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In Silico Dissection of Regulatory Regions of PHT Genes from *Saccharum* spp. Hybrid and *Sorghum bicolor* and Expression Analysis of PHT Promoters under Osmotic Stress Conditions in Tobacco

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Abstract: Phosphorus (P) is the second-most essential macronutrient required for the growth and development of plants. It is involved in a number of cellular processes that contribute to the plant's growth and development. This study investigated *Saccharum* spp. hybrid and *Sorghum bicolor* promoter regions of Phosphate transporters (PHT), viz., PHT1, PHT2, PHT3, PHT4, and PHO1, through in silico analysis. The transcription start sites (TSS), conserved motifs, and CpG islands were studied using various computational techniques. The distribution of TSSs indicated the highest promoter prediction scores (1.0). MSh2 and MSb4 were recognized as the common promoter motifs for PHT promoters, found in with 85 to 100% percentage of distribution. The CpG analysis revealed that the promoter regions of most PHT genes had low CpG density, indicating a possible tissue-specific expression. The PHT promoters were investigated for the presence of biotic- and abiotic-stress-associated transcription factor binding sites (TFbs) that revealed the presence of binding motifs for major transcription factors (TFs), namely, AP2/ERF, bHLH, bZIP, MYB, NAC, and WRKY. Therefore, the in-silico analysis of the promoter regions helps us to understand the regulation mechanism of phosphate transporter promoters and gene expression under stress management. The 5′ regulatory region of the EaPHT gene was isolated from Erianthus, a wild relative of the genus *Saccharum*. The promoter construct was prepared and transformed in tobacco wherein the promoter drove the expression of GUS. Analysis of GUS expression in transgenic tobacco revealed enhanced expression of GUS under salt-stress conditions. This is the first report of the isolation and characterization of a phosphate transporter gene promoter from *Erianthus* and is expected to be useful for the development of salt-stress transgenic crop plants.

Keywords: in silico; promoter; transcription start site; motif; CpG islands; transcription factor binding sites; sugarcane

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1. Introduction

Sugarcane (*Saccharum* spp.) is an important commercial crop, grown worldwide for biofuel production, and accounting for 80% of global sugar [1]. Environmental factors cause significant economic damage to sugarcane and affect growth and production by

30 to 60% [2]. Abiotic stress is acknowledged as an important environmental risk factors to agricultural plant productivity and quality. Drought, salt, severe temperatures, and nutritional deficits are among the common abiotic stresses recognized to occur worldwide [3]. Although plants have evolved to deal with a variety of stresses, crop efficiency remains severely constrained. The current scenario of changing climate and future weather changes are key challenges for researchers to easily understand the responses of plants such as signal cascade activation, transduction, and relevant gene expression with respect to single or combinations of biotic and/or abiotic stresses, eventually boosting agricultural output [4,5].

Transcription factors (TFs) are activated by various signalling pathways and can interact directly or indirectly with cis-elements to impact the transcriptional efficiency of targeted genes that act as important regulators for crop improvement [6]. Understanding the molecular processes behind plant stress response and gene function demands an in-depth understanding of gene expression regulation [7]. The gene promoter sequence is generally in charge of controlling gene expression. The promoter is a segment of non-coding DNA that contains various motifs or cis-acting regulatory elements (CAREs), as well as CpG islands, that regulate the expression of a gene [8]. The core promoter is generally found in the upstream region including the transcription start site (TSS) and is in charge of initiating transcription. CAREs are small conserved DNA motifs (five to 20 nucleotides) that have specialized DNA-binding proteins such as transcription factors (TFs) [9]. Over the last decade, tremendous developments have been made in understanding the significance of plant TFs as important environmental regulators in essential crops; yet, only a few studies have been performed on sugarcane.

Phosphorus (P) is a macronutrient that is required for the growth and development of plants. Crop production is severely hampered by a lack of inorganic phosphate (orthophosphate; Pi) in the soil, while excessive fertilizing has polluted the environment [10,11]. Pi acquisition and homeostasis are dependent on transport mechanisms regulated by Phosphate transporters (PHT), which have so far been classified into five families: PHT1, PHT2, PHT3, PHT4, PHT5, and PHO [12,13]. The expression of these transporters is controlled by both transcriptional and post-transcriptional levels. In AtPHR1, an MYB TF, binding to the P1BS (GNATATNC) or P1BS-like motif in PHT1 promoters up-regulates the gene expression at low P concentrations [14]. The WRKY transcription element regulates PHT1 gene expression by interacting with the W-box (TTGACT/C) cis-acting region in PHT1 promoters [15].

Salinity has been regarded as the most severe abiotic stress factor in sugarcane, causing severe dehydration and an imbalance of inorganic solutes in important crop species, eventually limiting development and output. As a result, the phosphate ions in the soil tend to form an insoluble phosphate that limits P availability, and the use of P fertilizer in sugarcane plantations has increased year over year [16–18].The development of salt tolerance in plants results from the combined effects of salinity stress and Pi starvation. Promoter technology makes it much easier to successfully use transgenic technology by allowing gene expression to be altered at the desired level [19,20]. In plants, only in potato (*Solanum tuberosum* L.) have the regulatory elements of glucan endo-1,3-beta-glucosidase gene been reported to date [21]. To the best of our knowledge, no study has evaluated sugarcane in this aspect. Therefore, the aim of this study is to predict the promoter and regulatory elements of the PHT gene family that are associated with regulating gene expression in response to abiotic stress challenges and provide vital indications for creating stress-tolerant sugarcane cultivars. Further, the study is an extension of our prior research, which has shown that the EaPHT1;2 gene showed increased expression in the root under salinity stress [22]. As a result, the current work intended to estimate the Transcriptional Start Sites (TSSs), conserved motifs, CpG islands, and TFbs of the promoter regions of phosphate transporter genes in sugarcane. We used the closely related *Sorghum bicolor* as a reference genome. In order to determine the functional characteristics of the EaPHT1-2 promoter, the T1 generation tobacco seedlings were subjected to salinity stress.

2. Materials and Methods

2.1. Retrieval of Promoter Regions and Determination of TSSs

The 2.0 kb promoter sequences of each PHT gene of *Saccharum* spp. hybrid (Sh) cultivar R570 and *Sorghum bicolor* (Sb) (v3.1.12) were retrieved from Sugarcane Genome Hub and Phytozome to determine their respective transcription start sites (TSSs), using the Neural Network Promoter Prediction (NNPP version 2.2) toolset with the minimum standard predictive score (between 0 and 1) cut-off value of 0.8 for eukaryotes [23]. If the promoter regions contained two or more representative TSS positions, the one with the highest prediction score was chosen to have reliable and valid predictions.

2.2. Documentation of Common Candidate Motifs and TFs

The MEME web server (https://meme-suite.org/tools/meme, (accessed on 12 August 2022)) was utilized to identify the potential motifs that act as transcription factor binding sites (TFbs) of the phosphate transporter gene family in *Saccharum* spp. hybrid. The MEME output with potential motifs was further analyzed using TOMTOM, a component of MEME Suite that compares the motifs published in public databases of known motifs [24]. JASPAR Core Plants database is a comprehensive collection of plant transcription factor binding models that was utilized as a reference database for this study with the default settings.

2.3. Gene Ontology Analysis

The GOMo (Gene Ontology for Candidate Motifs) version 5.0.1 search was performed to evaluate the functional characteristics of the recovered motifs [25]. GOMo (https://meme-suite.org/meme/tools/gomo, (accessed on 18 August 2022)) evaluates all promoters using the nucleotide patterns and is used to discover significant motifs associated with genes with one or more Genome Ontology (GO) keywords. The motifs' biological activities may be deduced using the necessary GO keywords. In a list of ranked genes, the program searches for enriched GO keywords related to high-ranking genes.

2.4. Search for CpG Islands for PHT Gene Promoter Regions

To find CpG islands, the Takai and Jones method was utilized with severe search criteria: length $\geq$ 200 bp, GC content $\geq$ 50%, and Obs CpG/ExpCpG $\geq$ 0.60 [26]. The CpGi130 (http://dbcat.cgm.ntu.edu.tw/, (accessed on 19 August 2022)) CpG island searcher tool was employed for the analysis. As a second method the MSpI restriction enzyme site was determined using the CLC-Genomics Workbench (ver.3.6.5).

2.5. Analysis of TFbs in Sugarcane Promoter Region

To identify transcription factor binding sites (TFbs) in ShPHT gene promoters, the PlantPAN 3.0 programme (http://plantpan.itps.ncku.edu.tw/, (accessed on 20 August 2022)) was utilized [27]. To determine the presence of the same TFbs in different promoter areas, the multiple promoter analysis tool (http://plantpan.itps.ncku.edu.tw/gene_group.php?#multipromoters, (accessed on 23 August 2022)) was employed. The upstream 2000 bp promoter region of each ShPHT gene was investigated to discover their interactions with distinct transcription factor groups during the regulation of stress signaling gene expression.

2.6. Development of Transgenic Tobacco and Screening under Salt Stress

The 5′ regulatory region of the *EaPHT1-2* gene was isolated using the RAGE genome walking method and fused to the GUS reporter gene into the pCAMBIA 1305.1 vector by replacing the CaMV35s promoter. The transgenic tobacco plants were generated using the leaf disc transformation method using recombinant *Agrobacterium* as described previously [28]. The putative transgenic plants were selected on MS medium supplemented with hygromycin (25 mg/L). The *EaPHT* transgenic tobacco plants were individually harvested in the T0 generation. One-month-old seedlings of transgenic tobacco were subjected to salt stress by being placed in MS medium containing different concentrations of NaCl (50 mM, 100 mM, 150 mM, and 200 mM) for 24 h, and GUS activity was analyzed [29]. The

stress samples were immersed individually into GUS staining buffer (50 mM NaH_2PO_4 (pH = 7.0), 10 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 2 mM X-glucuronide, 10% Methanol, and 0.1% Triton X-100) and incubated at 37 °C for 24 h in the dark. Then, 70% ethanol was used to bleach the chlorophyll of stained samples, and the seedlings were then imaged.

2.7. Quantitative RT-PCR Analysis

Total RNA extraction using the TRIzol method and cDNA synthesis was carried out using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. The quantitative real-time PCR (qRT-PCR) analysis was conducted using the StepOne real-time PCR system (Applied Biosystems, Burlington, ON, Canada). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference control [30–37]. The qRT-PCR profile was as follows: 10 min of denaturation at 95 °C, followed by 40 cycles 15 s of denaturation at 95 °C, 1 min of annealing and extension at 60 °C in a final volume of 25 µL reaction [26,29,30]. The relative expression of the EaPHT1-2 promoter was measured using GUS as a reporter gene and calculated using the $2^{-\Delta\Delta Ct}$ method [38].

3. Results

3.1. Identification of Transcription Start Sites in Promoter Sequences of the PHT Gene Family

The transcription start sites (TSSs) predicted in promoter sequences of each PHT gene in both sugarcane (ShPHT) and *Sorghum bicolor* (SbPHT) species ranged from one to nine and the regulatory element of the ShPHT3-4 gene from sugarcane recorded the highest TSSs. For TSSs, PHT genes in both species achieved the highest promoter prediction scores of 1.0. In *Saccharum* spp., five genes (PHT1-1, PHT1-4, PHT1-5, PHT3-3, and PHT3-4) received a 1.0 score, whereas two genes (PHT4-4 and PHT4-5) had the lowest promoter prediction scores (0.8) (Table 1). Furthermore, the promoter predictions for *S. bicolor* sequences resulted in ten genes (PHT1-3, PHT1-7, PHT1-9, PHT1-11, PHT1-12, PHT3-2, PHT3-5, PHT4-4, PHO1-1, and PHO1-2) with a score threshold of 1.0, whereas the least score of 0.91 was obtained for SbPHT3-1 (Table 2). In addition, 78.3% and 88.8% of the genes had multiple transcription start sites, whereas 21.7% and 12.0% had just less than two transcription start sites in sugarcane and Sorghum, respectively. TSSs of *Saccharum* spp. sequences were generally located between −110 and −1868 bp relative to the translation start codon (ATG), and 82.6% of the TSS were located below −1000 bp.

3.2. Identification of Common Motifs in PHT Promoter Regions

The majority of the PHT gene sequences employed in the present investigation shared promoter regions, revealing that *Saccharum* spp. had 100% coverage at MSh 2 (Motif 2) with an E-value of 7.1×10^{-19} and a width of 29 nucleotides (Table S1). The motifs found in the majority of PHT promoter regions of *Saccharum* spp. and *Sorghum bicolor* were chosen to discover functionally relevant motifs (Figure 1). As a result, in the ShPHT promoter regions, MSh2 was identified as a similar promoter element throughout the ShPHT promoter sequences for all (100%) genes and acts as a binding site for transcription factors responsible for the control of the phosphate transporter gene's expression. Similarly, 85.18% of *S. bicolor* promoter sequences contained a conserved motif MSb4 (Motif 4) with an E-value of 1.0×10^{-30} and a length of 29 nucleotides (Table S2). Figure 2 shows the sequence logo of potential motifs identified in promoter regions of sugarcane MSh2, Sorghum MSb4, and the combination of both species. Further, the MSh2 and MSb4 motifs were analyzed in comparison with the reported motifs in JASPAR, a public database, to discover if any putative motif resembled any of the previously reported regulatory motifs for transcription factors using the TOMTOM online tool. As a result, MSh2 resembled 11 of the 841 known patterns, whereas MSb4 resembled 42 of the 841 available motifs. The top ten matching motifs were chosen based on their assessed statistical significance ratings (Table 3 and Table S3).

Table 1. Number of TSSs and predictive score for ShPHT genes of *Saccharum* spp. hybrid.

Promoter ID	Number of Identified TSS	Predictive Score	Location of the Best TSS from Start Codon (ATG)
ShPHT1-1	5	1.00, 0.97, 0.95, 0.93, 0.81	−1645
ShPHT1-2	4	0.99, 0.99, 0.91, 0.87	−1662
ShPHT1-3	7	0.97, 0.94, 0.94, 0.94, 0.90, 0.84, 0.81	−951
ShPHT1-4	6	1.00, 0.97, 0.94, 0.93, 0.85, 0.81	−737
ShPHT1-5	2	1.00, 0.90	−446
ShPHT1-6	3	0.94, 0.89, 0.88	−609
ShPHT1-7	2	0.97, 0.90	−331
ShPHT1-8	2	0.99, 0.92	−294
ShPHT2-1	2	0.93, 0.90	−456
ShPHT3-1	2	0.99, 0.90	−908
ShPHT3-2	2	0.99, 0.83	−319
ShPHT3-3	5	1.00, 0.95, 0.95, 0.92, 0.88	−380
ShPHT3-4	9	1.00, 1.00, 0.99, 0.98, 0.92, 0.89, 0.88, 0.84, 0.82	−751
ShPHT4-1	1	0.94	−329
ShPHT4-2	1	0.97	−237
ShPHT4-3	6	0.98, 0.96, 0.96, 0.83, 0.83, 0.81	−1686
ShPHT4-4	1	0.83	−1194
ShPHT4-5	3	0.86, 0.84, 0.81	−473
ShPHT4-6	3	0.99, 0.91, 0.84	−856
ShPHO1-1	1	0.94	−486
ShPHO1-2	1	0.95	−219
ShPHO1-3	4	0.99, 0.93, 0.91, 0.82	−524
ShPHO1-4	2	0.91, 0.91	−110

Table 2. Number of TSSs and predictive score for SbPHT genes of *S. bicolor*.

Promoter ID	Number of Identified TSS	Predictive Score	Location of the Best TSS from Start Codon (ATG)
SbPHT1-1	3	0.99, 0.99, 0.98	−171
SbPHT1-2	3	0.99, 0.98, 0.97	−884
SbPHT1-3	7	1.00, 0.95, 0.95, 0.92, 0.92, 0.89, 0.88	−226
SbPHT1-4	4	0.98, 0.88, 0.88, 0.83	−796
SbPHT1-5	1	0.95	−421
SbPHT1-6	8	0.99, 0.99, 0.98, 0.95, 0.94, 0.91, 0.88, 0.85	−1780
SbPHT1-7	7	1.00, 0.99, 0.99, 0.95, 0.90, 0.87, 0.85	−384
SbPHT1-8	1	0.92	−562
SbPHT1-9	7	1.00, 1.00, 0.99, 0.95, 0.94, 0.93, 0.82	−1685
SbPHT1-10	6	0.99, 0.99, 0.97, 0.94, 0.82, 0.81	−280
SbPHT1-11	4	1.00, 0.99, 0.96, 0.83	−1868
SbPHT1-12	4	1.00, 1.00, 0.97, 0.97	−1501
SbPHT2-1	4	0.97, 0.88, 0.86, 0.83	−800
SbPHT3-1	2	0.91, 0.88	−265
SbPHT3-2	3	1.00, 0.99, 0.86	−798
SbPHT3-3	4	0.96, 0.94, 0.93, 0.83	−647
SbPHT3-4	5	0.99, 0.94, 0.90, 0.85, 0.80	−113
SbPHT3-5	4	1.00, 1.00, 0.92, 0.90	−743
SbPHT3-6	2	0.99, 0.89	−445
SbPHT4-1	1	0.99	−253
SbPHT4-2	2	0.92, 0.81	−279
SbPHT4-3	5	0.99, 0.95, 0.95, 0.91, 0.81	−158
SbPHT4-4	8	1.00, 1.00, 0.95, 0.93, 0.90, 0.84, 0.83, 0.81	−406
SbPHT4-5	6	0.99, 0.99, 0.95, 0.93, 0.92, 0.91	−618
SbPHT4-6	4	0.99, 0.94, 0.91, 0.85	−1510
SbPHO1-1	6	1.00, 1.00, 0.94, 0.91, 0.90, 0.89	−218
SbPHO1-2	3	1.00, 0.95, 0.88	−181

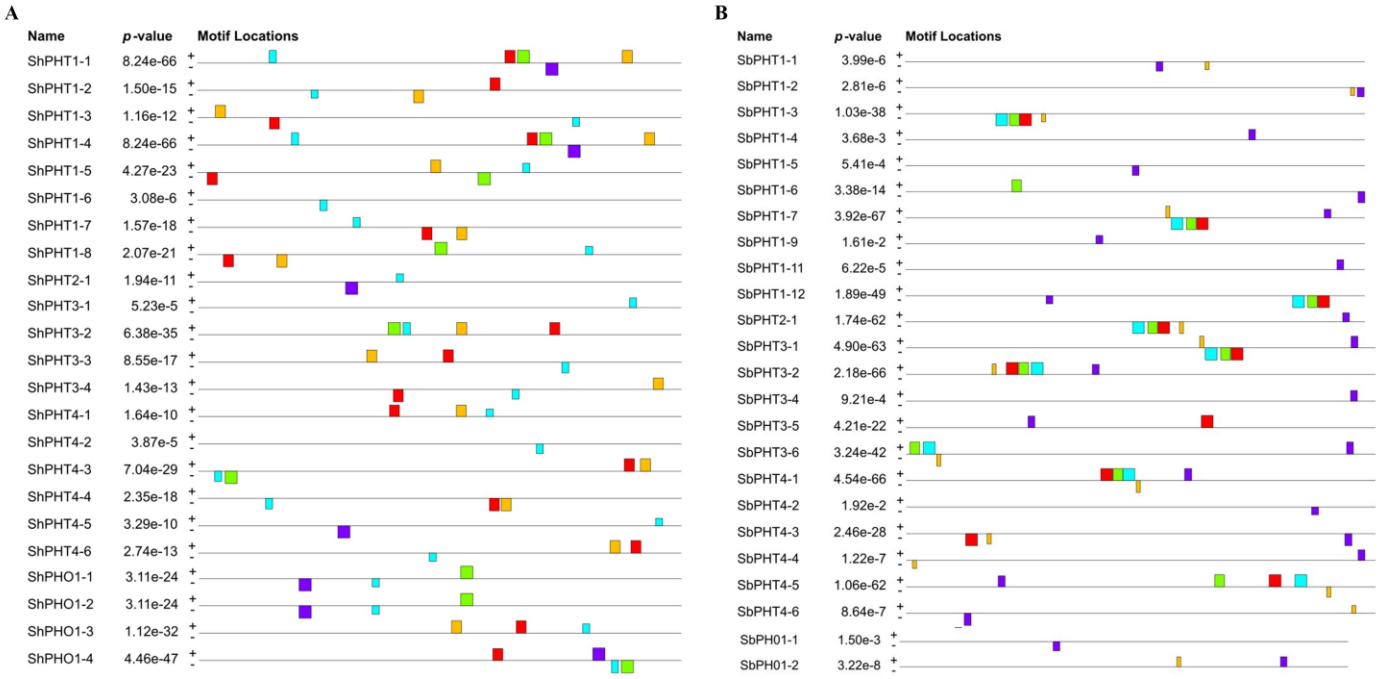

Figure 1. Schematic representation of putative conserved motifs identified with the MEME program. (**A**) Putative conserved motifs shared by sugarcane PHT promoter sequences; Motif 2—Light Blue represents the MSh2 motif. (**B**) Putative conserved motifs shared by Sorghum PHT promoter sequences. Five motifs are indicated by different colored boxes: Motif 1—Red, Motif 2—Light Blue, Motif 3—Yellow, Motif 4—Violet and Motif 5—Green. Motif 4—Violet represents the MSb4 motif.

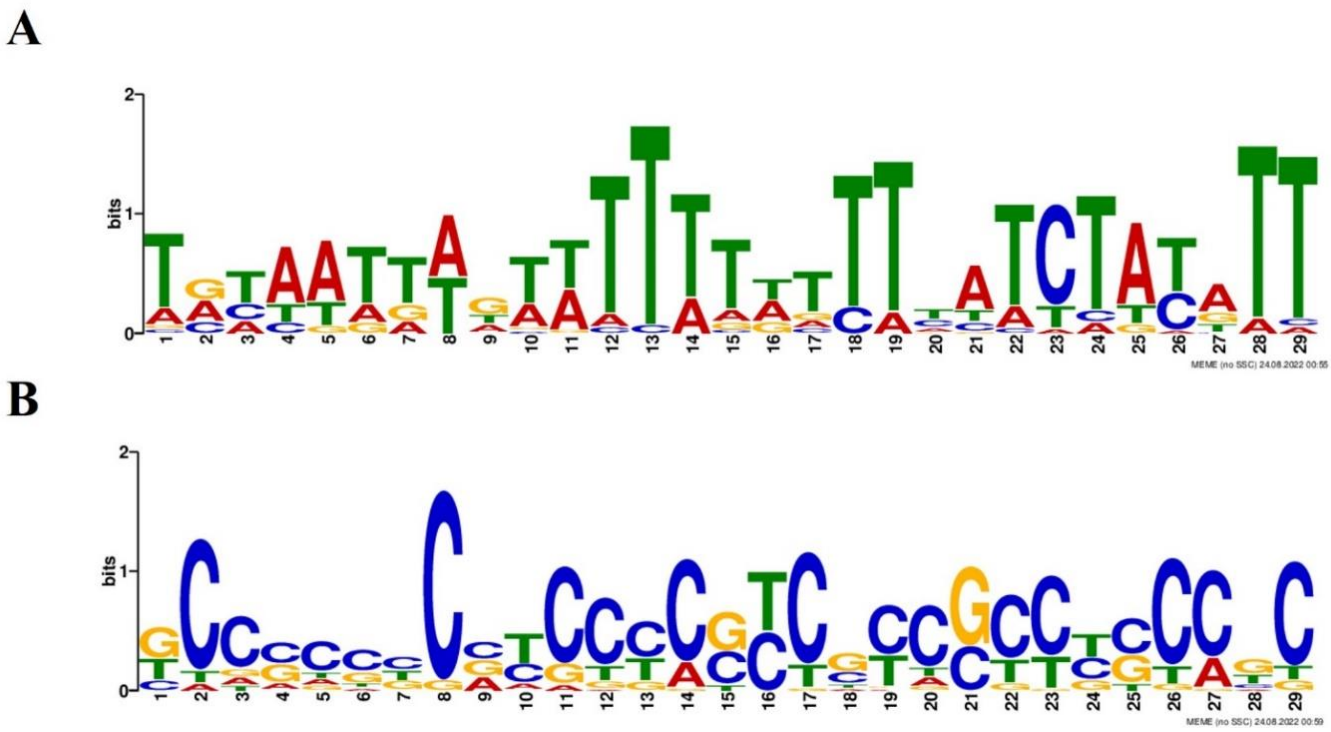

Figure 2. Sequence logo representation of identified motifs. (**A**) Common motif MSh2 identified using MEME in sugarcane PHT promoter. (**B**) Common motif MSb4 identified using MEME in Sorghum PHT promoter. (**B**) Logo of the motif found with MEME analysis (below, named motif 1) with the first five TFBSs found by TOMTOM analysis against the JASPAR core (2014) database.

Table 3. List of matches to the query motif from the database JASPAR2018_CORE_vertebrates_non redundant for *Saccharum* spp. hybrid.

Matrix ID	Gene Name	E-Value	Overlap	Offset	Orientation	TF Family/Class
MA1125.1	ZNF384	8.48×10^{-1}	12	−11	Reverse Complement	C2H2 zinc finger factors
MA1104.2	GATA6	9.63×10^{-1}	13	−13	Normal	C4-GATA-related
MA0041.2	FOXD3	1.85×10	16	−13	Reverse Complement	FOX
MA1978.1	ZNF354A	1.90×10	18	−11	Reverse Complement	C2H2 zinc finger factors
MA1970.1	TRPS1	4.28×10	12	−14	Normal	C4-GATA-related
MA1606.1	Foxf1	5.72×10	11	−17	Reverse Complement	FOX
MA0035.4	GATA1	6.14×10	11	−15	Normal	C4-GATA-related
MA0029.1	Mecom	6.95×10	14	−11	Reverse Complement	C2H2 zinc finger factors
MA0602.1	Arid5a	7.85×10	12	−17	Reverse Complement	ARID-related
MA0157.3	Foxo3	8.36×10	12	−16	Reverse Complement	FOX
MA0465.2	CDX2	9.53×10	12	−10	Reverse Complement	HOX

3.3. Gene Ontology Analysis for the Identified Sequence Motifs

The current investigation discovered gene ontology (GO) keywords for the common putative motifs MSh2 and MSb4 in the PHT gene promoter sequences of *Saccharum* hybrid and *S. bicolor* (Figure 3). A search for the common motifs in the MEME suite yielded domains with established molecular activities and biological processes.

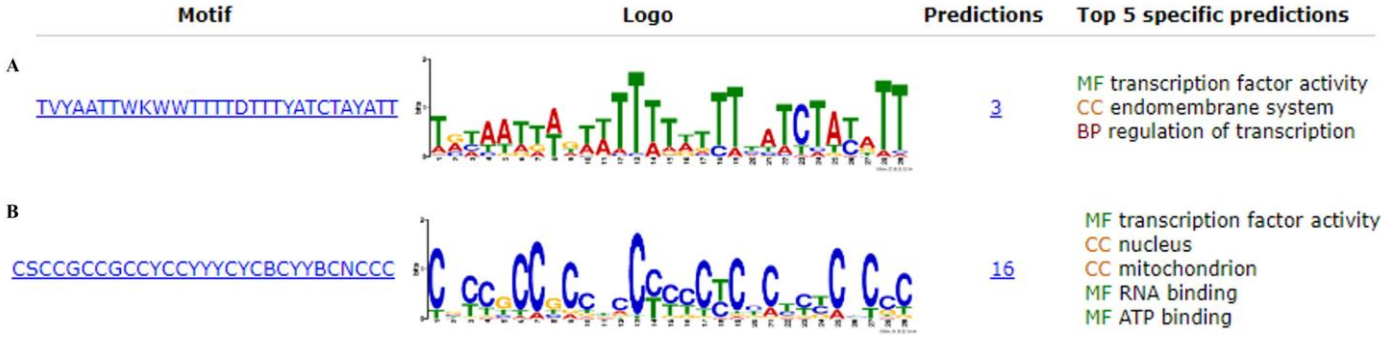

Figure 3. Sequence logo representation of GOMo analysis using the MEME Suite. (**A**) MSh2 motif predicted GO terms; (**B**) MSb4 motif predicted GO terms. MF represents Molecular Function, CC represents Cellular Component, and BP represents Biological Process.

3.4. Analysis of CpG Islands in Phosphate Transporter Gene Promoter Regions

The promoter region CpG island analysis was explored in the current study utilizing two algorithms, CpG island Finder and CLC Workbench 3.6.1. According to Takai and Jones [26], among 27 SbPHT promoters, 13 (48.14%) sequences, namely, SbPHT1-1, SbPHT1-4, SbPHT1-5, SbPHT1-6, SbPHT3-2, SbPHT3-4, SbPHT3-6, SbPHT4-4, SbPHT4-5, SbPHT4-6, SbPHTO1-1, and SbPHTO1-2 CpG islands were observed (Figure 4). Compared to Sorghum, only nine (ShPHT1-2, ShPHT1-3, ShPHT1-7, ShPHT3-1, ShPHT4-1, ShPHT4-3, ShPHT4-6, ShPHO1-3, and ShPHO1-4) out of twenty-three (39.13%) showed CpG islands in the promoter regions of *Saccharum* spp. On the other hand, MspI (C/CGG sequence) restriction sites were analyzed in the CLC workbench and this revealed that all the promoter regions in *Saccharum* spp. hybrid (Table 4) have at least one CpG island, whereas the SbPHT1-2 and SbPHT1-10 promoter regions of *S. bicolor* lack CpG islands (Table S4; Figure S1).

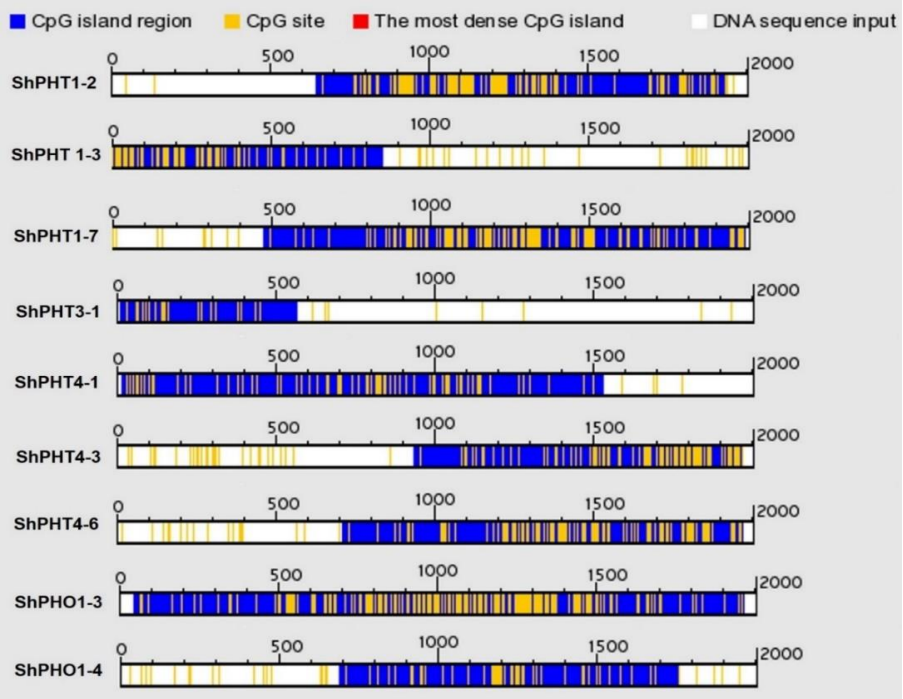

Figure 4. Visualization of promoter-associated CpG islands in the ShPHT gene promoter regions using CpGi130. Numbers shown along the *x*-axis designate the length of the promoter sequence.

Table 4. MspI cutting sites and fragment sizes for promoter regions of ShPHT genes in *Saccharum* spp. hybrid.

Promoter ID	No. of Cut Sites	Nucleotide Positions of MspI Sites
ShPHT1-1	0	-
ShPHT1-2	1	816
ShPHT1-3	1	-
ShPHT1-4	0	-
ShPHT1-5	1	106
ShPHT1-6	0	-
ShPHT1-7	1	1049
ShPHT1-8	0	-
ShPHT2-1	0	-
ShPHT3-1	1	147
ShPHT3-2	1	968
ShPHT3-3	0	-
ShPHT3-4	2	498, 799
ShPHT4-1	0	-
ShPHT4-2	0	-
ShPHT4-3	0	-
ShPHT4-4	2	1081, 1256
ShPHT4-5	3	1062, 1186, 1200
ShPHT4-6	1	876
ShPHO1-1	0	-
ShPHO1-2	0	-
ShPHO1-3	2	744, 1061
ShPHO1-4	3	82, 638, 1252

3.5. Abiotic-Stress-Related TFbs Analysis

The ShPHT gene promoter regions were examined for transcription factor binding sites (TFbs) using PlantPAN3.0. As a result, 67,897 TFbs were generated by the database from input searches (Table S2). Several TFs, namely, AP2/ERF, AT-Hook, bHLH, bZIP,

C2H2, DOF, GATA, MYB, MADS-Box, NAC, TBP, and WRKY, involved in the regulation of growth and development, light-responsive elements, and biotic and abiotic stimuli were discovered. Among all the discovered TFs, we emphasize the TF families implicated in abiotic stress responses. AP2/ERF has the most TFbs with 5263 binding sites, followed by MYB, which has 4805 binding sites across the ShPHT promoter region (Figure 5). Other TFs including the bZIP-binding site, WRKY, bHLH-binding site, and NAC were found to have 3495, 2393, 2376, and 1168 TFbs sites, respectively (Figures 6 and 7).

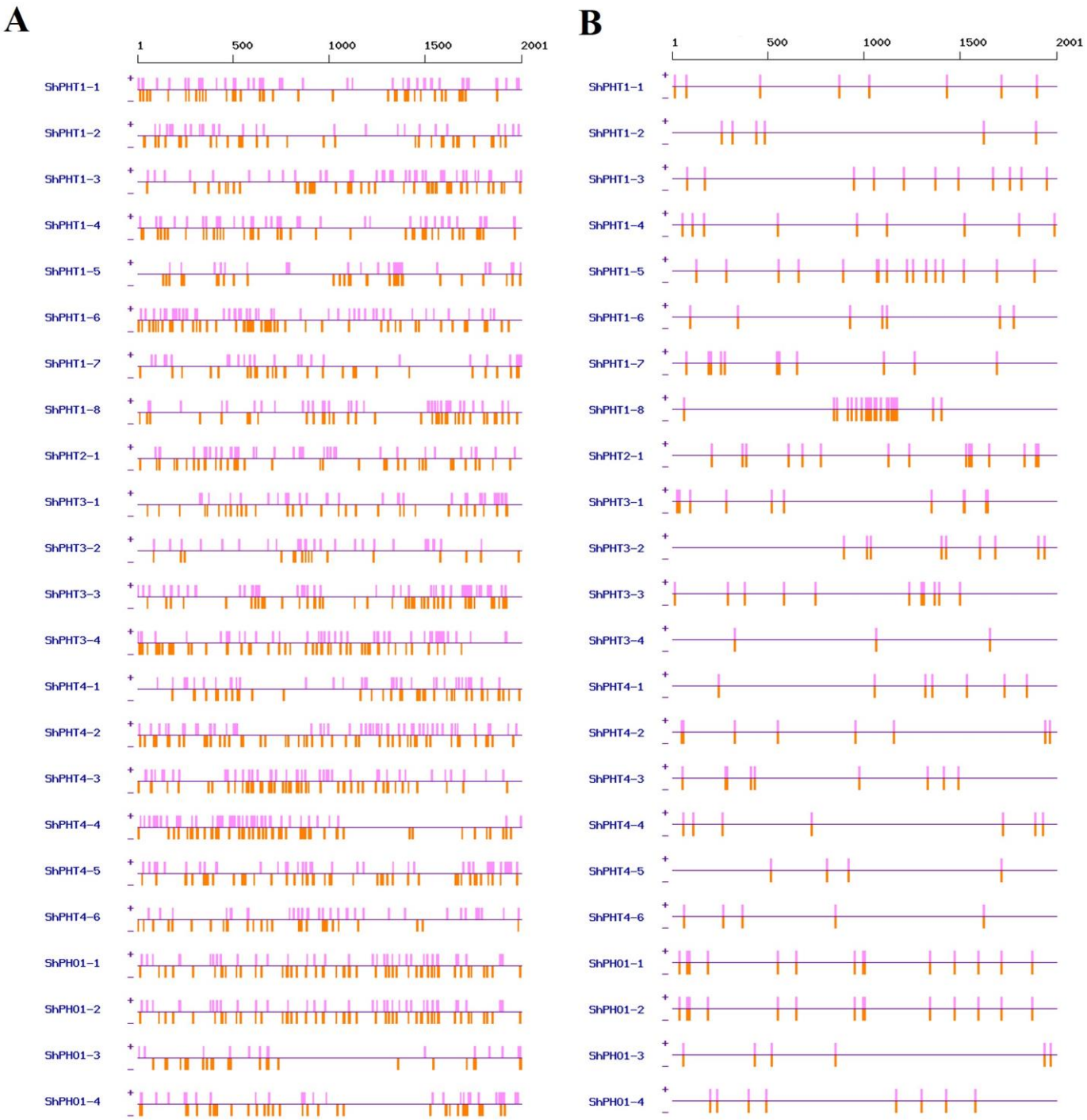

Figure 5. Distribution of transcription factor binding sites (TFbs) in 23 ShPHT promoter regions. (**A**) Putative AP2/ERP TFbs and (**B**) MYB TFbs. The pink- and orange-colored bars represent the locations in the promoter region on both positive and negative strands, respectively.

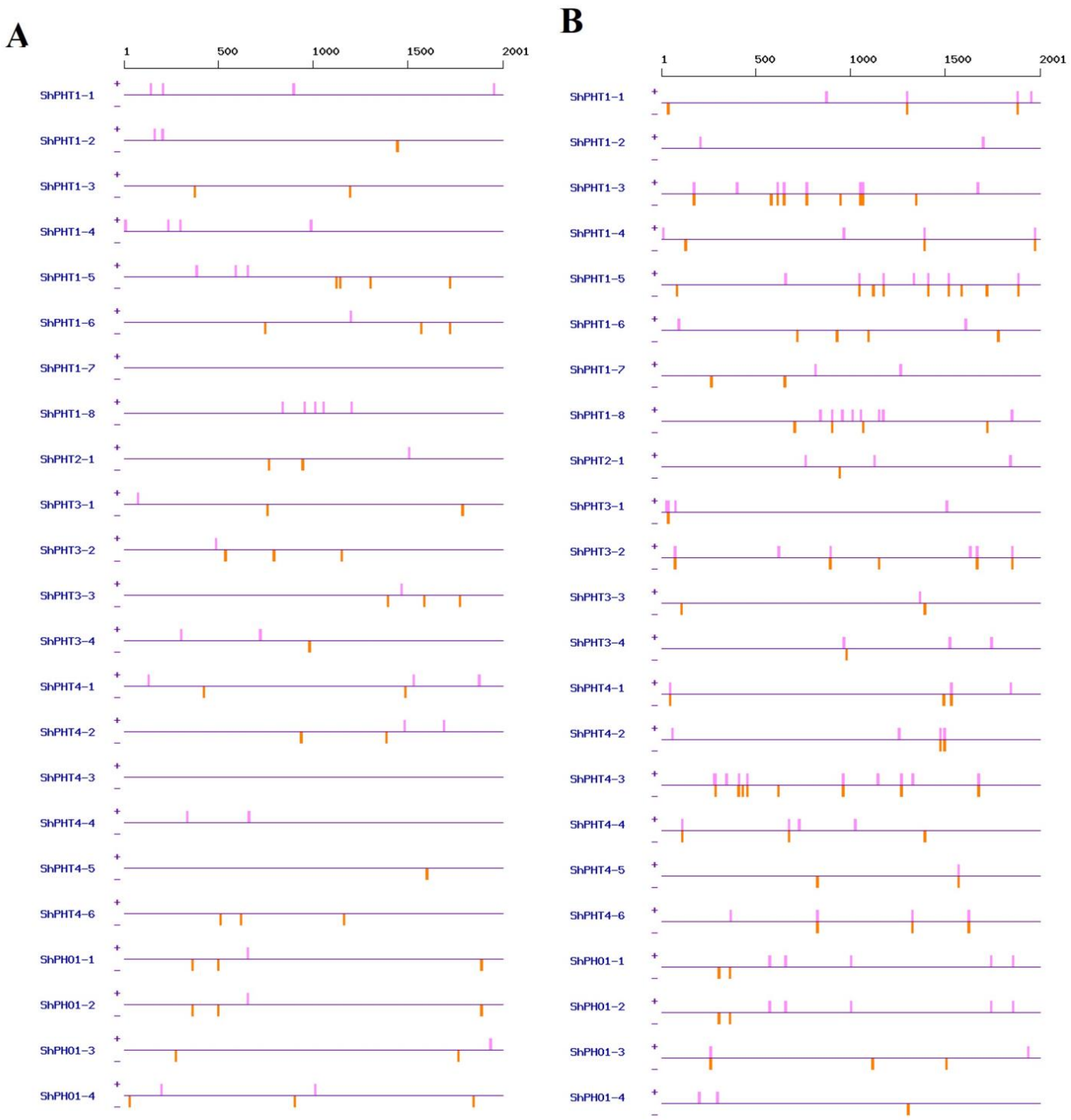

Figure 6. Distribution of transcription factor binding sites (TFbs) in 23 ShPHT promoter regions. (**A**) Putative WRKY TFbs and (**B**) bZIP TFbs. The pink- and orange-colored bars represent the locations in the promoter region on both positive and negative strands, respectively.

Figure 7. Distribution of transcription factor binding sites (TFbs) in 23 ShPHT promoter regions. (**A**) Putative bHLH TFbs and (**B**) NAC TFbs. The pink- and orange-colored bars represent the locations in the promoter region on both positive and negative strands, respectively.

3.6. GUS Activity of Transgenic Tobacco EaPHT Promoter under Osmotic Stress

To investigate GUS expression under control and stressed conditions of the EaPHT promoter ten independent transgenic lines were randomly screened in the T1 generation using histochemical GUS staining. High GUS activity was recordedin all parts of two-week-old transgenic tobacco seedlings, including leaves, stems, and roots, as measured by

histochemical staining. The analysis showed intense GUS activity in the NaCl-treated transgenic, but no significant differences between different concentrations of NaCl. The GUS activity suggested that this might be due to the presence of a number of stress-responsive cis-elements that regulate the EaPHT promoter expression under various stresses. Three independent transgenic lines with consistently high levels of GUS expression were selected for further analysis (Figure 8A). RT-PCR was used to evaluate the normal and stress-induced expression of the GUS reporter transcripts driven by the EaPHT promoter under salt stress. T1 tobacco transgenic lines with high levels of GUS expression were utilized for qRT-PCR analysis. Figure 8B depicts the amplification of the relative transcript abundance in transgenic plants under both normal and stressful conditions. The GUS expression level increased with NaCl treatment and this finding provided evidence that the EaPHT gene promoter has strong expression under salt-stress conditions.

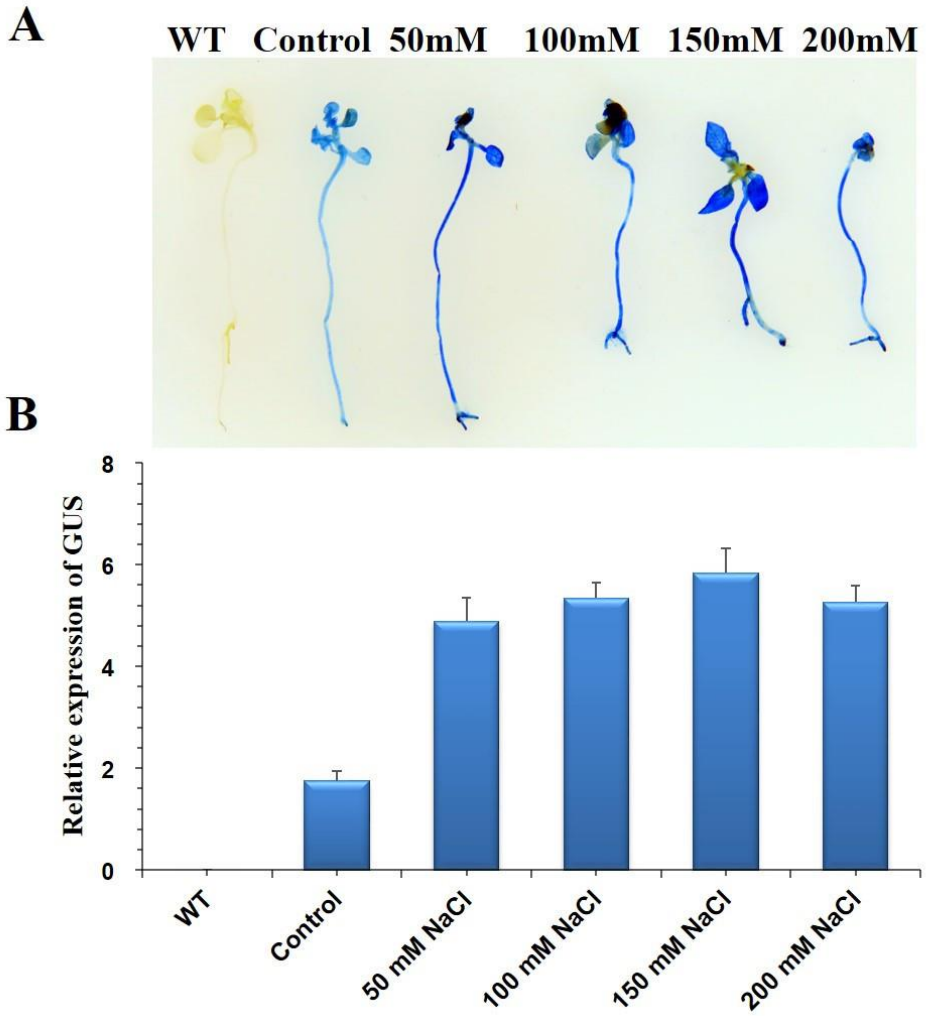

Figure 8. GUS activity of EaPHT transgenic tobacco with 50 mM, 100 mM, 150 mM, and 200 mM NaCl. (**A**) GUS histochemical staining analysis. (**B**) GUS expression pattern of EaPHT, determined by qRT-PCR. WT—Wild type, Control—Untreated EaPHT transgenic event.

4. Discussion

In the present study, the comparative analysis of 50 PHT promoter regions of *Saccharum* spp. Hybrid (ShPHT) and *S. bicolor* (SbPHT) using the in silico analysis of transcription start sites (TSS), motif discovery, CpG islands, and Transcription Factor binding sites (TFbs) have demonstrated their versatility in phosphate transporter gene regulation under stress response. The current study's use of the NNPP server produced a wide range of TSSs, which may indicate their role in regulatory systems. Studies have shown that the TSS is highly

important and improves the accuracy of predicting promoters [39], since the promoter region that includes transcription factor binding sites is often located right upstream of 1 kb. As a result, ShPHT gene promoters typically contained a single transcription start site, with the number of TSSs ranging from one to nine. Furthermore, only the ShPHT3-4 gene possesses up to nine TSSs (Table 1). Similarly, the majority of genes in Sorghum contained at least one TSS, and SbPHT4-4 had a maximum of eight TSSs. Most studies, however, have discovered that genes with multiple TSSs have an increased likelihood of transcription initiation and contribute genes in response to changes in the environment [40]. The current analysis also indicated that the sites of 47.5% and 51.9% of the TSSs were less than −500 bp from the ATG in sugarcane and Sorghum, respectively [21]. The gene expression pattern was regulated by the transcriptional factors by binding with the short motif present in the promoter region of a gene [41]. In this study, the MEME web server was used to discover the common and putative motifs in the PHT gene promoters of sugarcane and Sorghum. As a result, five motifs that were predicted in each promoter region shared at least 30.4% and 37.0% of common motifs in the ShPHT and SbPHT promoters, respectively. Among the five motifs, motif 2 (MSh2) and motif 4 (MSb4) were revealed as the most common motifs with 100% and 85% of the higher percentage of distribution among promoter sequences (Tables S1 and S2, and Figure 2). Therefore, an extensive study on the MSh2 and MSb4 motifs was carried out using TOMTOM to obtain more information on the motifs with previously published and publicly accessible databases to check whether they resembled known regulatory motifs for TFs. As a result, C2H2-Zinc finger transcription factor families were predominantly found to bind to the MSh2 and MSb4 motifs and thereby be involved in the regulation mechanism of PHT gene expression (Table 3 and Table S3). Furthermore, the C2H2-ZF-type transcription factor TaZFP15 was up-regulated under starved-Pi stress, while TaZAT8 was reported in modulating tolerance to Pi deficiency by regulating P acquisition [42,43]. On the other hand, the C2H2-type TFs act as a positive regulator under salt stress and regulate plant responses to abiotic stress by interacting with stress response factors [44,45]. In addition to transcription factors, promoters are also regulated by CpG islands and these CpG islands have been demonstrated to restrict transcription factor access to the promoter region, hence reducing gene expression [46,47]. Two methods were used in this present study to predict CpG islands in the promoter region. It has been proposed that the phosphate transporter promoter region has a low number of CpG islands (<50%) in both the *Saccharum* and Sorghum genomes, indicating that the PHT gene family might have tissue-specific gene expression.

To better understand plant signalling regulation during stress, it is important to study the transcription factors and cis-acting regions involved in the signalling cascade. Some transcription factors perform various functions in response to diverse stress conditions. In the present study, bioinformatics analysis predicted several TFs associated with stress management, such as MYB TF, WRKY TFs, NAC TFs, bHLH TF, AP2/ERF TFs, and bZIP. In our study the maximum number of MYB TFs was detected across the ShPHT promoter region. MYB transcription factors are commonly associated with signalling networks in various stress responses. In the PHT gene promoters, phosphate starvation response 1 (PHR1) is a typical MYB transcription factor that binds to the PIBS site during the Pi starvation response [48]. A total of 2393 WRKY TFs was discovered in the ShPHT promoter region (Figure 5), and the C2H2-ZF binding motif identified in the TOMTOM analysis revealed that PHT promoters are associated with stress-related expression. However, the identification of the promoter TFbs for WRKY TF was controlled by binding with the W-box (TTGACC). We found transcription factors including MYB, WRKY, and bZIP that have roles in Pi starvation and other environmental stress signalling.

In this study, we identified the characteristics of the EaPHT1;2 promoter in transgenic tobacco seedlings grown in four different NaCl concentrations. The EaPHT1;2 promoter provides an efficient GUS reporter gene expression and qRT-PCR analysis showed a three-fold higher expression, suggesting that it is a strong promoter under salinity stress. In poplar, regardless of the phosphate levels, the PtPHT1;2 gene showed enhanced expression

during drought conditions [49]. Additionally, the PHT1;2 promoter in wheat showed predominant expression in root tissue under Pi-starvation [50]. Thus the EaPHT1;2 promoter can be an alternate plant promoter for CaMV35S and may provide an efficient means of conferring high levels of transgene expression in sugarcane under salinity stress.

5. Conclusions

In the present study, phosphate transporter (PHT) gene promoter regions in *Saccharum* spp. hybrid and *S. bicolor* were comparatively analyzed. Using in silico strategies, the transcription start sites (TSSs), common promoter motifs, CpG islands, and transcription factors and their binding sites were analyzed. The majority of the PHT genes had more than two TSSs. As a result, among all the detected TSSs, the one with the highest predictability score was chosen to define the promoter regions. Furthermore, five common potential motifs were identified in both of the genomes. Among these motifs, MSp2 and MSb4 were explored as the common promoter elements. As a consequence, the overall findings indicate that the identified potential motifs and interacting transcriptional factors are predicted to contribute extensively to gene expression under diverse stress responses. The presence of several types of TFbs was found in varying frequencies among 23 ShPHT promoter regions, and only six major TFs involved in biotic and abiotic stresses were focused on in this study. Although ShPHT promoters exhibited a higher number of TFbs related to various stress management, further research is needed to confirm these ShPHT promoters for later use in plant genetic engineering. The results suggested that PHT promoters from sugarcane are a potential foreign gene expression system for genetic transformation. Therefore, the overall results suggest that the identified common candidate motifs and binding transcription factors through bioinformatics approach can contribute to a better understanding of PHT gene family expression and help to identify the gene regulatory elements in promoter regions. The in silico analysis together with the evidence of enhanced expression of the EaPHT1;2 promoter provide insights into responsiveness of the promoter under salinity stress. Therefore, in the future, the PHT promoter could serve as a valuable source of promoters for the development of transgenic sugarcane and other crops for stress tolerant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15021048/s1, Figure S1: Visualization of promoter-associated CpG islands in the SbPHT gene promoter regions using CpGi130. Numbers shown along the *x*-axis designate the length of the promoter sequence. Table S1: Identified common candidate motifs in PHT gene promoter regions of *Saccharum* spp. hybrid; Table S2: Identified common candidate motifs in PHT gene promoter regions of *Sorghum bicolor*; Table S3: List of matches to the query motif from the database JASPAR2018_CORE_vertebrates_non redundant for *Sorghum bicolor*; Table S4: MspI cutting sites and fragment sizes for PHT genes in the promoter regions in *Sorghum bicolor*.

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