to sterile microcentrifuge tubes. To this, 300µL Buffer PL2 was added and mixed thoroughly. Then RNase A solution (10µL) was added to each tube and the suspension was incubated for 30-60 min at 65°C. Next, 75µL Buffer PL3 was added and mix thoroughly and then the tubes were incubated for 5 minutes on ice. Now the lysate was loaded on to NucleoSpin® Filter Column-Collection Tube assembly and centrifuged for 2 min at 11,000 x g. The flow through collected was added with 450µL Buffer PC; mixed by pipetting. Next, this solution was loaded on to the NucleoSpin® Plant II Column Collection Tube assembly and spun for 1 min at 11,000 x g. The resultant flow-through was discarded, the spin column was retained and washed with Buffer PW1 (400µL) and Buffer PW2 (700µL). Then the clean DNA from the membrane was eluted with buffer PE (50µL).

Quantification of DNA

The quality of the DNA was confirmed by Agarose Gel electrophoresis, Figure No.1. The agarose gel (1 %) was prepared in 1X Tris Acetate EDTA buffer (TAE). 5µl of DNA was mixed with 2µl of Gel loading dye; type III (6X) [Bromophenol blue (0.25 %) Xylene cyanol (0.25 %) and glycerol (30%)] and was loaded carefully into the gel matrix. A current of 120 Volts was applied for approx. 45 min to segregate the DNA. Ethidium bromide stain was already incorporated in the gel matrix while casting the gel and hence the gel was documented immediately after the run in a gel documentation system (E gel imager, Life Technologies) equipped with a UV transilluminator and camera.

Primer design

Primer blast tool used to design the required primers for MYB genes[7,18]

Steps

To utilize Primer-BLAST, simply input the sequence or the NCBI accession number corresponding to the gene of interest

It is important to provide the PCR product length.

Establish the primers' preferred melting temperature (Tm) (the minimum, optimal, maximum difference between the set)

Select "Primer must bridge an exon-exon junction" as the action.

Choose Refseq mRNA as the database for the search.

Make a choice on the organism you will be BLAST.

Check the primers' placement and the other variables.

From the preceding actions S1F - S1R, S2F - S2R, S3F - S3R, and S4F - S4R are the four pairs of primers (both forward (FP) and reverse (RP)) that were created, ordered, and obtained from Barcode Biosciences. When the primers' annealing temperatures were examined, it was discovered that all of them had high GC contents and high Tm. (Table No.1).

PCR - Polymerase chain reaction[19,20,21]

PCR was done in 20µl reaction volume containing 1µl DNA (10-50ng),

Addition of 1µl each of Forward and Reverse primers (10 picomoles µl-1)

10µl Emerald Amp GT PCR master mix (Takara).

During the optimization, additives were added appropriately (BSA, DMSO and MgCl2).

The cycling conditions used are as follows

Initial denaturation done at 94°C for 2 min.

Subsequently 30 cycles of denaturation at 94°C for 30 seconds.

Annealing performed for 40 seconds at 52.2°C, 58°C, or 60°C.

Extension at 72°C for 1.5 min and a final extension for 10 minutes at 72°C.

PCR optimization

PCR trials were done with the following conditions[22,23]

Different annealing temperatures for all four primers (3 different temperatures for each primer)

With DMSO (to reduce the annealing temperature and to compensate for the GC difference between primer pairs)

Addition of BSA, followed by MgCl2 and finally Combination of DMSO, BSA and MgCl2

The success of the PCR reaction was confirmed by running 5µl PCR product on 1% agarose gel (impregnated with ethidium bromide) at 120 V; for ~45min in 1X TAE Buffer. The image of the gel was recorded with the UV gel documentation system.

PCR product purification

Unincorporated primers and dNTPs that might obstruct succeeding reactions and result in an unintelligible sequence must also be removed from the reaction mixture. Prior to sequencing, the optimum procedure for preparing the PCR product should have 0% sample loss, be straightforward to carry out, and yield reliable findings. To solve these problems, USB Corporation created ExoSAP-IT® for PCR product cleanup. ExoSAP-IT® is a powerful replacement for conventional techniques for cleaning up PCR products before sequencing. The PCR products were further treated with ExoSAP-IT PCR Product Clean-up Reagent and were used as a template for sequencing PCR[24].

Steps

ExoSAP-IT® is used to degrade primers and remove phosphates from dNTPs in PCR.

The treatment is done for 15 minutes at 37°C, then it is incubated for 15 more minutes at 80°C to turn off both enzymes completely.

PCR products can be used directly for sequencing without requiring any additional steps

Sanger sequencing PCR

The BigDye™ Terminator v3.1 Cycle Sequencing Kit is a suitable option for a range of sequencing assignments, such as starting from scratch sequencing, repeating sequencing, and wrapping up, utilizing sources such as the result of PCR reactions, circular DNA, fosmid clones, and Bacterial Artificial Chromosomes[25,26] Sequencing PCR was done with ABI PRISM Big Dye terminator ready reaction mix (Life Technologies, USA). The cycle extension products were purified following 70% ethanol/0, 5M EDTA/ sodium acetate precipitation and the sequencing standard was done with Applied Biosystems ABI 3730xl DNA Analyzer. The sequences were quality-checked and trimmed using the software Sequencher V4.10.1 (Gene Codes Corporation, Ann Arbor, MI USA). Gene Codes Corporation is a global company that makes bioinformatics software for analyzing DNA sequences. Their user-friendly software tools are widely used in the DNA sequencing field. The trimmed Sequence was shared. S1 results were analyzed and sequenced. The sequenced results were blasted with NCBI against Sorghum bicolor and the results were noted[27].

Open Reading Frame Finder (ORF)

ORF finder searches for open reading frames (ORFs) in the DNA sequence you enter. The program returns the range of each ORF, along with its protein translation. Use ORF finder to search newly sequenced DNA for potential protein encoding segments[12].

Primarily, to obtain nucleotide sequences from a DNA sequence, the input must be in FASTA format. Once the input is in the correct format, the

user can select a minimal Open Reading Frame (ORF) length of 75. An Open Reading Frame refers to a sequence of nucleotides that can be translated into a sequence of amino acids. After setting the minimal ORF length, the software will analyze the input and shortlist all ORFs that contain more than 100 amino acids. This means that any ORF that has fewer than 100 amino acids will not be included in the output. The output of this analysis will be presented as a list of ORFs that meet the criteria specified by the user. This list of ORFs will include their corresponding nucleotide sequences and their corresponding amino acid sequences. Overall, this analysis provides valuable information on the protein-coding potential of the DNA sequence entered.

ScanProsite

The utilization of this form presents the opportunity to conduct a comprehensive scan of proteins, in which matches can be identified not only against the PROSITE collection of motifs but also against patterns that are unique to the user. This approach enables a thorough exploration of potential matches and increases the likelihood of uncovering meaningful results. ScanProsite is a web-based tool for identifying motifs and domains in protein sequences. The tool is based on the Prosite database, which contains a collection of protein domains, families, and functional sites[28,29].

LocNES

Reliable prediction of classical nuclear export signals (NESs) remains challenging, despite several attempts made in the last decade, due to the diverse and complex nature of the signals. NESs are short cognate peptides that direct proteins out of the nucleus via the CRM1-mediated export pathway, and CRM1 is responsible for regulating the localization of hundreds of macromolecules involved in various cellular functions and diseases[30,31]. After the protein file has been uploaded, predict NES an assessment can be made to determine whether NES, a specific component of interest, is present and the results were noted.

RESULTS AND DISCUSSION

DNA Isolation and electrophoresis results of DNA quality

The isolated DNA from the MN plant kit and the quality of the DNA confirmed by Agarose Gel electrophoresis were shown in (Figure No.1).

PCR trial and Optimization results

PCR trials were done with the following conditions: Different annealing temperatures for all four primers (3 temperatures for each primer). With DMSO (to reduce the annealing temperature and to compensate for the GC difference between primer pairs). Addition of BSA, MgCl2 and Combination of DMSO, BSA and MgCl2. The success of the PCR reaction was confirmed by running 5µl PCR product on 1% agarose gel (impregnated with ethidium bromide) at 120V; for ~45min in 1X TAE Buffer. The image of the gel was recorded with the UV gel documentation system Figure No.2-4). 100bp DNA ladder (Thermo Scientific) was loaded parallel to PCR products as a size marker.

PCR amplification was there for two Primer Pair S1F-S1R and S4F-S4R. (For the 2nd set primer along with the amplicon; some nonspecific amplifications were also found along with the sample). S2F-S2R and the 4th primer pair S3F-S3R yields non-specific amplification. The PCR bands for primer S1 and S4 were purified and proceeded to Sanger sequencing**.**

Sequenced results in FASTA and ABI files of the S1

CATGCCACTATTGCTCCCTGTAAACGTCAGT
GTGGAGGTAGGTGACTCCA
GGTCTATCTTTGCACCCTGCCATGCATGCAG
CACGTCAAGGCATGGAGAA
GCAGCAGAGGCAGGTACATTCGTTGGCTGC
GCATGTAGTGCAGTTGGTGT
TGGCGTTGCTGTTCGAATCTTTGATTCTCGA
GCCAGCCGTGCCTCAGCCT
CAAGGCGGGCACTCTCCCATTGTGCCATGT
GGCTGAGGCTTGCCGCGGCC
TTGGCTGATTTGTTGTTTGTGGTGCCAGTGA
GAGTGCCACTGATAGATTT
GTGGGTGACAGGATCGATCCCCATCTTGGC
CAGCCTTTTCTTGAGGTGTG

TGTTCCAGTAGTTCTTGATCTCATTGTCTGT
GCGATTTGGTAGATGCGTT
GCAATGGCCGACCACCTGAACAGTGCCATG
AACAAACCTATGATTTAAAA
TTAACATCTTGTTTTCATCTACCATGTATGC
CGAAATTCTATGTTCTGAG
GGAAGGGGAGATGAGATGGCAAATTAAAA
AGTACAAACACCATGCCATAA
CTC

NCBI: BLAST

BLAST, which stands for Basic Local Alignment Search Tool, is a program that compares nucleotide or protein sequences to sequence databases. Once the program performs this comparison, it is able to identify regions of similarity between biological sequences and calculate the statistical significance of the matches[31].

Sequence Homology and Function through NCBI BLAST Analysis

After running a sequence analysis using NCBI BLAST, the resulting data revealed a high degree of similarity between the query sequence and the mRNA for the Sorghum bicolor transcription factor MYB 106 (LOC8076068), with a percentage identity of 98.56%. This suggests that the query sequence may be a homolog of MYB 106 and may share similar functions and/or structural features. Further analysis and experimentation will be needed to confirm these findings and explore their potential implications.

Open Reading Frame Finder: (ORF) Finder

Finder ORF OUTPUT

>lcl|ORF5

MALFRWSAIATHLPNRTDNEIKNYWNTHLKK
RLAKMGIDPVTHKSISGTL
TGTTNNKSAKAAASLSHMAQWESARLEAEA
RLARESKIRTATPTPTALHA
QPTNVPASAASPCLDVLHAWQGAKIDLESPTS
TLTFTGSNSGM

After generating the ORF frame, we utilized Scanprosite to examine its sequence and to confirm the presence of the MYB domain.

ScanProsite output

The analysis of the Open Reading Frames (ORFs) using computational tools revealed that one of the ORFs had the presence of the MYB domain. Specifically, this ORF was found to contain a start codon at position 429 and a stop codon further downstream with a length of 429 nucleotides and corresponding 142 amino acids. The MYB domain is a common DNA-binding domain present in a wide range of proteins involved in gene regulation, and its presence in the identified ORF suggests that it may play a role in the regulation of gene expression in the organism under study. Further investigation and experimentation will be required to confirm this hypothesis and determine the specific biological functions of this MYB-containing ORF.>100 shortlisted proteins using ScanProsite and checked for MYB domain,

>~out: 429 to 1: Frame 2 143 aa.

Table No.1: Designed primers sequence FP and RP

S.No	SCMYB	GenBank ID	Primer	FP	RP
1	ScMYB52	HF679466	S1	ATGGGGCGGTCGCCGT GCTGCGAG	TCAGTTGGACTCACCCCAG CATTCTG
2	ScMYB53	HF679474	S2	ATGGGGAGGCACTCCT GCTGTTACAAGC	TAATGTTAAAAAAGTTAAT GGTGAA
3	ScMYB56	HF679484	S3	ATGGGCCGTAGCCCGT GCTGCGA	CGGCAGGCTTTTTTCCAGC AGCAG
4	ScMYB57	HF679480	S4	ATGGCGAGCCCGAGCC CGAGCTGCC	GCTGCAGCTCCAAGGCGCA CGCGC

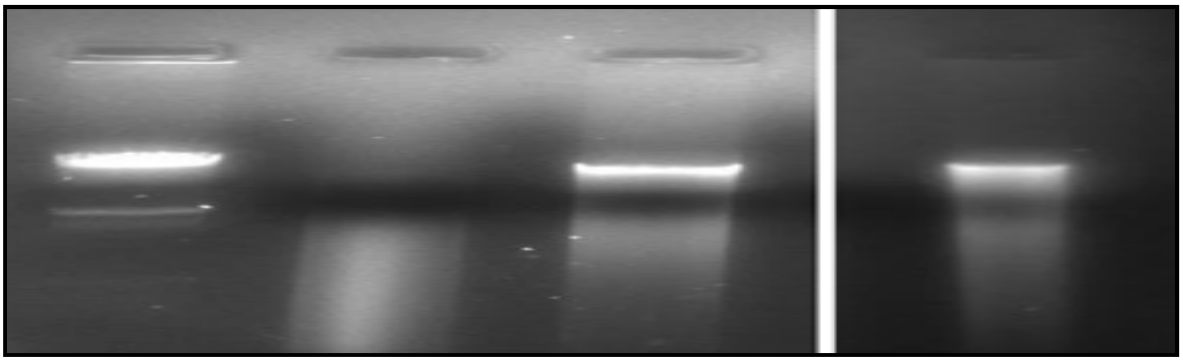

Figure No.1: Well 1: Lambda DNA Hind III marker; Well 2: Leaf DNA; Well 3: Stem DNA; Well 4: Leaf DNA repeat; Remarks: Good quality DNA was obtained for both

Figure No.2: (a)100bp, (b)LS1B, (c)LS1D, (d)LS2B, (e)LS2D, (f)SS1B, (g)SS1D, (h)SS2B, (i)SS2D S1, S2, S3, S4 stands for – PRIMERS, L stands for leaf, S stands for stem, B stands for BSA, D stands for DMSO

Figure No.3: S1, S2, S3, S4 stands for – PRIMERS, L stands for leaf, S stands for stem. B stands for BSA and D stands for DMSO

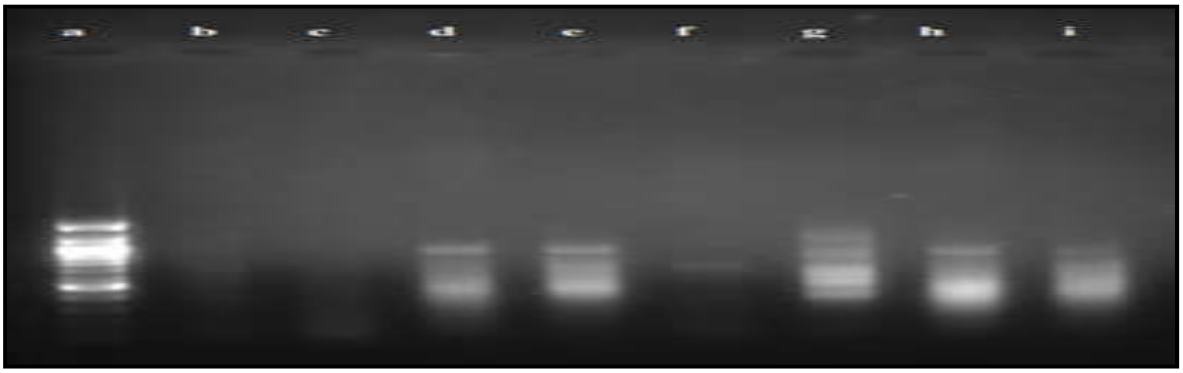

Figure No.4: (a)100bp, (b)LS3B, (c)LS3D, (d)LS4B, (e)LS4D, (f)SS3B, (g)SS3D, (h)SS4B, (i)SS4D

Figure No.5: Presence of HTH MYB Domain from ORFs

LocNES: Location of classical NESs

Protein Name	Position	Sequence	Score
>LocNES60604233_0	24-38	YWNTHLKKRLAKMGI	0.110
>LocNES60604233_0	122-136	GAKIDLESPTSTLTF	0.348

Figure No.6: Localization of Nuclear export signals from sequence protein

CONCLUSION

The DNA isolated from the *Saccharum officinarum* Co86032 plant was subjected to PCR amplification using four designed primers specific for MYB genes. Primers 1 and 4 produced an amplicon, and the amplicon produced by primer 1 was sequenced, yielding approximately 555 nucleotides. The sequenced nucleotides were compared to the NCBI sorghum bicolor plant and showed 98.56% identity with Sorghum bicolor TF MYB106 (LOC8076068). ORF finder was used to check for the presence of ORF, and Scanprosite indicated the presence of an MYB domain in frame 2 of 142 amino acids. Finally, LocNES analyzed the presence of nuclear export signals (NES) and found them in positions 24-38 and 122-136. In addition, this study identified the presence of an MYB gene in *Saccharum officinarum* Co86032, which plays an important role in abiotic stress. The gene was amplified using specific primers, and the resulting amplicon was sequenced, showing high similarity to Sorghum bicolor TF MYB106 (LOC8076068). The gene was found to contain an MYB domain and nuclear export signals. The study not only confirmed the presence of a MyB domain in the *Saccharum officinarum* Co86032 MYB gene but also sought to investigate the presence of nuclear export signals (NES) within this gene. NES are short amino acid sequences that regulate the movement of proteins out of the nucleus of a cell. The analysis, which utilized LocNES software, revealed the presence of NES in two specific regions of the MYB gene, namely positions 24-38 and 122-136. These findings suggest that the *Saccharum officinarum* Co86032 MYB protein may be transported out of the nucleus via these NES, potentially playing a role in the regulation of gene expression or other cellular processes. Additional studies could explore the functional significance of these NES in the context of abiotic stress response in *Saccharum officinarum* Co86032. In summary, this study provides valuable insights into the structure and function of the *Saccharum officinarum* Co86032 MYB gene, which could aid in the development of more resilient crops.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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