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Comparative Transcriptomic Analysis of AtBBX29 Transgenic and Wild Type Sugarcane Exposed to Drought Stress

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ABSTRACT

Previously, we produced drought-tolerant transgenic sugarcane plants that overexpressed the *AtBBX29* gene, which encodes a transcription factor (TF) B-box protein. These plants displayed delayed senescence, were able to maintain photosynthesis, and accumulated high levels of antioxidants and osmolytes when exposed to water deficit stress compared with wild type (WT) plants. To unravel the molecular mechanisms underlying the enhanced drought tolerance in these plants, in the current study, we compared the transcriptomes of the *AtBBX29* transgenic and WT plants exposed to water deficit stress using RNA sequencing. Using comparative transcriptome analysis, we identified up to 4039 differentially expressed genes (DEGs) in the stressed WT and transgenic plants compared to their non-stressed controls. A further 131 DEGs were identified when comparing the stressed WT with the stressed transgenic plants. Notably, under stress, DEGs were linked to complex stress perception and signaling, various TFs, photosynthesis and nitrogen metabolism, senescence, and oxidative stress detoxification. In the transgenic plants, TFs likely linked to the abscisic acid (ABA)-independent stress response pathway (HSF, DREB, GTE7, and AP2/ERF) and glutathione regulation were upregulated, while transcripts in the KEGG pathways linked to the photosystem I (PSI) were downregulated compared to the non-stressed plants. In the WT plants, some TFs linked to the ABA-dependent stress response pathway were downregulated (HTH-MYB domain and BBX24), while transcripts linked to senescence were uniquely upregulated in the stressed WT plants, and KEGG pathways mapping amino acid metabolism were upregulated. The differentially expressed profiles between WT and AtBBX29 overexpressing sugarcane established by this study provide important insights into the molecular mechanisms behind the AtBBX29-mediated drought-tolerant phenotype of the transgenic plants.

1 | Introduction

Sugarcane (*Saccharum* spp. hybrids) is a perennial grass, belonging to the Poaceae family, that produces tall fibrous stalks rich in sucrose. The sugarcane industry is responsible for the production of almost 80% of the world's sugar, and bagasse and cane juice are used for biofuel production (De Miranda and Fonseca 2020). Other value-added products derived from sugarcane are the use of bio-waste as feedstock, bagasse to manufacture pulp and paper, and the production of cane vinegars

and biomolecules (Eggleston et al. 2023; Lavarack 2023; Mohan et al. 2023).

Sugarcane growth and development is optimal in tropical and subtropical regions where it grows well in hot and humid climates, with water requirements of around 1500 mm distributed over its growing season (FAO, Land and Water—Sugarcane 2023). The dependency of its biomass yield on high moisture renders it susceptible to drought. The climate change currently experienced across the globe accelerates the intensity and duration of droughts due to increased temperatures, which enhance evaporation, resulting in dry soils and reduced water resources (Heino et al. 2023). Water deficit is reported to be the single most important abiotic stress affecting sustainable sugarcane production by directly affecting cane biomass (Ferreira et al. 2017). Therefore, there is a need for sugarcane cultivars with traits enabling growth and development under water-deficient conditions.

In a previous study, we reported on the development of transgenic sugarcane overexpressing a B-Box (BBX) transcription factor gene that resulted in enhanced drought tolerance (Mbambalala et al. 2021). B-Box proteins are zinc finger proteins whose folding structure is stabilized by binding zinc ions. These proteins contain one or two conserved B-Box domains and sometimes also CONSTANS, CO-like, and TOC1 (CCT) domains, which allow them to interact with DNA, RNA, or other proteins (Gendron et al. 2012; Cao et al. 2023). The BBX family of proteins has been characterized to some extent in several plant species, including Arabidopsis, rice, maize, sorghum, tomato, pear, apple, and sugarcane (Khanna et al. 2009; Huang et al. 2012; Cao et al. 2017; Liu et al. 2018; Shalmani et al. 2019; Bu et al. 2021; Medina-Fraga et al. 2023; Wu et al. 2023).

Functional annotation of numerous BBX proteins revealed active roles in plant growth and development linked to photoperiod regulation, flowering, seed germination, shade avoidance response, and hormone signaling (reviewed by Cao et al. 2023). More recently, functional studies of BBX proteins revealed their roles in abiotic stress responses (Bandara et al. 2022; Cao et al. 2023; Song et al. 2022; Wu et al. 2023).

Through transcriptional control, BBX proteins play a key role in regulatory networks participating in the plant's stress response. Transcriptomic data sets and qPCR analysis revealed the activation of numerous BBX genes when plants experience abiotic stress (Bandara et al. 2022). Furthermore, when BBX genes have been overexpressed in transgenic plants, these plants displayed enhanced abiotic stress-tolerant phenotypes. For example, enhanced salt stress tolerance in Arabidopsis and populus was observed when overexpressing STO (salt tolerance protein), which likely binds to a MYB transcription factor, resulting in enhanced root growth; and overexpressing the *BBX15* gene from Gingko resulted in high sugar accumulation and higher peroxidase activity (Nagaoka and Takano 2003; Huang et al. 2021), respectively. Besides, *sth2* mutants (BBX25 known as a salt tolerance homolog [STH]) displayed hypersensitivity to NaCl treatment (Xu et al. 2014). Drought-tolerant plant phenotypes have also been linked to heterologous expression of BBX genes through different pathways. For example, the stress tolerance phenotype in *CmBBX22* transgenics has been attributed to increased expression of *ABA INSENSITIVE 13, 15* (*AB*) and *ELONGATED HYPCOTYLS 5* (*HY5*) (Liu, Li, et al. 2019; Liu, Chen, et al. 2019). Overexpression of *CmBBX24* leads to improved tolerance partly through regulation of genes involved with compatible solutes and carbohydrate metabolism (Yang et al. 2014). *MdBBX10* overexpression in Arabidopsis resulted in enhanced root growth, while at the same time, the expression of antioxidant genes increased (Liu, Li, et al. 2019; Liu, Chen, et al. 2019). When *OsBBX4* was overexpressed in Arabidopsis, enhanced drought tolerance was linked to the upregulation of the stress-related genes *KIN*, *RESPONSIVE TO DESICCATION 29A* and *22* (*RD*) (Yan et al. 2012). In a recent study conducted by Zhang et al. (2022), the *IbBBX24* gene from sweet potato was found to activate the expression of the *IbPRX17* peroxidase gene by binding to its promoter and interacting with the APETALA2 (AP2) protein IbTOE3. Overexpression of *IbBBX24d* led to enhanced tolerance of sweet potato to salt and drought stresses through increased peroxidase activity and lower H_2O_2 accumulation by modulating the expression of genes encoding ROS antioxidant scavenging enzymes, such as peroxidases. The overexpression of the *MdBBX7* gene from apple has been shown to enhance drought tolerance with direct targets such as the ETHYLENE RESPONSE FACTOR (ERF1), EARLY RESPONSIVE TO DEHYDRATION 15 (ERD15), and GOLDEN2-LIKE 1 (GLK1). Moreover, in Arabidopsis, a drought escape response can be induced through the ABA-dependent activation of the *flowering locus T* (FT) gene, which requires CONSTANS (CO) intervention (Riboni et al. 2016). Promoter regions of BBX genes also indicate transcriptional activity under abiotic stress conditions. For example, through chromatin immunoprecipitation-sequencing (ChIP-seq) analysis, the CCTTG cis-element and its binding motif, the T/G box (CACGTT/G), known BBX recognition motifs, were identified in the promoter of *MdBBX7* (Chen et al. 2022). In *MdBBX10*, abiotic stress-responsive elements such as MBS and ABRE, a MYB binding site, and an abscisic acid-responsive site were detected (Liu, Li, et al. 2019; Liu, Chen, et al. 2019). In tomato, 11 predicted cis-elements related to stress responses were identified in the BBX family, of which the majority of the BBX genes, more than 77%, contained the MYB, STRE (stress-related elements), and WUN binding sites in their promoter regions (1.5 kb) (Bu et al. 2021). However, the drought regulation of BBX genes and how they interact with other genes are not yet fully discovered and understood.

In this study, we describe the transcriptomic profiling of transgenic sugarcane overexpressing the *AtBBX29* gene under water deficit stress. The primary objective of the study was to enhance our understanding of the role played by BBX TFs in the plant's stress response. The transgenic plants displayed an enhanced drought-tolerant phenotype through maintenance of photosynthetic processes for an extended period, adjusted osmotic regulation, increased antioxidants accumulation, and the limitation of ROS and oxidative damage (Mbambalala et al. 2021). We identified 131 differentially expressed genes in the transgenic plants exposed to water deficit, of which 59% showed significant identity to other plant genes. Several of these identified DEGs were linked to the ABA-independent signal transduction pathway and enhanced antioxidant activity in the transgenic plants. The discovery of interactive gene expression activity will provide novel insights into AtBBX29-mediated abiotic stress tolerance in sugarcane.

2 | Materials and Methods

2.1 | Plant Material and Sample Collection

Wild type (WT) sugarcane plants from the *Saccharum* sp. hybrid cultivar NCo310 and transgenic sugarcane overexpressing the *AtBBX29* transcription factor gene (accession number At5g54470) were planted in the glasshouse in 20 cm pots containing a homogenous mixture of peat (Starke Ayres), vermiculite (Rosarium) and sand (ratio 2:1:1) under natural light at 26°C ± 2°C. The two transgenic lines, T1.1 and T1.6, used in this experiment were obtained from our previous study (Mbambalala et al. 2021): the *AtBBX29* transgene was expressed under control of a dual constitutive maize ubiquitin/35S promoter and cauliflower mosaic virus (CaMV) terminator. The plants were allowed to grow for 4 months, during which time they received water daily and were fertilized every second week with Hygrotech Generic Fertilizer (Hygrotech) containing 2.5 g/L $Ca(NO_3)^2$.

Plants in the tillering stage (eight plants per genotype) were moved to a growth room where temperature and light parameters were carefully controlled. Night and day temperatures varied between 20°C and 26°C, and the photoperiod was set at 16/8 h light/dark provided by OrbitX 150 W, 15,000 lm IP65 LED lights with an average light intensity of 561 μmol/m^2/s. The plants were allowed to acclimate in the growth room for 1 week. Thereafter, all plants were well-watered and excess water was allowed to drain for 3 h. Leaf tissue was then harvested from four plants of each of the genotypes, which included the WT and the two *AtBBX29* transgenic lines (T1.1. and T1.6). From each plant, the top visible dewlap (TVD) leaf and the two leaves just above and below the TVD leaf (TVD + 1 and TVD-1) were harvested, flash frozen in liquid nitrogen, and stored at −80°C until used. For plants assigned to the stress treatment (four plants for each genotype), water was withheld to induce water deficit stress for 12 days, after which leaf tissue was harvested. The soil moisture was measured prior to and after 12 days without receiving water by inserting a ProCheck soil moisture probe (Decagom Devices) 10 cm deep into the soil of each pot included in the trial, taking three readings around the plant stem.

2.2 | RNA Isolation, cDNA Library Construction and Sequencing

Total RNA was extracted from the leaf tissue harvested from four unstressed and stressed transgenic and WT plants each using the Maxwell16 LEV Plant RNA Kit (Promega), which included a DNaseI treatment. The quality of the RNA was determined using an Agilent TapeStation (Agilent Technologies) and the libraries were constructed using RNA samples with an integrity number > 7.0. A total of 24 cDNA libraries were constructed and sequenced on the Illumina MiSeq system at the Agricultural Research Council, Biotechnology Platform, South Africa.

2.3 | Sequence Assembly and Mapping

The paired-end raw reads were checked/processed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) to ensure the data quality for downstream analysis. The FastQC results were summarized using MultiQC (Ewels et al. 2016). Next, the adaptor contamination, low-quality reads (< 20), and the ambiguous "N" sequences were removed using Trimmomatic v0.32 (Bolger et al. 2014). Trinity v2.14.0 was used to assemble the de novo transcriptome of the raw reads (Grabherr et al. 2011). Then, we mapped back the raw reads to the transcriptome using Bowtie2 (Langmead and Salzberg 2012) and quantified the transcript using RSEM (Li and Dewey 2011). Samtools was used to sort and manipulate the assembled bam file (Li et al. 2009). Finally, the candidate coding regions within transcript sequences were identified using TransDecoder (Haas and Papanicolaou 2019).

2.4 | Differential Gene Expression Analysis and Functional Annotation

The differential expression was performed using EdgeR (Chen et al. 2016) and transcripts per kilobase million (TPM) values, and genes with an adjusted p-value ≤ 0.05 were considered as differentially expressed genes (DEGs).

The differentially expressed transcripts were annotated against the UniProt database. Briefly, a local database was constructed using the sequences of *Viridiplantae*, downloaded from the UniProt database (The UniProt Consortium 2008). Then, the blastx of the DIAMOND program was performed in the *Viridiplantae* database (Bagcı et al. 2021). In addition, the functional information of the *Viridiplantae* was retrieved from UniProt and assigned to the homologous transcript using the Python program.

The Gene ontology (GO) identity associated with the annotated transcripts was used to enrich the GO terms of the DEGs using the topGO Bioconductor package of the R program. The Fisher statistics test was applied with a p-value cut-off < 0.05. Accordingly, Molecular Function (MF), Biological Process (BP), and Cellular Component (CC) terms associated with the DEGs were rendered using the ggplot2 package of R (Wickham 2016; R Core Team 2023). The DEGs were further annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (Kanehisa 2016). Briefly, the peptide sequences of the DEGs were incorporated for homology search in the KEGG database using the GhostKOALA (Suzuki et al. 2014; Kanehisa et al. 2016). GHOSTX score > 99 was considered to be a significant threshold, and accordingly, the annotation data (K number) was assigned to the DEGs. The K-number was used to reconstruct the KEGG pathways.

2.5 | Validation of DEGs by Quantitative PCR

We randomly selected five DEGs detected by the Illumina RNA-seq analysis for gene expression validation by quantitative reverse transcription PCR analysis (qPCR), namely the transcription factors *GTE7* and *bHLH62* as well as *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN*, *NITRATE REDUCTASE*, and *GLUTAMINE SYNTHETASE*. Primers listed in Table S1 were designed using the Primer3web online tool (https://primer3.ut.ee/) and were synthesized by Inqaba Biotec. Three reference genes, *ACTIN* (NCBI: AY742219.1), *GLYCERALDEHYDE-3-PHOSPHATE*

DEHYDROGENASE (GAPDH) (CA254672) and *UBIQUITIN* (CA094944) were included in the analysis (Table S1). The qPCR analysis was performed with the same set of RNA samples that were used for transcriptome analysis, which included four biological repeats for each genotype and treatment. cDNA was synthesized from 1 μg RNA by reverse transcription using the RevertAid H Minus reverse transcriptase kit (Thermo Scientific). The reactions were carried out using the QuantStudio 3 Real-Time PCR System and PowerUp SYBR Green Master Mix Assay (Applied Biosystems). The total reaction volume was 10 μL, containing 1 μL cDNA template, 5 μL of 2X iQ SYBR Green mix, and 300 nM of each primer. The relative gene expression levels were calculated by $2^{-\Delta\Delta Ct}$ analysis (Livak and Schmittgen 2001). The sugarcane housekeeping gene *UBIQUITIN* (Table S1) was chosen as the reference gene to normalize the expression of the target genes as it complied with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines (MIQE Guidkelines) (Bustin et al. 2009). The significance of the relative expression between the combinations was determined at $p \leq 0.05$ based on Dunnett's (two-sided) multiple comparisons test.

3 | Results

To better understand how *AtBBX29* influences water deficit stress tolerance, we performed an RNA-seq analysis on four-month-old AtBBX29 transgenics, lines T1.1 and T1.6, and WT plants after withholding water for 12 days. During this time, the volumetric soil moisture content in all the pots included in the trial decreased on average 8.4-fold; from 0.32 m^3/m^3 in well-hydrated soil on day 0 of the trial to 0.04 m^3/m^3 ($n = 4$), almost completely dried, at day 12 of the trial. This was linked to an average decrease of 30% in the relative moisture content of the plant leaves (Figure S1). All the stressed plants displayed signs of wilting, especially the WT plants, but they remained mostly green, with only a few dead leaves observed in the WT plants and some dried leaf tips in the T1.6 transgenic plants. From 24 paired reads, we assembled 457 Mb of a reference transcriptome for sugarcane, comprising 276,613 predicted genes and 541,734 transcripts with an average GC content of 47.92% (Bioproject: PRJNA1127307). About 194,370 genes were predicted to be protein-coding. The N50 value for the transcripts was 1409 bp in length. The differential expression was estimated by the transcript level (logFC cut-off 1; Figure S2).

3.1 | Comparing Each Genotype When Stressed vs. Unstressed

3.1.1 | The Differentially Expressed Profile in Genotypes Under Stressed vs. Unstressed Conditions

Under stress conditions, 1054 up and 819 down, 1676 up and 1888 down, and 2106 up and 1087 down, transcripts were differentially expressed (DE) in transgenic plants T1.1, T1.6, and WT, respectively, when compared to their corresponding unstressed plants (Figure 1; Supporting Information files S1, S2, S3).

3.1.2 | GO Term and KEGG Pathway Enrichment Analysis of DEGs Between Stressed vs. Unstressed Conditions

The DEG of the transgenic and WT plants after the stress treatment, compared to the unstressed plants, displayed mostly similar gene ontology patterns in terms of biological processes (Figure S3). The exception is the enriched biological process response to lipid ($p \leq 0.001$), which is unique to the stressed transgenic plants. In addition, the differentially expressed transcripts linked to the biological process of the response to wounding ($p \leq 0.001$) are more enriched in the stressed WT plants than in the transgenic plants. Expected biological process reactions, such as a response to abscisic acid (ABA), are seen in all the genotypes exposed to stress. Gene ontology related to molecular functions such as geranyltransferase activity, starch synthase activity, DNA-binding transcription factor activity, FMN binding, and glutamate-5-semialdehyde dehydrogenase activity are unique to the stressed transgenic plants ($p \leq 0.001$). Unique to the stressed WT is only the regulation of response to stimuli ($p \leq 0.001$) as a biological function. However, the stressed WT plants had several unique molecular functions, including racemase and epimerase, UDP-glucose 4-epimerase, MAP kinase, dihydropyrimidine dehydrogenase (NADP+), phosphoprotein phosphatase, and

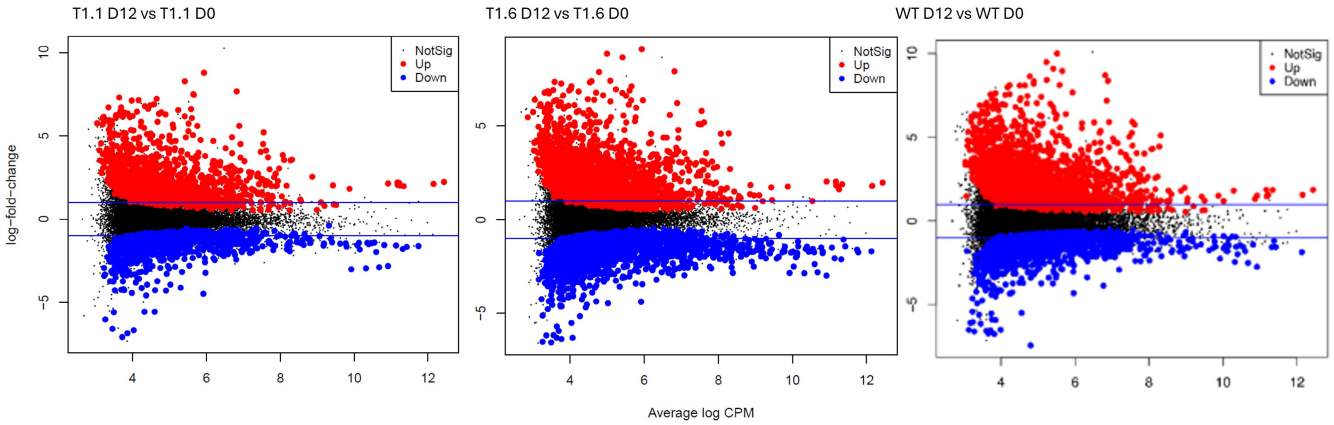

FIGURE 1 | Differential expression in *AtBBX29* transgenic (T1.1 and T1.6) and WT plants when exposed to 12 days (D12) of water deficit compared to plants from each genotype that were well watered (day 0; D0). MA plot of differentially expressed transcripts at $p \leq 0.05$.

polyamine oxidase activity, and flavin adenine dinucleotide binding ($p \leq 0.001$); and cellular components such as peroxisome, microbody, intracellular organelle, organelle, and cellulose synthase complex ($p \leq 0.05$; Figure S2). In the stressed transgenic plants, unique cellular components included the plastoglobuli, various sections of the endoplasmic reticulum ($p \leq 0.001$), organelle sub-compartment, the chloroplast stroma, and the plastid stroma ($p \leq 0.01$).

3.2 | Comparing Stressed Genotypes

3.2.1 | The Differentially Expressed Profile Between Stressed Genotypes

Under stress conditions, no genes were differentially expressed between the two transgenic lines, T1.1 and T1.6. However, when comparing the stressed transgenic plants with the stressed WT plants, 68 up and 54 down in T1.6, and 69 up and 62 down in T1.1, regulated transcripts were identified (Figure 2a). There was a total of 131 DE genes in the transgenic plants (both T1.1 and T1.6) compared to the WT plants under stress (Supporting Information File S4), including the *AtBBX29* transgene.

3.2.2 | GO Term and KEGG Pathway Enrichment Analysis of DEGs Between Stressed Genotypes

Gene ontology (GO) analysis indicated that the DEGs related to biological processes in the stressed transgenic plants, compared to the stressed WT exposed to water deficit, were enriched in cellulose biosynthesis and metabolism ($p \leq 0.001$), glucan biosynthesis and metabolism, polysaccharide and carbohydrate biosynthesis, anatomic structure, developmental maturation, reproductive processes, and transposition ($p \leq 0.01$), while processes involved in the response to heat, transposition, and cell proliferation were downregulated ($p \leq 0.01$; Figure 2b). Upregulated molecular functions in the transgenic plants related to cellulose synthase, glucosyltransferase, acyl-desaturase, oxidoreductase, sucrose synthase, transcription factor binding, and regulation ($p \leq 0.001$), hexosyltransferase, methyltransferase (histone and lysine), and serine peptidase and hydrolase activity

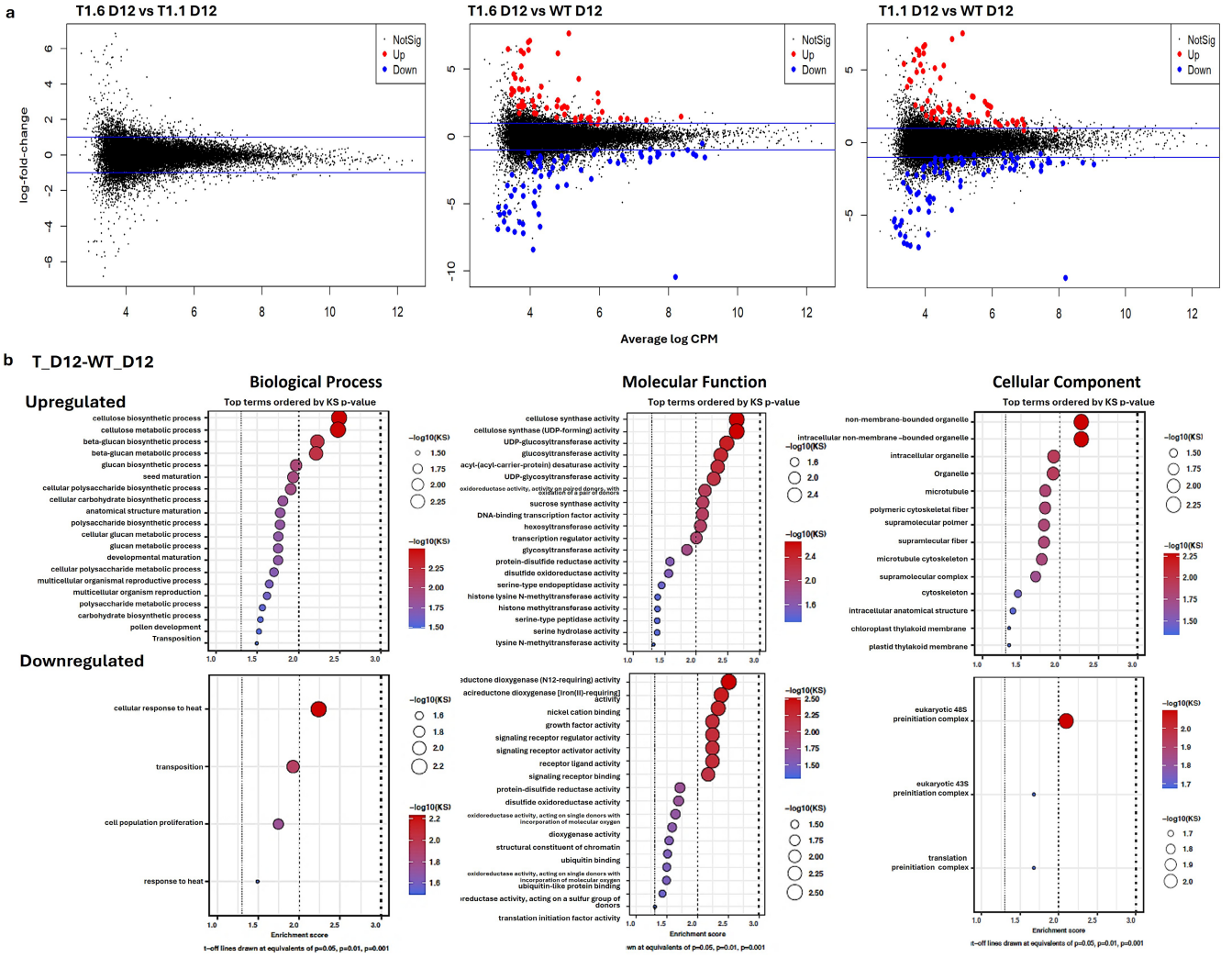

FIGURE 2 | Differential expression between (a) *AtBBX29* transgenic (T1.1 and T1.6) and WT plants when exposed to 12 days of water deficit stress. (b) Gene ontology terms associated with the differentially expressed transcripts in stressed *AtBBX29* transgenic (T1.1 and T1.6) plants compared to stressed WT plants. Listed are gene ontology related to biological processes, molecular function and cellular components. Included are differentially expressed transcripts at $p \leq 0.05$.

($p \leq 0.01$; Figure 2b). In contrast, molecular functions that were downregulated in the transgenics were acireductone dioxygenase activities that require iron and Ni2 and nickel binding, receptor signaling, activation, binding, and ligand activity ($p \leq 0.001$), reductase and dioxygenase activity, ubiquitin and ubiquitin-like binding, and lastly, translation initiation activity ($p \leq 0.01$; Figure 2b). Upregulated cellular components in the transgenic plants under stress included non-membrane bound organelle ($p \leq 0.001$), organelle, supramolecular polymer and fiber, microtubule, the cytoskeleton, intro anatomical structures, and the chloroplast and plastic thylakoid membrane ($p \leq 0.01$).

A total of 47.6% of the differentially expressed transcripts, coding for proteins, in the stressed transgenic and WT genotypes were successfully annotated using KEGG categories ($p \leq 0.05$; Table 1). In the stressed plants, transgenic (T1.1 and T1.6) and WT, the DEGs were substantially enriched in KEGG categories involved in 75 versus 69 active metabolic pathways, respectively. Specifically, the KEGG pathway categories substantially enriched in the transgenic plants under water deficit stress were associated with the metabolism of carbohydrates, cofactors, and vitamins and energy, while the WT plants displayed enriched categories in lipid and amino acid metabolism. In the transgenic plants, energy metabolism was especially linked to photosynthesis and nitrogen metabolism (Figures 3 and 4). Unique transcripts involved in Photosystem I, electron transport, and ATP activity were seen in the transgenic plants (Figure 3a). Noteworthy, DEGs of the transgenic genotypes were classified into exclusive slim categories, such as folate biosynthesis, riboflavin, lipoic acid, cyanoamino acid, glyoxylate and dicarboxylate, eter lipid, and sulfur metabolism. The DEGs in the WT plants associated with amino acid metabolism were upregulated for cysteine and methionine, arginine and proline, glycine, serine and threonine, and beta-alanine metabolism. Stressed plants from both genotypes also showed activities associated with the metabolism of lipids, the WT plants linked to linoleic acid and glycerolipid metabolism and fatty acid degradation, while the transgenic plants were enriched in steroid biosynthesis. Overall, complex stress-related signaling in which several TFs played a role was activated in both the transgenic and WT plants exposed to water deficit (Figure 5). These findings provide valuable insights into the molecular mechanisms underlying the response to water deficit stress controlled by the BBX29 TF gene.

3.3 | Validation of RNA-Sequencing Results

Considering the qPCR data (Figure 6), the validated genes displayed similar transcriptional profiles (up- or down-regulation) between stressed and non-stressed genotypes, consistent with those identified by RNA sequencing (normalized TMM values), which supports the reproducibility and accuracy of the transcriptomic profiles presented in this study. For example, as shown in Figure 6, the GTE7 TF was 1.94-fold upregulated in T1.6 (TRINITY_DN28240_c3_g1_i3; logFC = 1,73), whereas the bHLH62 TF was downregulated (TRINITY_DN9262_c0_g1; T1.6 logFC = −2357 and T1.1–1748) when exposed to stress. In addition, the qPCR analysis confirmed the AtBBX29 transgene expression in the two genotypes (TRINITY_DN18506_c0_g1_i13; normalized TMM = 23.00 to 37.93).

4 | Discussion

4.1 | Stress Perception and Signalling, and the Involvement of Transcription Factors in the Activation of Stress Response Genes

In the study conducted by Mbambalala et al. (2021) in our laboratory, the overexpression of the *Arabidopsis thaliana* B-box gene (*BBX29*) in sugarcane resulted in enhanced drought tolerance, as indicated by higher relative water content and delayed senescence of the leaves under water-deficit conditions when compared to the wild-type plants (Figure 7). In the current transcriptomic study, we compared the expression of genes in these transgenic and wild-type plants under water-deficit stress. It was found that the transcripts of the two pools of sugarcane genotypes were similar when the plants were well-watered but different when stressed (Figures 1 and 2). As illustrated by the transcriptomic analysis of the plants, once water became limited, the sugarcane plants perceived the stress through membrane receptors, including receptor-like kinases, resulting in altered cellular Ca^{2+} levels and the formation of secondary molecules, such as the hormone abscisic acid (ABA) and reactive oxygen species (ROS) (Apel and Hirt 2004). These Ca^{2+} ions are sensed by intracellular calcium sensors, such as CBL (You et al. 2023), which were significantly upregulated in all the stressed plants (UniProt accessions: A0A1D6JMA2, A0A1D6NN61, CBL-INTERACTING SERINE/THREONINE-PROTEIN KINASE 9 and 8; Supporting Information Files S1, S2, S3). The secondary messengers initiate a protein phosphorylation cascade by various kinases, such as calcium-dependent protein kinases, protein kinases, and protein phosphatases (Movahedi et al. 2023). Several of these proteins were seen to be significantly upregulated in the stressed WT and transgenic plants (Figure 5; Supporting Information Files S1, S2, S3; for example, UniProt accessions: A0A804PE00, C5YEK7, K7U9U2, A0A8T0NA71, A0A1D6JBQ1, K7U9U2, CALCIUM-DEPENDENT PROTEIN KINASE 28, 4; C5X396, PYRIDOXAL PHOSPHATASE; A0A3L6FZA0, A0A1D6E0D8, CALCIUM-TRANSPORTING ATPASE 5, 9, PLASMA MEMBRANE-TYPE; A0A317YAQ3, A0A1W0VZ26, A0A0E0I957, B8B8Q0, C5XDR2, A0A1Z5RD26, C5XL91, A0A804P1U3, A0A0E0H6X1, A0A0E0HWB5, A0A0E0FRR9, B8B8Q2, A0A1Z5R9E0, A2ZN24, A0A1B6QAZ4, A0A804P1U3, B9FTI1, C5Y7I4, A0A804M6B9, A0A0E0H101, A0A1W0VZ26, A0A1Z5RE13, A0A1B6PRI2, C5XNA6, A0A0E0G1T2, A0A194YST5, PROTEIN KINASE DOMAIN-CONTAINING PROTEIN; C5XHU8, A0A8T0TDA2, CALCIUM-BINDING PROTEIN CML36, 21; A0A1D6DU14, A0A1D6DU14, ADENYLATE KINASE; C5XAJ1, SULFITE EXPORTER TAUE/SAFE FAMILY PROTEIN 3; K7W5B8, K7W5B8, K7W5B8, A0A1D6I5A9, ROOT-SPECIFIC KINASE 1; A0A1D6EQX9, putative ETHANOLAMINE KINASE; A0A1D6H144, putative METAL-NICOTIANAMINE TRANSPORTER YSL6; A0A1D6J747, putative MAPKKK family protein kinase; A0A1D6FZZ9, A0A1D6KFX6, A0A1D6J1W9, MAP KINASE FAMILY PROTEIN; A0A3L6FCS1, putative INACTIVE RECEPTOR KINASE; A0A1D6FC73, GLYCOGEN SYNTHASE KINASE 3 MsK-3; A0A1D6PMP8, putative GLYCOGEN SYNTHASE KINASE FAMILY PROTEIN; A0A1D6N8D8, LEUCINE-RICH REPEAT PROTEIN KINASE FAMILY PROTEIN; C5XL20, A0A8T0WL00, TRANSMEMBRANE PROTEIN; A0A3L6DLL3,

TABLE 1 | Significantly enriched KEGG categories represent differentially expressed genes ($p \leq 0.05$) of AtBBX29 transgenic and wild type genotypes under water deficit stress. The amount of involved DEGs indicated by a color change from blank to dark brown, 0 to 7.

ID	Pathway categories	Number of genes	
		WT	Transgenic
	Lipid metabolism		
Map00062	Fatty acid elongation	1	2
Map00071	Fatty acid degradation	2	0
Map00100	Steroid biosynthesis	1	3
Map00561	Glycerolipid metabolism	2	0
Map00564	Glycerophospholipid metabolism	4	3
Map00565	Eter lipid metabolism	0	1
Map00600	Sphingolipid metabolism	1	0
Map00591	Linoleic acid metabolism	7	1
	Nucleotide metabolism		
Map00230	Purine metabolism	1	2
Map00240	Pyrimidine metabolism	4	2
	Amino acid metabolism		
Map00250	Ala, Asp, Glx metabolism	3	2
Map00260	Gly, Ser, Thr metabolism	4	2
Map00270	Cys, Met metabolism	5	1
Map00280	Val, Leu, Ile metabolism	2	1
Map00330	Arg, Pro metabolism	5	1
Map00340	His metabolism	1	0
Map00350	Tyr metabolism	1	0
Map00360	Phe metabolism	1	3
Map00380	Trp metabolism	1	0
Map00410	Beta-Ala metabolism	5	0
Map00460	Cyanoamino acid metabolism	0	3
Map00480	Glu metabolism	2	1
Map00310	Lys degradation	2	0
Map00400	Phe, Tyr, Trp biosynthesis	1	3
	Carbohydrate metabolism		
Map00010	Glycolysis	4	5
Map00030	Pentose phosphate pathway	1	3
Map00051	Fructose and mannose metabolism	1	2
Map00052	Galactose metabolism	4	5
Map00500	Starch and sucrose metabolism	6	7
Map00520	Amino sugar and nucleotide sugar metabolism	3	5
Map00630	Glyoxylate and dicarboxylate metabolism	0	3
Map00562	Inositol phosphate metabolism	1	2
	Energy metabolism		
Map00190	Oxidative phosphorylation	1	2
Map00195	Photosynthesis	3	7

(Continues)

TABLE 1 | (Continued)

ID	Pathway categories	Number of genes	
		WT	Transgenic
Map00196	Photosynthesis-antenna	3	4
Map00680	Methane metabolism	2	3
Map00910	Nitrogen metabolism	1	5
Map00920	Sulfur metabolism	0	1
	Metabolism of cofactors and vitamins		
Map00760	Nicotinate and nicotinamide metabolism	1	0
Map00740	Riboflavin metabolism	0	1
Map00785	Lipoic acid metabolism	0	1
Map00790	Folate biosynthesis	0	1
Map00860	Porphyrin metabolism	3	4
Map00770	Pantothenate and CoA biosynthesis	3	0
	Metabolism of terpenoids and polyketides		
Map00904	Diterpenoid biosynthesis (incl Gibberillin)	1	0
Map00902	Monoterpenoid biosynthesis	0	1
Map00906	Caretenoid biosynthesis	4	3
Map00908	Zeatin biosynthesis	0	1
	Biosynthesis of other secondary metabolites		
Map00940	Phyenylpropanoid biosynthesis	2	1
Map00945	Stilbenoid, diarylheptanoid, gingerol biosynthesis	1	0
Map00941	Flavonoid biosynthesis	2	1
Map00946	Degradation of flavonoids	0	1
Map00950	Isoquinoline alkaloid biosynthesis	1	0
Map00960	Tropane, piperidine and pyridine alkaloid biosynthesis	3	2
Map00405	Phenazine biosynthesis	1	0
	Signal transduction		
Map02020	Two component system	0	1
Map04016	MAPK signaling pathway—plants	7	5
Map04014	Ras signaling pathway	0	1
Map04066	HIF-1 signaling pathway	2	1
Map04371	Apelin signaling pathway	0	1
Map04068	FoxO signaling pathway	0	1
Map04070	Phosphatidylinositol signaling system	1	2
Map04072	Phospholipase D signaling pathway	0	1
Map04020	Calcium signaling pathway	1	0
Map04071	Sphinogolipid signaling pathway	0	1
Map04075	Plant hormone signal transduction	12	7

MITOGEN-ACTIVATED PROTEIN KINASE 7; SA0A1D6K8Q4, SERINE/THREONINE-PROTEIN KINASE SRK2C). In a study conducted in transgenic chrysanthemum plants overexpressing the *CmBBX24* gene, two transcripts encoding proteins linked to signaling mediated by Ca^{2+} ions and a transcript encoding a protein kinase, considered an important secondary messenger in eliciting the abiotic stress response, were also upregulated (Yang et al. 2014).

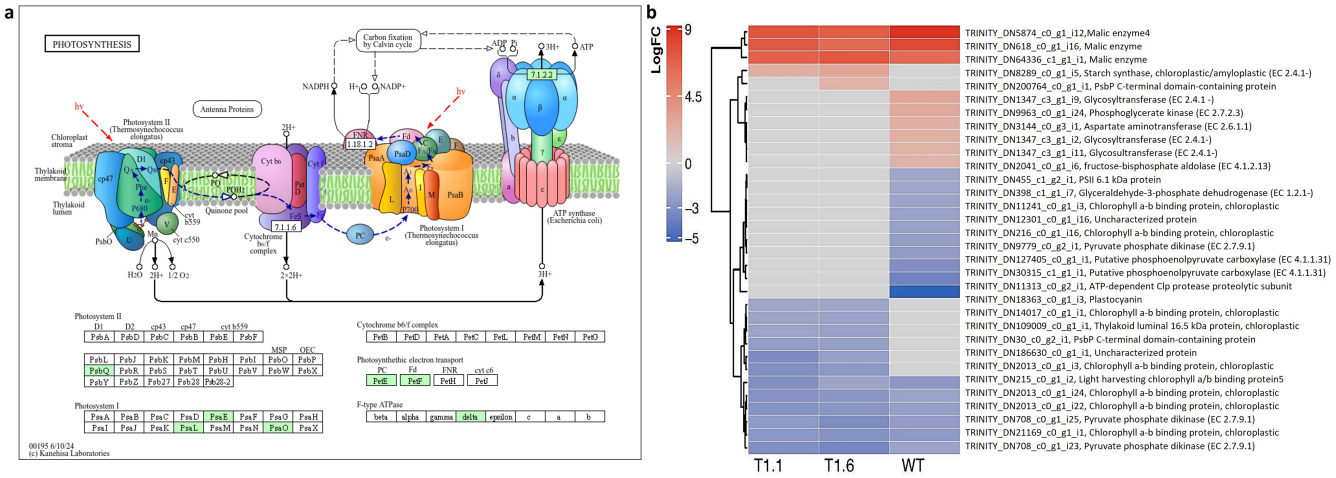

FIGURE 3 | Unique transcripts linked to photosynthesis as seen in the (a) KEGG Map00195. (b) Heatmap of the associated DEGs in WT and transgenic sugarcane overexpressing the AtBBX29 transgene when exposed to water deficit stress. Heatmaps were constructed using the log2 fold change values, and the genes in red and blue represent up- and down-regulated genes, respectively.

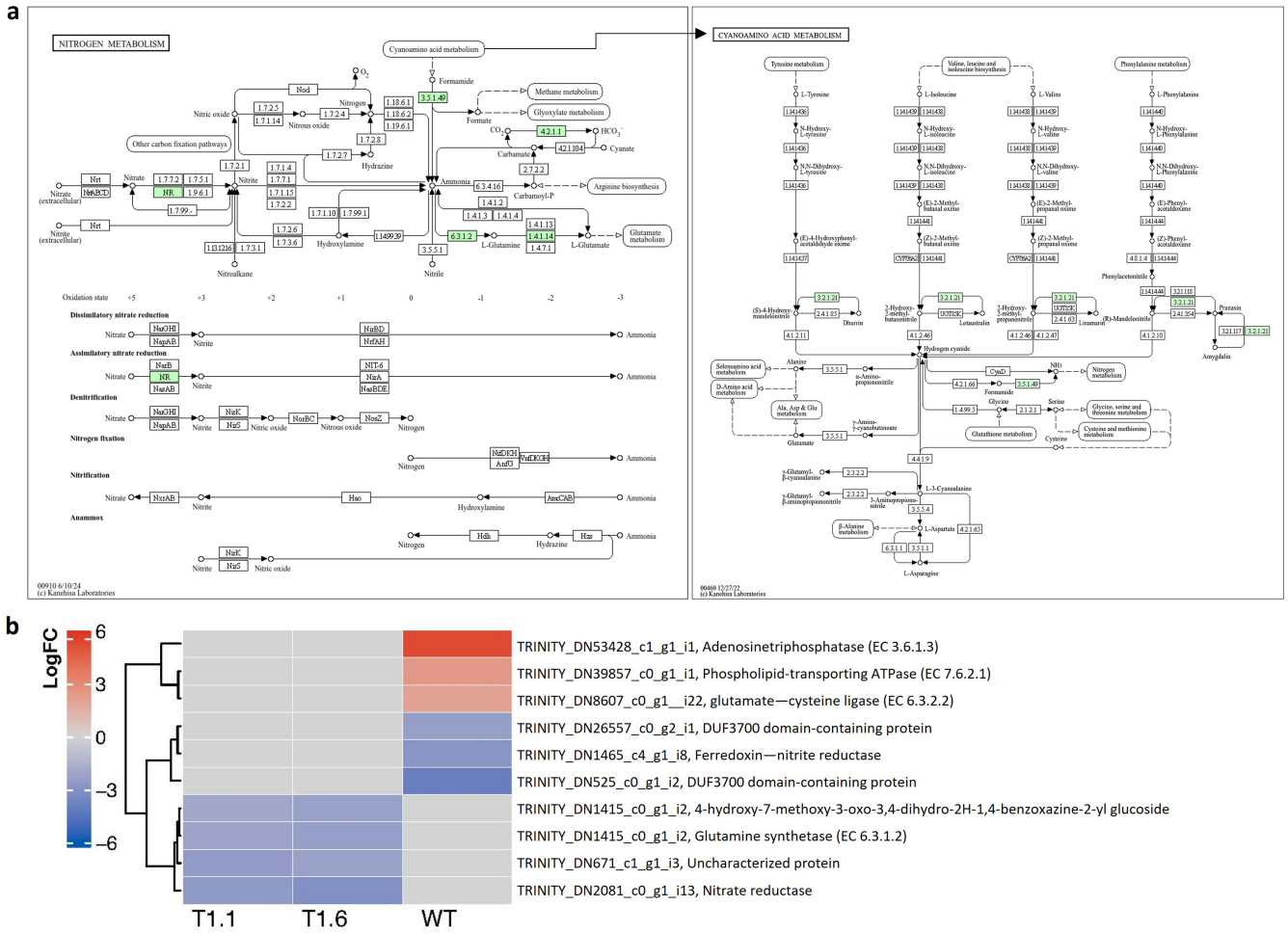

FIGURE 4 | Differentially expressed genes in transgenic plants exposed to water deficit stress display unique transcripts linked to nitrogen metabolism and cyanoamino acid metabolism as seen in the (a) KEGG Map00910 and Map00460. (b) Heatmap of the associated DEGs in WT and transgenic sugarcane overexpressing the *AtBBX29* transgene when exposed to water deficit stress. The heatmap was constructed using the log2 fold change values, and the genes in red and blue represent up- and down-regulated genes, respectively.

The stress perception and signaling commence with the biosynthesis of ABA (Ferreira et al. 2017), as seen in the activation of the ABA RESPONSIVE ELEMENT BINDING FACTOR 1 (ABRE) transcription factor (Supporting Information Files S1, S2, S3; UniProt accession: K4GNP0) in the stressed WT and transgenic plants. Abscisic acid mediates drought stress

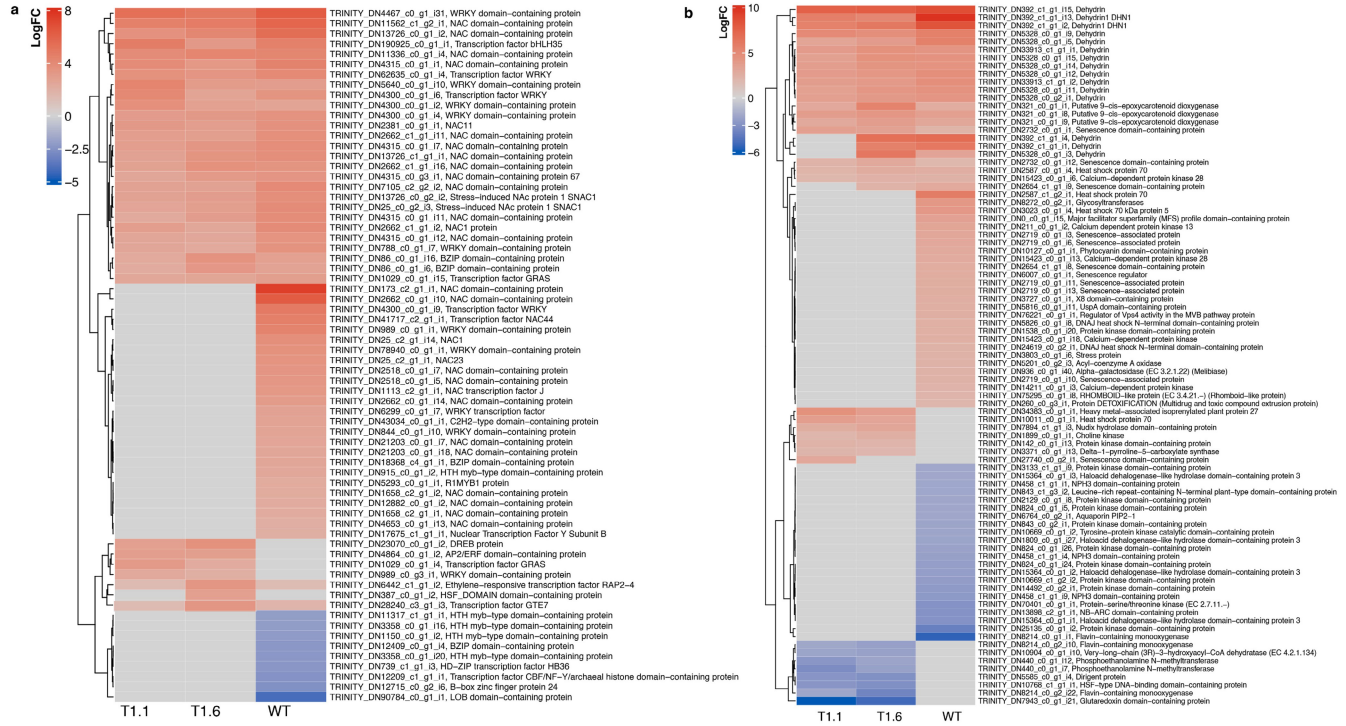

FIGURE 5 | Heat map analysis of the expression patterns of genes related to (a) transcription factors and (b) signaling in transgenic (T1.1 and T1.6) and WT sugarcane under water deficit stress. The expression patterns of DEGs were mapped by log2(FC), and row standardized. Red is upregulated expression and blue is downregulated expression.

responses in plants by regulating stomatal closure and stress-responsive gene expression in both ABA-dependent and independent signaling pathways (Tuteja 2007). Involved in this, a cascade of TFs was upregulated in both the WT and transgenic stressed plants, which included members of the bZIP, WRKY, GRAS, C2H2-ZF, NF-YB, and mostly downregulated MYB and BBX transcription factor families known to typically act in ABA-dependent stress response pathways (Figures 5 and 7). Similarly, when analyzing transgenic chrysanthemum plants overexpressing the *CmBBX24* gene exposed to drought, Yang et al. (2014) found members of the WRKY and bZIP families were upregulated, while we did not observe upregulation of the MYB members. Transcription factors have diverse roles in controlling gene expression in plants exposed to environmental stress (Hussain et al. 2021). In addition, ABRE is also known to act as an upstream binding site for TFs of MAPKKK18 expression, which was upregulated in all the stressed sugarcane plants (UniProt accession: A0A1D6J747). The MAPKK family protein kinases are known to play a role in long-term ABA response during drought stress and leaf senescence (Zhao et al. 2023).

Elevated ABA levels and activated ABA signaling due to abiotic stresses are known to promote senescence (Sah et al. 2016). Mbambalala et al. (2021) reported that the WT and AtBBX29 transgenic sugarcane displayed signs of senescence (wilting, leaf yellowing, and drying), a decrease in chlorophyll, and a decrease in stomatal conductance in all the plants after 14 days of water deficit stress. However, the transgenic plants displayed less senescence and tissue damage, and maintained significantly higher levels of stomatal conductance compared with the WT plants at this stage of the water deficit pot trial (Figure 7). Transcriptomic analysis of the stressed plants indicates upregulation of a few senescence-linked transcripts (Figure 5). Some transcripts encoding SENESCENCE-ASSOCIATED PROTEINS were uniquely upregulated in the stressed WT plants (UniProt accessions: A2YVP0, A0A8T0PPE1), while some were upregulated in both the stressed WT and transgenic plants (UniProt accessions: A0A1B6QPE1, C5Z1W6) when compared to non-stressed plants.

Several drought-responsive genes are involved in ABA signaling; however, most drought-induced genes do not react to ABA treatment, implying the presence of ABA-independent drought-response pathways (Kazuo and Yamaguchi-Shinozaki 2000). In the current study, a DREB (UniProt accessions: C5YKT7), an AP2/ERF (UniProt accession: B9FIJ2), two HSF-type DNA-binding domain-containing proteins (UniProt accessions Q109P2, A0A804R696), a GTE7 (UniProt accession: A0A1B6PG63), and an ethylene-responsive transcription factor RAP2-4 (UniProt accession: A0A317YFY6) were uniquely upregulated or differentially expressed in the stressed transgenic plants (Figures 5 and 6; Supporting Information Files S1—S4). Dehydration-responsive element binding genes (DREBs) are key plant TFs that regulate the expression of several stress-inducible genes, mostly in an ABA-independent manner, and play a vital role in improving osmotic stress tolerance in plants by interacting with a DRE/CRT cis-element present in the promoter region of numerous genes (Lata and Prasad 2011; Hussain et al. 2021). The APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family TFs are known as key regulators of various stress responses, forming interconnected stress regulatory networks and can respond to plant growth regulators, specifically ABA and ethylene, to improve plant survival during stress conditions (Xie et al. 2019). Specifically, the ETHYLENE RESPONSIVE RAP2.4 protein is capable of binding to both the ethylene-responsive

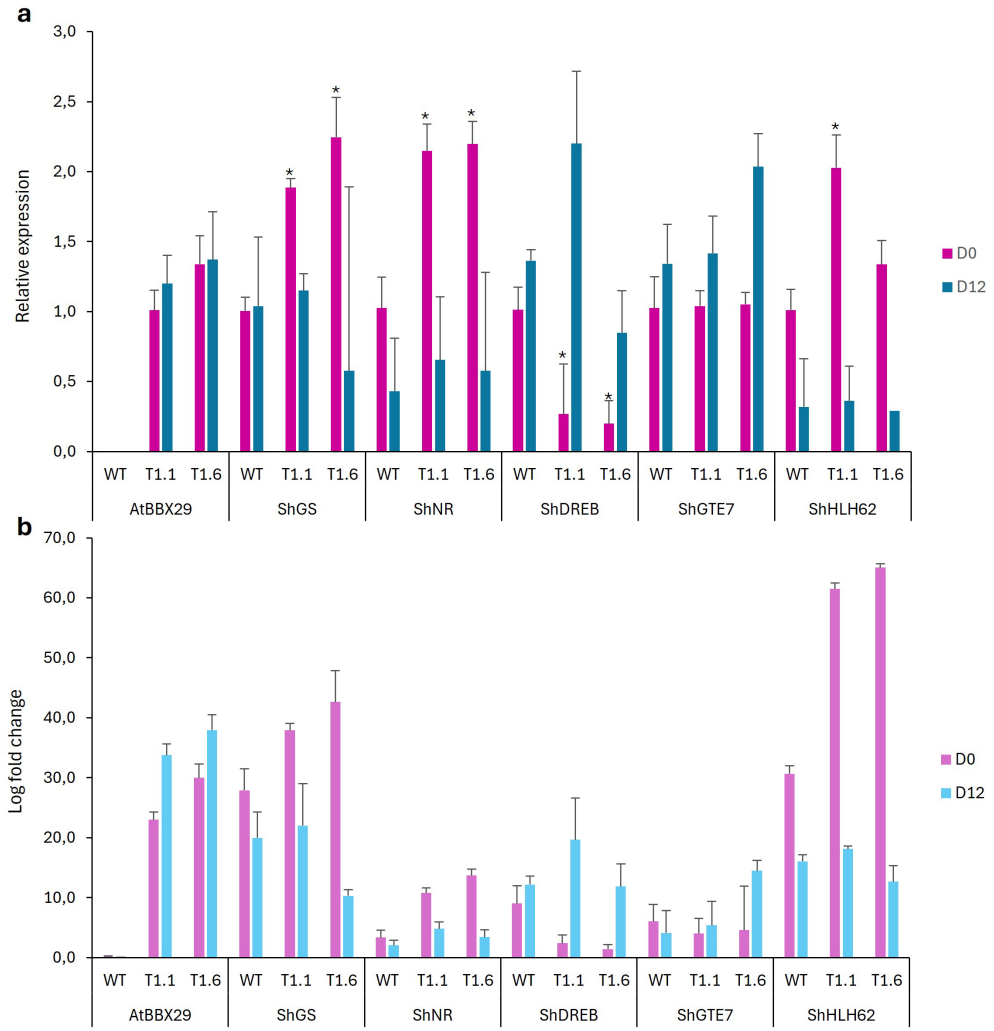

FIGURE 6 | Gene expression validation. (a) Quantitative real-time PCR (qPCR) analysis in comparison to the (b) estimated ratio of RNA production using trimmed mean of M values (TMM; Robinson and Oshlack 2010) values. Leaf transcriptional changes are represented for both transgenic (AtBBX29; T1.1 and T1.6) and WT sugarcane genotypes under well-watered (D0) and water deficit stress (D12). Data are presented as mean values with the standard error of the mean ($n = 4$). Statistical significance ($p \leq 0.05$) values compared to the non-stressed WT and indicated by the asterisk (*).

GCC-box and the dehydration-responsive element (DRE) in the promoter region of genes (Yang et al. 2020).

HEAT SHOCK FACTORS are TFs that play a critical role in plants when exposed to abiotic stresses by regulating the expression of stress-responsive genes, such as heat shock proteins (HSP; Swindell et al. 2007). In the current study, several HSP were upregulated in both the stressed WT and transgenic plants, but two HSF-TYPE DNA BINDING DOMAIN CONTAINING PROTEINS, namely UniProt accession Q109P2 and A0A804R696, were differentially expressed in the transgenic plants exposed to water deficit stress (Figure 5; Supporting Information File S4). When the stress is relieved, the HSR is decreased by surplus, mostly HSP 70 kDa (HSP70), heat shock-related proteins that repress the transcriptional activity of HSFs by binding to them and converting back to the original inactive form. Various overexpressing HSF plants are more resistant to salt, osmotic, oxidative, and anoxic stresses (Xiang et al. 2018; Ni et al. 2021; Samtani et al. 2022). Some HSFs are also known to act as ROS sensors and in ABA signaling cascades (Huang et al. 2016). As mentioned above, a few HSP70 and HEAT SHOCK COGNATES (HSC) were upregulated in all the stressed plants (UniProt accessions: A0A1D6G8N2, HSP70, A0A1B6PKD4, and A2Y5F9 HSC 70 kDA PROTEIN); some were uniquely upregulated in the stressed WT plants (A0A1D6IW15, HSP70-6 CHLOROPLASTIC, B8AC06 and A2XF47, HSC 70 KDA PROTEIN, A0A1D6PDJ4, HSP70-5) and some uniquely upregulated in the stressed transgenic plants (Q9S986, HSP70, C5YU66 and A0A194YRT0, HSP70-14 and -17; Supporting Information Files S2, S3, S4). HSP70 are known for their chaperone activity through the mediation of protein folding, translocation, and driving the movement of proteins across membranes as part of plants' defense response (Berka et al. 2022), while HSC are HS protein family members known to be expressed in the absence of heat stress (Aghaie and Tafreshi 2020). Furthermore, a GLOBAL TRANSCRIPTION FACTOR GROUP E7 (GTE7; UniProt accession A0A1B6PG63) was DE in the stressed transgenic plants. These TFs are also classified as bromodomain-containing proteins, which are highly conserved protein domains that specifically bind to acetylated lysine residues in histones, reading the chromatin epigenetic state, thereby activating transcription of target

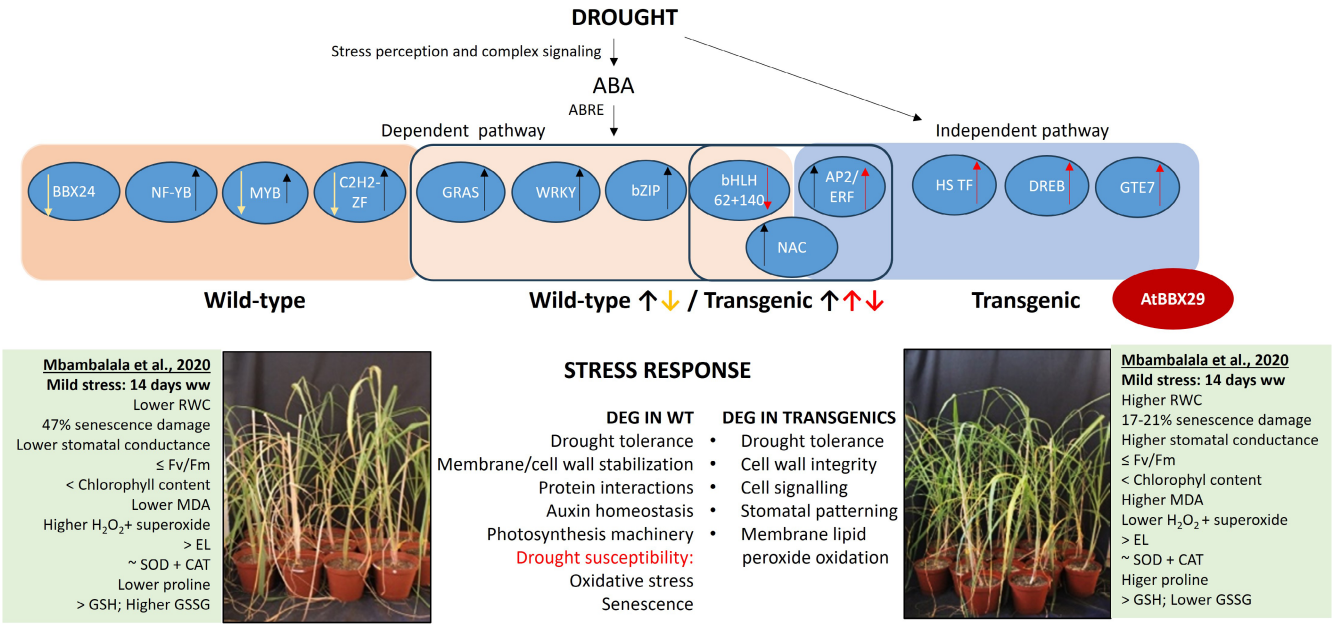

FIGURE 7 | Schematic illustration of the drought response in the WT and AtBBX29 transgenic sugarcane plants exposed to mild water deficit stress. Drought signal perception leads to activation of both abscisic acid (ABA)-dependent and ABA-independent stress response pathways, after activation of a complex stress signal transduction network. ABA-independent pathways are mostly activated in the transgenic plants. Drought tolerance is mainly attributed to reduced oxidative damage, maintenance of the photosynthetic machinery and higher levels of osmolytes. Transcription factor networks are based on published data (Yoshida et al. 2014; Singh and Laxmi 2015; Hussain et al. 2021). Physiological and biochemical analysis of the transgenic and WT plants as analyzed by Mbambalala et al. (2021).

genes (Bardani et al. 2023). Very recently, 37 of these GTE and GTE-like genes have been identified in *Saccharum spontaneum* (Jiang et al. 2023). Functional annotation of these TFs is still very limited, but the *SsGTEL3a* was shown to be significantly upregulated in drought-tolerant versus drought-sensitive sugarcane varieties exposed to stress. When overexpressed, this gene also improved the drought tolerance in Arabidopsis through improved ROS scavenging abilities (Jiang et al. 2023). These TFs have also been shown to assist in the mitotic cell cycle maintenance during development, ensuring proper leaf development, involvement in ABA signaling, hormone signaling, and upregulation under salt stress (Chua et al. 2005; Misra et al. 2018; Zhou et al. 2022; Abiraami et al. 2023).

In contrast, one C2H2-type TF was uniquely upregulated (UniProt accession: B9GCH7), and two were downregulated (UniProt accession: A0A1Z5REM2, A0A194YS73; Supporting Information File S1) in WT stressed plants. It is known that many C2H2-type zinc finger proteins enhance plant tolerance to osmotic stress through ABA-mediated signaling pathways. In addition to high salt, AZF2 (Sakamoto et al. 2004) and StZFP1 (Tian et al. 2010) respond rapidly to ABA, suggesting that they improve osmotic tolerance via an ABA-dependent pathway. In addition, several members of the MYB TF family were uniquely downregulated in the stressed WT plants. The MYB-TYPE HELIX-TURN-HELIX (HTH) domain proteins are known to bind to cis-elements of promoters of target genes. In Arabidopsis, around 51% and 41% of MYB genes are up-or downregulated, respectively, in response to drought (Katiyar et al. 2012). The MYB TFs regulate drought tolerance through the expression of genes coding for metabolites, such as flavonoids, wax, and cuticle formation. Relatedly, in transgenic Arabidopsis plants overexpressing the *AtBBX29* gene, MYB12 linked to flavonoid biosynthesis, was upregulated in a light-dependent manner (Medina-Fraga et al. 2023). These TFs can also participate in stomatal movement, mainly through ABA signaling. In addition, the transcriptional activity and protein stability of MYB proteins is known to be regulated by post-translational modifications such as phosphorylation, which influences the stability of these proteins in response to environmental stimuli (Wang et al. 2021).

Uniquely downregulated in the stressed WT plants is the BBX24 transcript (UniProt accession: A0A3L6FYI4). In Arabidopsis, research suggested that BBX24 reduces ABA sensitivity, leading to improved abiotic stress tolerance (Chiriotto et al. 2023). Overexpression of *Camellia oleifera BBX24* in Arabidopsis positively regulated drought tolerance through delayed leaf senescence (Liu et al. 2023). Downregulation of this TF in the WT sugarcane analyzed in this study might have contributed to the leaf senescence seen in these plants under water deficit stress (Figure 7). The basic Helix–Loop–Helix 62 and 140 (bHLH62/140; UniProt accessions: A0A1D6I7Y0, A0A1D6ESM3, and A0A1D6JZE9) TFs were the only ABA-dependent pathway TF that was uniquely downregulated in the stressed transgenic plants (Figure 7; Supporting Information Files S2 and S3). The bHLH TF family is the second largest family in plants after the MYB TF family, and recently, 37 (101 total alleles) bHLH genes were identified in sugarcane (Ali et al. 2021). The specific function of the bHLH62 and 140 genes is unclear, but bHLH-domain containing proteins, in general, have diverse functions in cellular differentiation and development (Chezem and Clay 2016), light signal transduction and photo-morphogenesis (Gallemi and Martinez-Garcia 2016), hormone-mediated signaling (Kazan and Manners 2013; Goossens et al. 2017), secondary metabolism (Alessio et al. 2018), and are differentially regulated in response to abiotic stresses such as salinity, drought, low temperature, and nutrient deficiency (Zhang et al. 2009; Sun et al. 2018; Radani

et al. 2023). Lastly, in the transgenic sugarcane, a DEG coding for the GLYCINE-RICH A3 PROTEIN (A0A8T0U219) was identified. In plants, GLYCINE-RICH PROTEINS (GRPs) are characterized by the presence of a glycine-rich domain consisting of Gly-X repeats. In several plant species, the expression of these genes is regulated by abiotic factors, and RNA binding is involved in post-transcriptional regulation of target transcripts. For example, the expression of the *GRP3* gene in rice enhanced drought tolerance by regulating ROS-related genes (Shim et al. 2021). The Gly-rich protein identified in this study contains an intrinsically disordered region (aa 1–111), which is known to act in itself as an RNA binding domain (Järvelin et al. 2016).

One defense system employed by plants to survive water deficit stress involves the regulation of ROS (Singh et al. 2019). The equilibrium between the detoxification and generation of ROS is maintained by both enzymatic and non-enzymatic antioxidant defense systems under abiotic stress (Hasanuzzaman et al. 2020). Mbambalala et al. (2021) reported that the AtBBX29 transgenic plants accumulated less ROS under mild and severe stress, and higher SOD and catalase activity under severe water deficit stress when compared to the WT plants.

In the stressed sugarcane transgenic plants, compared with the stress WT plants, a DEG encoding for GLUTATHIONE S-TRANSFERASE (GST) N-terminal domain-containing proteins (UniProt accession: A0A1B6QNZ1) was identified, while six (UniProt accessions: A0A0P0V328, A0A804LD57, C5X303, A0A1D6GZI4, A0A3L6F483, A0A1D6H4U6) GLUTATHIONE TRANSFERASES (F9 and T1) were significantly upregulated in the stressed versus unstressed transgenic plants (Supporting Information Files S2 and S3). Only one GLUTATHIONE TRANSFERASE (UniProt accession: C5Z557) was significantly upregulated in the stressed WT compared with the unstressed WT plants (Supporting Information File S1). GLUTATHIONE S-TRANSFERASES are ubiquitous enzymes encoded by a large gene family with a wide range of cellular functions, catalyzing a wide range of reactions involving the conjugation of glutathione (GSH). Glutathione is a small intracellular thiol molecule that is believed to be a strong non-enzymatic antioxidant (Hasanuzzaman et al. 2017). Their involvement in the detoxification of endogenous or xenobiotic compounds and oxidative stress metabolism, and the regulation of redox homeostasis in plants under adverse environments, such as drought, is well known (Choudhury et al. 2017; Estevez and Hernandez 2020). For example, ectopic expression of the *CsGSTU8* gene from the tea plant in Arabidopsis resulted in enhanced drought tolerance in the transgenics with improved scavenging of excess amounts of ROS under drought conditions (Zhang et al. 2021). The identified GST proteins in the sugarcane could also play a role in glutathionylation. This is a reversible post-translational modification where a disulfide is formed between a free thiol of a protein and glutathione, which can also protect proteins from oxidation and modify their activity. This PTM mostly occurs in response to the excessive accumulation of ROS or an increase in oxidized glutathione (Michelet et al. 2005).

4.2 | Photosynthesis

One of the traits that the transgenic plants displayed, likely contributing toward the higher level of tolerances, was the better protection of their photosynthetic machinery (Mbambalala et al. 2021). Mapping of the DEGs between the stressed transgenic (T1.1 and T1.6) and WT plants in this study indicated the upregulation of metabolic pathways linked to photosynthesis (Table 1). According to KEGG pathway analysis, these DEGs play a role in photosystem I and II, photosynthetic electron transport, and ATPase (Figure 3).

When water is limited, plant leaves may lose turgor and become wilted, curled, and stomata close due to higher levels of ABA, which decreases CO_2 influx and thus limits photosynthesis yield (Medrano et al. 2002). Photosystem II, as the start point for the photosynthetic chain, captures photons and uses the energy to extract electrons from water molecules. Photosystem I receives electrons from the chloroplast electron transport chain and uses light energy to transfer them across the membrane to ferredoxin on the stromal side (Tikkanen et al. 2014). Both photosystems are affected by drought, although at different stages. The operative quantum efficiency of PSII (ΦPSII) is very sensitive to drought and is used as a parameter for early detection of drought stress. As water becomes scarce, PSII antenna and light-harvesting complex II (LHCII) detach from PSII supercomplexes and degrade if the water limitations persist (Huang et al. 2019). Under severe drought stress, the PSI antenna size is reduced, the PSI-LHCI super complexes disassemble, and non-functional PSI protein complexes form (Hu et al. 2023).

The involvement of BBX genes in the photosynthetic processes has been documented; for example, potatoes overexpressing the *AtBBX21* gene had higher rates of photosynthesis with a significant increase in photosynthetic gene expression and reduced photoinhibition compared to WT plants (Crocco et al. 2018). In the current study, transcripts coding for PsaO, PsaE, PsaL, PetE, PetF, and ATPF1D (Uniprot accessions: A0A067YFZ8, A0A811R298, B6T097, A0A811SC03, A0A1B6Q1J7, A0A811R8A1, A0A811PKV0, A0A811PR24, and A0A811PCP5), mainly involved in PSI (Figure 3), were downregulated in the stressed transgenic plants (Supporting Information Files S2 and S3). In addition, in the stressed plants, genes coding for PHOSPHOENOLPYRUVATE CARBOXYLASES (PEPC) and CHLOROPHYLL A–B BINDING PROTEINS were downregulated, while several GLYCOSYLTRANSFERASES were upregulated in the WT plants. However, some transcripts encoding PHOSPHOENOLPYRUVATE CARBOXYLASES (UniProt accession: A0A804R6J4) and a PSII_PBS31 DOMAIN-CONTAINING PROTEINS (A0A8T0MCL6) were DE in the stressed transgenic plants compared with the stressed WT plants (Figure 3; Supporting Information File S1). This variation in gene expression likely contributed to the maintenance of the photosynthetic machinery in the stressed transgenic plants compared to the WT plants (Figure 7).

The transcriptomic analysis of the stressed transgenic and WT sugarcane plants also indicated differential expression of transcripts linked to N metabolism (Figure 5). In rice, N metabolism has been associated with the tolerance of photosynthesis to osmotic stress through changes in CO_2 diffusion, antioxidant capacity, and osmotic adjustment (Zhong et al. 2017). In this study, KEGG pathway analysis indicated that *NITRATE REDUCTASE* was downregulated in the stressed transgenic plants, also verified by the qPCR analysis (Figure 6), which was not seen in the

stressed WT plants (Table 1; UniProt accession: A0A317Y6B2). In addition, *GLUTAMINE SYNTHETASE* (UniProt accession: A0A1D6JTN2) was DE in these stressed transgenic plants compared with the stressed WT plants (Figure 6; Supporting Information File S4). The reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which is then reduced to ammonia (NH_4^+), is catalyzed by NITRATE REDUCTASE (NR) and finally assimilated into the amino acids and the nitrogen compounds of the cell. High concentrations of ammonium are toxic to plant cells (Hoai et al. 2003), and the high capacity to assimilate ammonium could be an important factor in alleviating the consequences of stress. Photosynthesis is recognized as one of the most efficient ways to increase nitrogen use efficiency (NUE) and crop yield (Kumar et al. 2006). Therefore, the majority of assimilated N in plants is invested in the photosynthetic machinery (Nunes-Nesi et al. 2010), and N is quite strongly positively linked with the photosynthetic rate (Makino et al. 2003). GLUTAMINE SYNTHETASE (GS) and GLUTAMATE SYNTHETASE (GOGAT) are the essential enzymes for the integration of inorganic N into amino acids in the cell, bridging carbon and nitrogen metabolism. Therefore, GS is involved in the pathway of ammonium assimilation and reassimilation (Zhong et al. 2017). This enzyme has been documented as a metabolic indicator of drought stress tolerance in cereal crops (Nagy et al. 2013). The adapted N metabolism seen in the AtBBX29 transgenic sugarcane plants might contribute to N homeostasis and enhance water deficit tolerance seen in these plants.

Many plant species have been reported to accumulate amino acids in response to drought stress (Fabregras et al. 2019). In the current study, most of the amino acid metabolism pathways (KEGG) were more upregulated in the WT plants than in the transgenic plants (Table 1). Amino acids play a vital role in drought stress adaptation by operating as osmolytes, controlling ion transport, modulating stomatal opening, enzyme synthesis, and influencing gene expression and redox homeostasis (Hammad and Ali 2014; Shim et al. 2023). An amino acid metabolic pathway that was uniquely upregulated in the transgenic plants was cyanoamino acid metabolism (Table 1). In the cyanoamino acid metabolism pathway, tyrosine and phenylalanine biosynthesis were upregulated in transgenic plants. Cyanoamino acid metabolism is involved in the biosynthesis of cyanogenic glycosides, which are known to act as a defense mechanism against herbivores and pathogens (Jeon et al. 2023). In addition, it has been suggested that the amino acids in this pathway may protect plant membranes from excess water loss by causing stomatal inhibition during water stress (Rai 2002; Laursen et al. 2016; Ackah et al. 2021).

5 | Conclusions

Overall, plant interaction with the environment is regulated by a multigenic response. This was also observed in the sugarcane plants, where numerous genes and their isoforms were differentially expressed in both WT and transgenic plants overexpressing the *AtBBX29* gene when exposed to water deficit stress, compared to their unstressed counterparts. However, not all DE transcripts could be identified, and further analysis will be required to characterize these. Stress perception and signaling responses were largely similar between WT and transgenic plants under stress. However, differentially expressed transcripts, particularly those involved in stress signaling and transduction through TFs in the ABA-independent pathway (such as HS, DREB, and GTE7), were upregulated in *AtBBX29* transgenic plants. This suggests that the encoded AtBBX24 protein may potentially bind to the promoter regions of other TFs involved in the ABA-independent stress response, a novel possibility that warrants further investigation. In contrast, ABA-dependent stress response pathways were affected in both WT and transgenic plants. Some TFs within this pathway, including BBX24, certain MYB family members, and C2H2 ZF proteins, were specifically downregulated in the WT plants. Furthermore, genes associated with senescence (SENESCENCE-ASSOCIATED PROTEINS; UniProt accessions: A2YVP0) were uniquely upregulated in the stressed WT plants, which may explain the delayed senescence observed in the transgenic plants. Protection against water deficit stress in transgenic plants was also supported by enhanced antioxidant accumulation, evidenced by the upregulation of GST domain-containing proteins (UniProt accessions: A0A1B6QNZ1; and A0A0P0V328, A0A804LD57, C5X303, A0A1D6GZI4, A0A3L6F483, A0A1D6H4U6). Further investigation into the role of the GST gene family in enhancing drought tolerance in sugarcane will be a focus of future studies. Differentially expressed genes in the energy metabolism KEGG pathways, map00195 (photosynthesis) and map00910 (nitrogen metabolism), will also be key targets for elucidating the mechanisms underlying AtBBX29-mediated control of photosynthetic integrity.

Author Contributions

Christell van der Vyver and Sanjib K. Panda conceived and designed the experiments; Christell van der Vyver, Preetom Regon, and Kristen Fulton performed the experiments, analyzed the data, and wrote the paper; Christell van der Vyver and Sanjib K. Panda improved the paper. All authors have read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw data presented in the study are publicly available. These data can be found here: Bioproject: PRJNA1127307, Biosample: SAMN41999931, SAMN41999932, and SAMN41999933.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.