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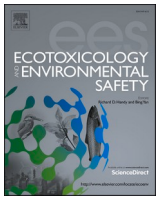

Exogenous 24-epibrassinolide boosts plant growth under alkaline stress from physiological and transcriptomic perspectives: The case of broomcorn millet (*Panicum miliaceum* L.)

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ABSTRACT

Land alkalization is an abiotic stress that affects global sustainable agricultural development and the balance of natural ecosystems. In this study, two broomcorn millet cultivars, T289 (alkaline-tolerant) and S223 (alkaline-sensitive), were selected to investigate the response of broomcorn millet to alkaline stress and the role of brassinolide (BR) in alkaline tolerance. Phenotypes, physiologies, and transcriptomes of T289 and S223 plants under only alkaline stress (AS) and alkaline stress with BR (AB) were compared. The results showed that alkaline stress inhibited growth, promoted the accumulation of soluble sugars and malondialdehyde, enhanced electrolyte leakage, and destroyed the integrity of broomcorn millet stomata. In contrast, BR lessened the negative effects of alkaline stress on plants. Transcriptome sequencing analysis showed that relative to control groups (CK, nutrient solution), in AS groups, 21,113 and 12,151 differentially expressed genes (DEGs) were identified in S223 and T289, respectively. Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed various terms and pathways related to metabolism. Compared to S223, alkaline stress strongly activated the brassinosteroid biosynthesis pathway in T289. Conversely, ARF, TF, and TCH4, associated with cell growth and elongation, were inhibited by alkaline stress in S223. Moreover, alkaline stress induced the activation of the mitogen-activated protein kinase (MAPK) pathway, the abscisic acid signaling pathway that initiates stomatal closure, as well as the starch and sucrose metabolism. The EG and BGL genes, which are associated with cellulose degradation, were notably activated. BR enhanced alkaline tolerance, thereby alleviating the transcriptional responses of the two cultivars. Cultivar T289 is better in alkalized regions. Taken together, these results reveal how broomcorn millet responds to alkaline stress and BR mitigates alkaline stress, thus promoting agriculture in alkalized regions.

1. Introduction

Large-scale land salinization brings inevitable and severe challenges to the sustainable development of global agriculture and an ecological environment (Montanarella et al., 2015). Land salinization has hindered the agricultural development of approximately sixty million hectares of irrigated farmland worldwide, resulting in a huge and significant loss of annual agricultural income (Abdelrahman et al., 2018; Abiala et al., 2018). Planting saline-alkaline tolerant plant crops is one of the most effective strategies for solving this problem. Alkaline stress is caused by alkaline salts (mainly Na_2CO_3 and $NaHCO_3$) and includes osmotic stress, ionic toxicity, and high pH toxicity (Ma et al., 2022), resulting in different damage to plant growth and development in alkaline stress versus NaCl (Chen et al., 2021). Therefore, enhancing the tolerance of crops to alkaline stress is of great significance in the agricultural development of saline-alkaline land.

Starch and sucrose metabolism are crucial plant abiotic stress responses (Ruan et al., 2010). Starch synthesis and degradation is regulated by abiotic stress to prevent carbon starvation or unproductive carbon sequestration (Thalmann et al., 2016). Metabolites produced by

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plants breaking down starch act as osmoprotectants and contribute to the defense of osmotic or oxidative stress (Zanella et al., 2016). Sucrose regulates carbon sources to adapt to abiotic stress, via the interaction of sugar signaling molecules with other metabolic pathways (Couée et al., 2006; Miller et al., 2010; Ruan et al., 2010). Therefore, the starch and sucrose metabolism pathways are important regulatory pathways that endow plants with abiotic stress tolerance. Additionally, appropriate adjustments to starch and sucrose metabolism can promote plant adaptation under abiotic stress. Similarly, phytohormones regulate plant responses to abiotic stress and play a critical role in plant resistance and yield. The SA, JA, and ABA synergistically regulate the growth and metabolism of plants to resist harsh environments (Ali and Baek, 2020; Bharti and Garg, 2019; Chen et al., 2020). JAs enhance the water retention capacity of plants by modulating the cuticle and compounds such as proline, lignin, and polyphenols (Wasternack and Hause, 2013). ABA regulates plant antioxidant defenses to mitigate the negative effects of copper on plants (Zehra et al., 2020). Brassinolides (BR) mitigate heavy metal toxicity by improving osmolyte levels and strengthening the antioxidant defense system (Guedes et al., 2021; Shah et al., 2020; Wani et al., 2017).

Broomcorn millet (*Panicum miliaceum* L.) is a recognized future smart food, as well as an excellent stress-resistant pioneer crop (Ma et al., 2021; Siddique et al., 2021). Therefore, studying broomcorn millet tolerance and mitigation of alkaline stress is crucial. Broomcorn millet has been shown to be highly tolerant to sodium chloride (NaCl) salt stress (Yuan et al., 2021a). Our previous study has shown that a variety of broomcorn millet germplasm have excellent adaptability to alkaline stress at the germination and seedling stages (Ma et al., 2021). However, the tolerance mechanism of broomcorn millet to alkaline stress is not fully understood. Brassinolide is a growth regulator that is widely used in agricultural production and has positive effects on plants resistance to abiotic stress (Talaat et al., 2015). Many studies have demonstrated that BR increased the tolerance threshold of plants to various stresses, such as palladium, cadmium, and salt stress (Guedes et al., 2021; Shah et al., 2020; Wani et al., 2017). However, research on BR's role in plant alkaline stress alleviation is still unknown. This study aims at exploring the complex response of broomcorn millet to alkaline stress and the role of BR in alkaline tolerance. Here, this study elucidates the mechanism of broomcorn millet response to alkaline stress and provides a theoretical basis for studying alkaline-tolerant crops.

2. Materials and methods

2.1. Plant material and treatment

Two broomcorn millet (*Panicum miliaceum* L.) cultivars, S223 (alkaline-sensitive) and T289 (alkaline-tolerant) (Ma et al., 2021), provided by the Minor Grain Crop Science and Technology Innovation Team of Northwest A&F University, were selected for this study. The seedlings were subjected to alkaline stress when they reached the three leaves with one-heart stage; 24-epibrassinolide (BR) was sprayed at 9:00 a.m. and 21:00 p.m. during the alkaline stress period, continuously for five days. Seedlings were cultivated by hydroponics. Four treatment groups were set up in the study: control group with nutrient solution (CK), nutrient solution with BR (CB), alkaline stress (AS), and alkaline stress with BR (AB). The alkaline stress solution had a concentration of 40 mmol·L^{-1}, with a molar ratio of 9:1 $NaHCO_3$:Na_2CO_3, as previously described (Ma et al., 2021). The amount of BR sprayed was approximately 40 mL/m^2 with an optimal concentration of 0.5 mg·L^{-1}, selected based on a previous report and trial experiments (Liu et al., 2022).

2.2. Determination of morphological traits and biomass

After seven days of treatment, three broomcorn millet seedlings were randomly selected from each treatment to measure the plant height, stem diameter, root length, green leaf area, plant phenotype, and stomatal morphology. The fresh weight (FW) and dry weight (DW) of the underground and aboveground parts of the seedlings were determined.

2.3. Determination of soluble sugar monomer content, malondialdehyde, and electrolyte leakage in leaves

Glucose, fructose, maltose, and sucrose content were determined by high performance liquid chromatography-mass spectrometry (HPLC-MS) (Vanquish & Q Exactive, Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Xu et al., 2020). Malonaldehyde (MDA) content and electrolyte leakage in the leaves were determined as described by Ma et al. (2021).

2.4. Field-emission scanning electron microscopy

Fresh leaves were harvested on the seventh day of treatment and rinsed with sterile water. Next, they were soaked in 4% glutaraldehyde for at least 6 h at 4 °C. The samples were then dehydrated and dried with solutions of increasing ethanol concentrations. The dried samples were sputtered with a 60:40 gold/palladium ratio and observed under a scanning electron microscope (S-4800, Hitachi, Tokyo, Japan) (Yuan et al., 2021b).

2.5. Transcriptome sequencing

Seedling leaves under different treatments were collected for transcriptome sequencing. Three biological replicates were used for each treatment group. Total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen, Beijing, China). Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess RNA concentration and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was selected to assess RNA quality. Transcriptome libraries were subjected to rRNA depletion using the TruSeqTM RNA sample preparation and Ribo-Zero Magnetic kits (Illumina, San Diego, CA, USA). Subsequently, the libraries were sequenced on an Illumina Novaseq 6000 system (Illumina, San Diego, CA, USA) to generate 150 bp paired-end reads. Low-quality reads were removed, and adapter sequences were filtered from raw reads using the FASTX toolkit (v 0.0.14). The sequence quality was assessed using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Yuan et al., 2021b). The cleaned and filtered reads were subsequently mapped to the *Panicum miliaceum* L. reference genome downloaded from the NCBI database using Tophat v2.0.10 (Trapnell et al., 2010). Aligned reference-based reads were assembled using Cufflinks pipeline (v2.1.1) (Trapnell et al., 2012). Transcript expression levels were calculated using the fragments per kilobase of exon model per million mapped reads (FPKM). Differentially expressed genes were identified using DESeq2 (v 1.24.0) with a false discovery rate (FDR) of < 0.05 and log2 | fold-change| ≥ 1. Differentially expressed genes (DEGs) in the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified using the GOseq R package (v1.12) and KEGG Orthology Based Annotation System software (KOBAS, v2.0), respectively.

2.6. Statistical analysis

The data were analyzed by one-way ANOVA using IBM SPSS 23.0 (Armonk, NY, USA). The means of different treatments were compared using Duncan's test ($P < 0.05$). Correlations between treatments were analyzed using Pearson's test. The figures were drawn using Origin Pro 2020 (OriginLab, Northampton, MA, USA), Adobe Illustrator CC 2019 (Adobe, USA), and the Gene Denove Bioinformatics Cloud Platform (https://www.omicshare.com/?lang=en).

3. Results

3.1. Effect of 24-epibrassinolide on growth and phenotype of seedlings under alkaline stress

Comparison of the growth status of broomcorn millet seedlings with different treatments after seven days of treatment showed the growth status of the seedlings in AB were considerable better than that in AS (Fig. 1A). Our data revealed that the plant height of T289 significantly exceeded that of S223 under alkaline stress, but no difference was observed between that of S223_AB and T289_AB. Moreover, the stem diameter in the AB groups significantly exceeded that in the AS groups. Additionally, root length of S223_AB significantly exceeded that of S223_AS. Further, BR significantly increased the green leaf area of T289 and S223 plants under alkaline stress (Fig. 1 B). From the aboveground and underground data, under alkaline treatment, the application of BR significantly enhanced the fresh weight of the aboveground and underground parts; therefore, those of S223_AB and T289_AB were significantly higher than those of S223_AS and T289_AS (Fig. 1 C). Comparable results were observed for the dry weight.

3.2. Effect of 24-epibrassinolide on soluble sugar component and membrane lipid peroxidation in leaves under alkaline stress

The application of BR significantly reduced the levels of glucose, fructose, maltose, and sucrose in the leaves of broomcorn millet seedlings exposed to alkaline stress. The effect of alkaline stress was more severe on S223 than on T289, and the highest glucose, fructose, maltose, and sucrose contents were found in S223_AS. Glucose was the most sensitive to alkaline stress, and the content of S223_AS and T289_AS increased 39.85- and 14.73-fold, respectively, compared with S223_CK and T289_CK. respectively. Under alkaline stress, BR reduced the glucose content of S223 and T289 by 67.85% and 26.11%, respectively (Fig. 2 A). BR significantly reduced the accumulation of MDA in seedling leaves under alkaline stress but had no effect on leaves without alkaline stress. Among all the treatments, S223_AS exhibited the highest malondialdehyde (MDA) content, followed by T289_AS, S223_AB, and T289_AB. In addition, BR application effectively reduced the electrolyte leakage rate of seedlings under alkaline stress. Compared with AS treatment groups, the electrolyte leakage rate of AB-treated groups of S223 and T289 decreased by 27.86% and 13.6%, respectively (Fig. 2 B).

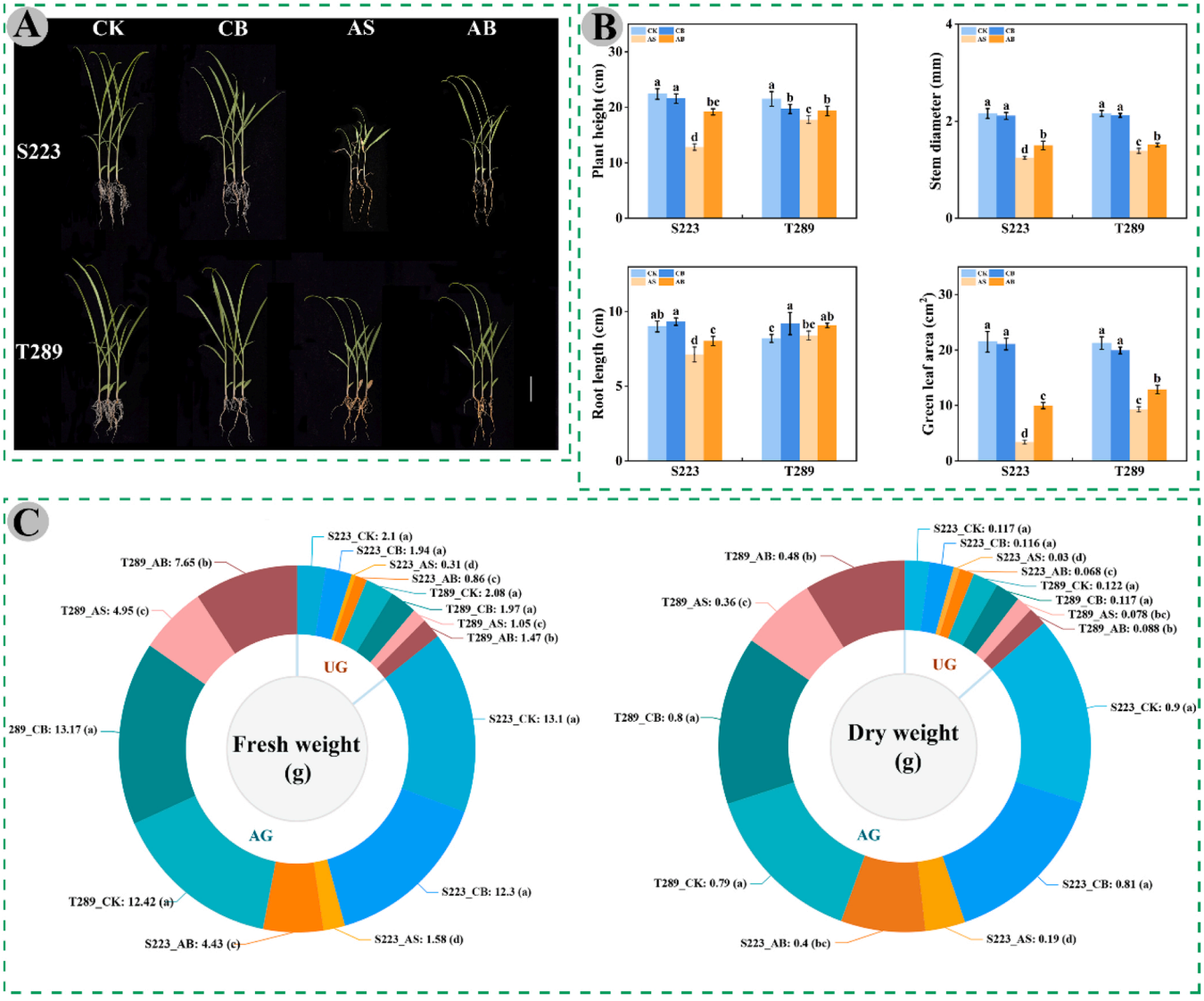

Fig. 1. Phenotypic differences and dry matter accumulation of broomcorn millet under different treatments. (A): the growth state of different treatments; (B): the plant height, stem diameter, root length, and green leaf area; (C): Fresh and dry weight of aboveground and underground parts. CK presents pure nutrient solution, CB presents nutrient solution + 0.5 mg·L^{-1} BR spray, AS presents alkaline stress, and AB presents alkaline stress + 0.5 mg·L^{-1} BR spray. AG presents aboveground, UG presents underground.The data represent the mean ± SD (n = 5 in B, and n = 10 in C). Different letters indicate significance at $P < 0.05$.

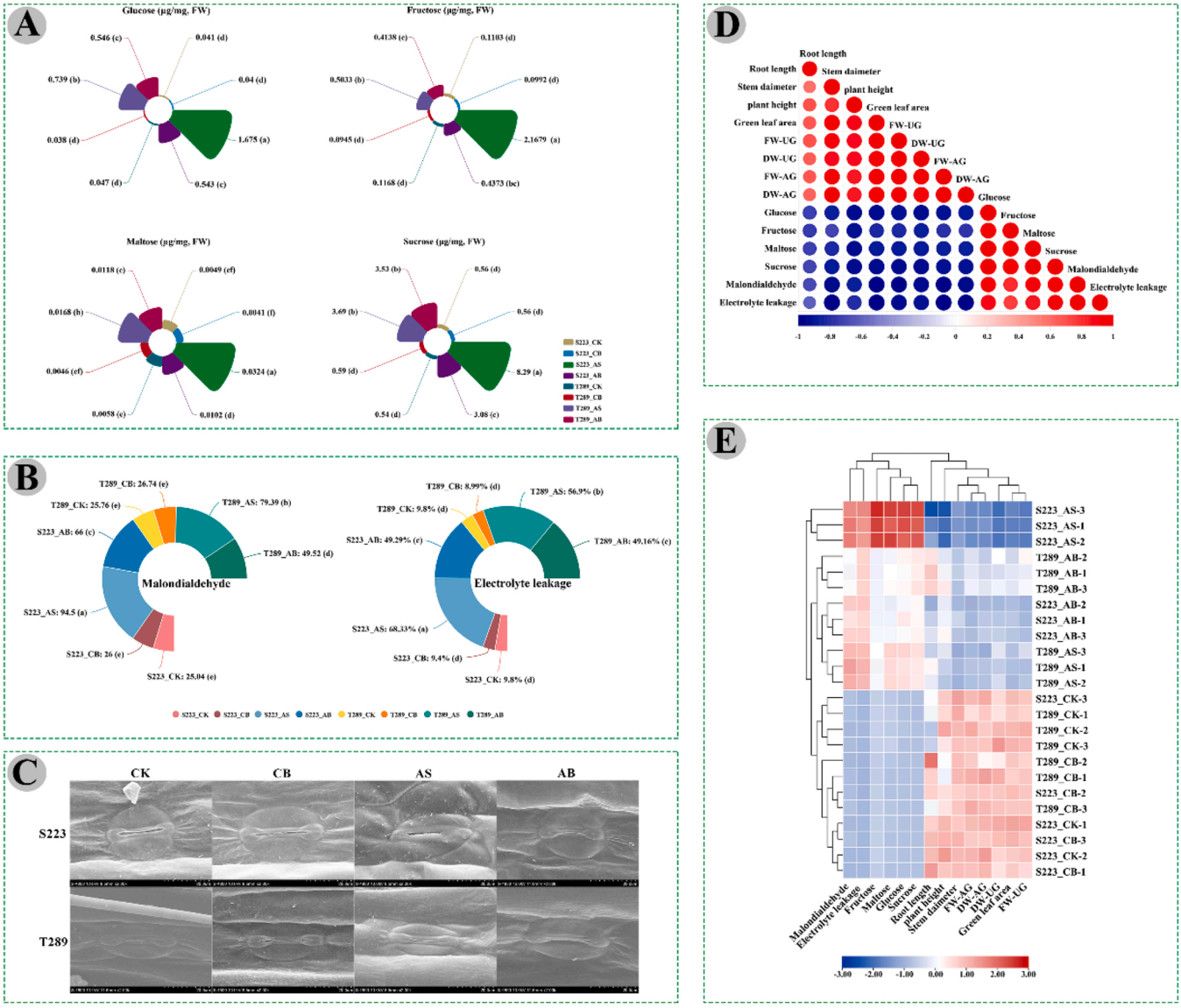

Fig. 2. Physiological indices study and chemometrics analysis for S223 and T289 under different treatments. (A): Soluble sugar monomers; (B): malondialdehyde content and electrolyte leakage rate; (C): differences in stomatal morphology; (D): Pearson`s correlation analysis of physiological indicators; (E): cluster heatmaps of physiological indicators and different treatments. CK presents pure nutrient solution, CB presents nutrient solution + 0.5 mg·L^{-1} BR spray, AS presents alkaline stress, and AB presents alkaline stress + 0.5 mg·L^{-1} BR spray. The data represent the mean ± SD (n = 3). Different letters indicate significance at $P < 0.05$.

These results demonstrate that BR protects leaves' cell membrane integrity under alkaline stress.

3.3. Effects of 24-epibrassinolide on the morphology of stomatal tissue in seedling leaves

Alkaline stress significantly affected the stomatal morphology of seedlings. In CK and CB groups of S223 and T289, guard cells and accessory cells of the stomata were full and swollen, and they maintained typical tissue morphology. In contrast, scanning electron microscopy showed that S223_AS seedlings were affected by alkaline stress, evident in the severely damaged stomatal morphology, which was disintegrated and deformed. Interestingly, BR application protected stomatal morphology under alkaline stress, but the stomata were closed. Compared with S223_AS, the stomata of T289_AS seedlings were closed but maintained intact morphology (Fig. 2 C). These results imply that BR effectively protects the morphological and structural integrity of the stomata under alkaline stress.

Correlation analysis and cluster analysis based on physiological data

The results of the correlation analysis of fourteen indicators (S. table 1) are shown in Fig. 2 D. Physiological indicators of the growth parameters showed significant negative correlations. Cluster analysis was performed on alkaline-stressed samples and physiological indicators according to normalized physiological data (Fig. 2 E). The 24 samples were divided into three clusters, and the 14 indicators were classified into two clusters. Cluster analysis revealed that the phenotypic differences among the samples were caused by alkaline stress.

3.4. Alkaline stress and 24-epibrassinolide induced transcriptomic responses in two broomcorn millet cultivars

Transcriptomic changes of the two cultivars under different treatments were compared. A total of 51,225,530–79,541,704 clean reads were obtained from all the samples by transcriptome sequencing (S. table 2). Q20 and Q30 bases exceeded 97.24% and 92.67%, respectively. The GC content ranged from 55.60% to 57.88%. The percentage of

mapped reads in each library ranged from 95.07% to 97.26%, and the range of unique mapped reads ranged from 92.04% to 93.77%. The cluster analysis results of DEGs of all samples were consistent with the clustering results of the physiological indicators above (S. Fig. 1 A). Principal component analysis (PCA) (S. Fig. 1 B) and Spearman correlation coefficient analysis (SCC) (S. Fig. 1 C) showed BR did not cause the separation of T289 AS and AB groups, but caused the separation of S223 groups, indicating that BR had a considerably greater effect on the alkaline-sensitive broomcorn millet cultivar. As in Fig. 3 A, 21,113 DEGs (11,146 upregulated and 9967 downregulated DEGs) were identified in S223_AS vs S223_CK, and 12,151 DEGs (7223 upregulated and 4928 downregulated DEGs) were identified in T289_AS vs T289_CK. Under alkaline stress, BR treatment caused 12,334 DEGs (5413 upregulated and 6921 downregulated) in S223 and 4261 DEGs (489 upregulated and 3772 downregulated) in T289. Moreover, Venn diagram analysis shows 10,978 and 3223 overlapping DEGs in the between-group comparison of S223 and T289, respectively.

3.5. GO functional classification and KEGG pathway functional analysis of DEGs

GO enrichment analysis was performed to classify and explore the potential functions of the DEGs in the comparative combinations of AS vs CK and AB vs AS, in S223 and T289 (S. table 3 and Fig. 3 B). GO results showed that the DEGs were mainly involved in molecular function (MP) and biological process (BP). For S223, the application of BR facilitated functional annotation of the generation of precursor metabolites and energy, carbohydrate metabolic process, and amino acid transport. For T289, BR promoted the functional annotation of protein phosphorylation, cellular protein modification process, and amino acid transport.

Fig. 3. Analysis of DEGs under different treatments. (A): Volcano and Venn diagrams of differentially expressed genes for AS vs CK and AB vs AS of S223 and T289. Green dots represent down-regulated genes and red dots represent up-regulated genes in the Volcano plot. (B): GO enrichment analysis. Blue and orange represent molecular function (MF) and biological process (BP), respectively. The first lap indicates the top 20 GO terms. The second lap indicates the number of this category in the background gene and P-values for gene enrichment for the specified GO terms, the more genes, the longer the bar, and the smaller the value, the redder the color. The third lap indicates bar graph of enriched genes. The fourth lap indicates the up- and down-regulated gene number of the enriched genes corresponding to the third circle. The fifth lap represents the rich factor of each category, and each cell of the background auxiliary line represents 0.1. GO represents gene ontology. (C): KEGG pathway enrichment analysis. Purple, green, blue, dark green, and dark blue represent metabolism (M), genetic information processing (GIP), organismal systems (OS), cellular processes, and environmental information processing (EIP), respectively. The first lap indicates the top 20 KEGG pathways. The second lap indicates the number of this category in the background gene and P-values for gene enrichment for the specified GO terms, the more genes, the longer the bar, and the smaller the value, the redder the color. The third lap indicates bar graph of enriched genes. The fourth lap indicates the gene number of the enriched genes corresponding to the third circle. The fifth lap represents the rich factor value of each category, and each cell of the background auxiliary line represents 0.1. KEGG represents Kyoto encyclopedia of genes and genomes.

KEGG enrichment analysis (S. table 4 and Fig. 3 C) showed that these DEGs were mapped to five biological pathways: metabolism, genetic information processing, organic systems, cellular processes, and environmental information processing. The top 20 KEGG pathways were analyzed, and the results showed that BR significantly enriched the biosynthesis of secondary metabolites, carbon metabolism, photosynthesis, and glutathione metabolism of S223 under alkaline stress. Additionally, eight metabolisms in T289 (alpha-linolenic acid metabolism, glutathione metabolism, isoquinoline alkaloid biosynthesis, tyrosine metabolism, fatty acid degradation, beta-alanine metabolism, linoleic acid metabolism, and phenylalanine metabolism) and three environmental information processes (ABC transporters, mitogen-activated protein kinase (MAPK) signaling pathway, and plant hormone signal transduction) were significantly enriched. These results indicated that different alkaline-tolerant broomcorn millets adopt different measures to cope with alkaline stress. Moreover, BR specifically regulated the adaptability of different broomcorn millet genotypes to alkaline stress.

3.6. Transcriptional differences in steroid hormone biosynthesis and brassinosteroid biosynthesis in broomcorn millet leaves

A total of 62 genes were identified to be involved in steroid hormone biosynthesis and brassinosteroid biosynthesis in broomcorn millet leaves (S. table 5 and Fig. 4). Among them, 38 genes encoded DWF1, CYP51, EBP, and the other 13 enzymes were involved in steroid hormone biosynthesis. Alkaline stress enhanced the expression of genes encoding the above-mentioned enzymes in S223, especially TGL4, DWF1, SMO1, CPI, and CYP710A, but did not significantly affect the expression levels of these genes in T289. The expression level of genes encoding TGL4 were strongly affected by alkaline stress and BR. Further, the expression level of genes encoding CYP710A changed significantly in S223 but were almost unaffected in T289 (Fig. 4).

Moreover, 24 DEGs were identified to be involved in the brassinosteroid biosynthesis pathway (S. Table 5 and Fig. 5). Alkaline stress disrupted the expression of most of the 24 DEGs. For S223, almost all genes were down-regulated by alkaline stress, except for certain genes encoding BR6OX1, CYP92A6, and BAS1. BR spraying significantly upregulated the expression of genes encoding CYP90A1, BR6OX1, and BAS1 in S223 leaves. However, the application of BR significantly downregulated the expression of genes encoding BR6OX1, CYP92A6, and BAS1 in T289. These results suggested that alkaline stress induces S223 and T289 to initiate a specific transcriptional program to defend against alkaline damage. Additionally, the application of 24-epibrassinolide provided the requirement of hormones in the leaves of broomcorn millet, thus reducing the expression of related genes in the biosynthetic pathway.

3.7. Transcriptional differences in plant hormone signal transduction and MAPK signaling pathways in broomcorn millet leaves

DEGs were identified in plant hormone signal transduction and MAPK signaling pathways (S. table 5 and Fig. 6). Under alkaline stress, the gene encoding AUX1 was downregulated in S223 and partially upregulated in T289. Of the AUX/IAA genes, eight and sixteen genes were significantly up- and downregulated, respectively, in S223.

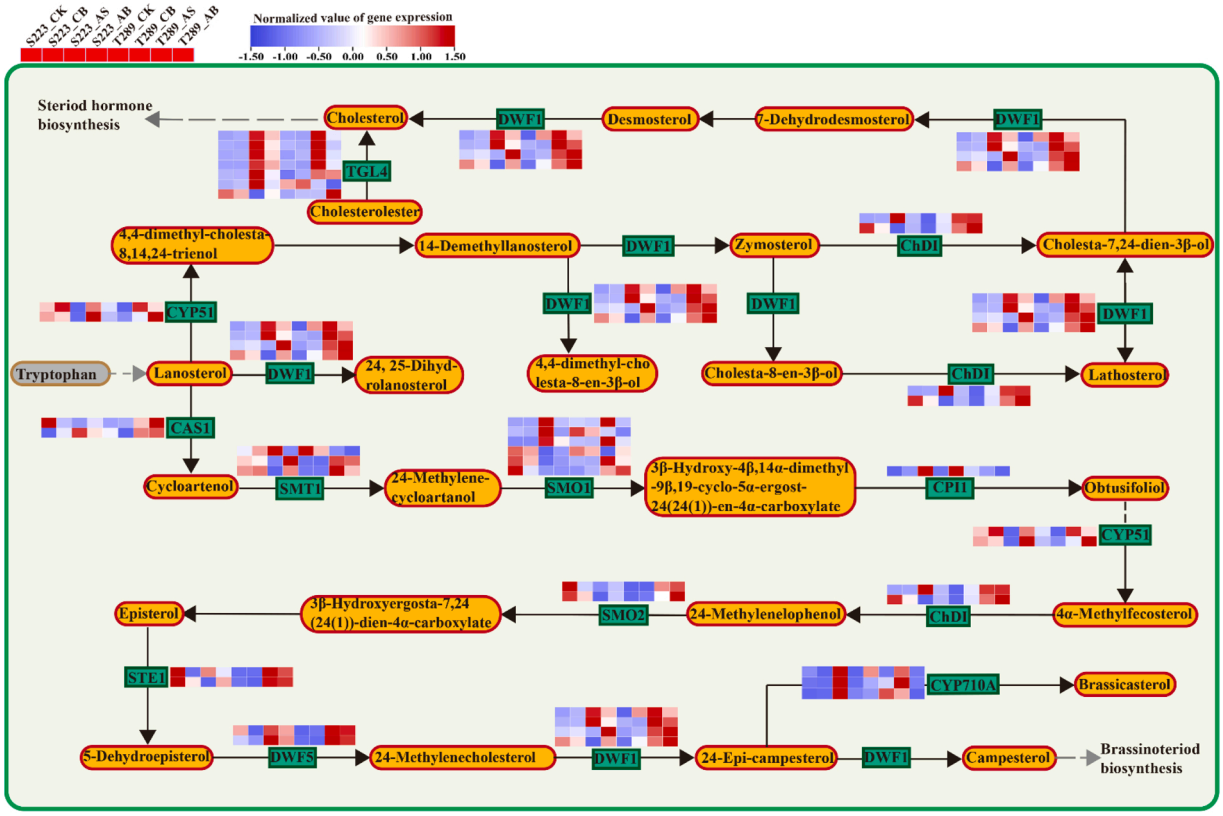

Fig. 4. Steroid biosynthesis of S223 and T289 under different treatments. The color block represents the normalized value of gene expression. Purple and Red indicate significantly downregulated and upregulated genes, respectively. For enzyme reactions, the arrows between two metabolites represented the directions of catalytic reactions. The transporter proteins and transcriptional factors are represented in green rectangles, whereas metabolites are in orange ellipses. DWF1, Delta24-sterol reductase; CYP51, sterol 14alpha-demethylase; ChDI, cholestenol Delta-isomerase; STE1, delta7-sterol 5-desaturase; CAS1, cycloartenol synthase; SMT1, sterol 24-C-methyltransferase; SMO1, plant 4,4-dimethylsterol C-4alpha-methyl-monooxygenase; CPI1, cycloeucalenol cycloisomerase; SMO2, plant 4alpha-monomethylsterol monooxygenase; CYP710A, sterol 22-desaturase; TGL4, TAG lipase / steryl ester hydrolase / phospholipase A2 / LPA acyltransferase; DWF5, 7-dehydrocholesterol reductase.

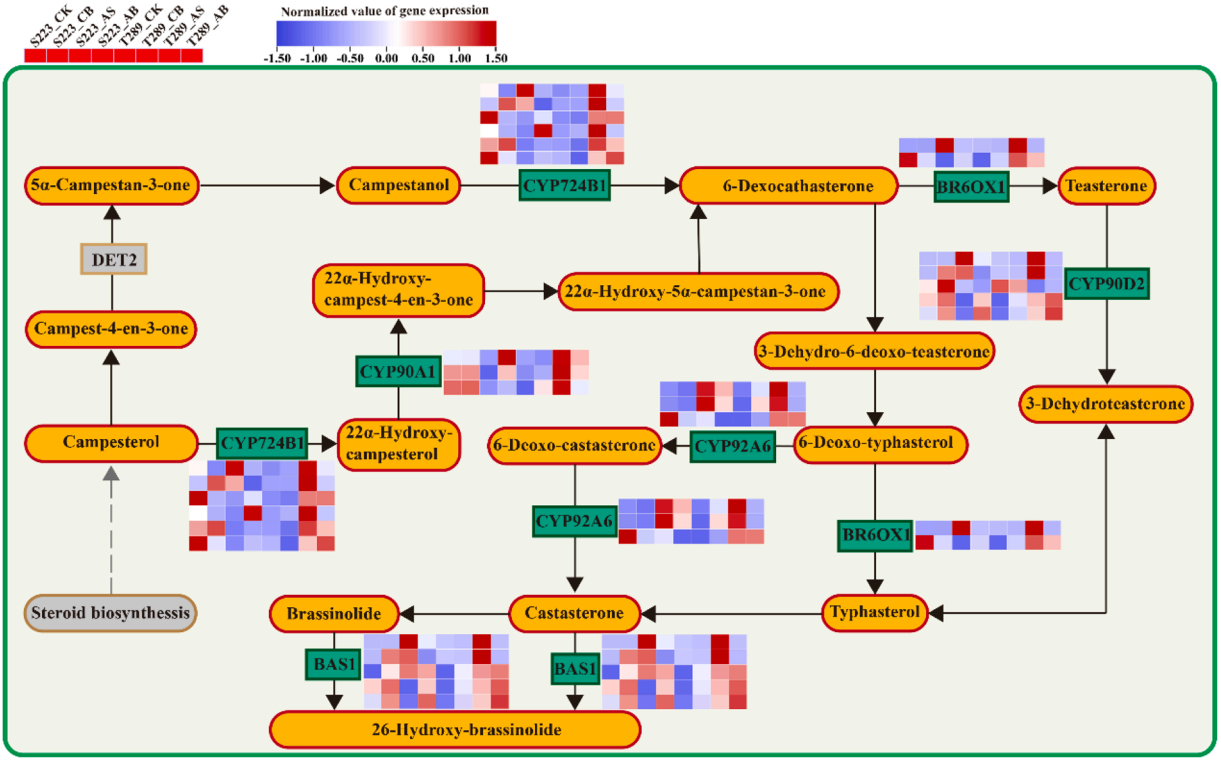

Fig. 5. Brassinosteroid biosynthesis of S223 and T289 under different treatments. The color block represents the normalized value of gene expression. Purple and Red indicate significantly downregulated and upregulated genes, respectively. For enzyme reactions, the arrows between two metabolites represented the directions of catalytic reactions. The transporter proteins and transcriptional factors are represented in green rectangles, whereas metabolites are in orange ellipses. CYP724B1, steroid 22S-hydroxylase; CYP90A1, 3beta,22alpha-dihydroxysteroid 3-dehydrogenase; BR6OX1, brassinosteroid-6-oxidase 1; CYP90D2, steroid 3-oxidase; CYP92A6, typhasterol/6-deoxotyphasterol 2alpha-hydroxylase; BAS1, PHYB activation tagged suppressor 1.

Additionally, eighteen genes were significantly upregulated in T289. Alkaline stress downregulated ARF genes in S223 but upregulated them in T289. T The application of BR seems to promote ARF genes expression in S223 under alkaline stress. Similarly, GH3 genes varied in S223 and T289. Under alkaline stress, SAUR genes in S223 showed more drastic changes in expression than those in T289. BR decreased the expression of most SAUR genes, whereas alkaline stress significantly enhanced the expression of GID1 in both S223 and T289. Two DELLA genes, one downregulated and one upregulated, were detected in both S223 and T289. Alkaline stress attenuated most expressions of the TF genes in S223, but BR improved their expression. The BRI1 and BSK genes were boosted by alkaline stress. Compared with AS, the expression levels of BRI1 and BSK genes were less in the AB groups. The expression of the TCH4 gene in S223 was inhibited by alkaline stress, whereas two TCH4 genes were upregulated in T289. Compared to CK, AS groups had one and three PYR/PYL genes promoted in S223 and T289, respectively. Alkaline stress stimulated high expression levels of PP2C, SnK2, and ABF genes in S223 and T289, and BR supplementation reduced these levels. Alkaline stress upregulated three and two CAT1 genes in S223 and T289, respectively. Additionally, it induced the expression of MAPKKK17/18 and MPK1/2 genes in S223 and T289. Interestingly, BR attenuated the expression levels of these genes under alkaline stress. Taken together, the above results demonstrate both the specificity and similarity of broomcorn millet cultivars in their transcriptional responses in plant hormone signal transduction and MAPK signaling pathways.

3.8. Transcriptional differences in starch and sucrose metabolism in broomcorn millet leaves

The starch and sucrose metabolic pathways were studied to explore the transcriptional response of broomcorn millet to alkaline stress (S. table 5 and Fig. 7). Under alkaline stress, the EG and BGL genes were highly expressed, and the application of BR had a negative effect. Gene expression of INV, malZ, FRK, HK, TPS, and TREH were also upregulated by alkaline stress. Remarkably, the expression of genes related to INV, FRK, TPS, TREH, and malZ were all attenuated in the BR groups compared with the AS groups. Under alkaline stress, the SUS gene expression was upregulated and the upregulation was inhibited by the application of BR. Although some SPP genes were upregulated under alkaline stress, the expression of the SPS gene was inhibited. Additionally, 4α-GTase was downregulated by alkaline stress in both S223 and T289. A total of ten DEGs of glgC were detected, and alkaline stress upregulated the expression of four genes in S223 and T289, but this was diminished or even lost in BR groups. The expression of other six genes was inhibited by alkaline stress; however, this inhibition was alleviated by BR. Alkaline stress upregulated two SS genes. The gene expression of AMY showed that a few genes were reduced under alkaline stress, but the expression of BMY varied slightly. These results demonstrate that alkaline stress promoted the decomposition and utilization of leaves carbohydrates, and this effect was reduced by BR.

4. Discussion

Alkaline stress is an important abiotic stress that limits global crop growth (Liu et al., 2021; Xiao et al., 2020) and inhibits the germination of broomcorn millet (Ma et al., 2021, 2022). Our knowledge of the physiological and molecular mechanisms underlying alkaline response in broomcorn millet remains limited despite it having its genome reported recently (Zou et al., 2019). In the present study, comparative analysis of the alkaline-tolerant cultivar T289 and the alkaline-sensitive cultivar S223 showed that osmotic homeostasis, cell membrane function, and stomatal morphology maintenance are important physiological mechanisms for alkaline stress tolerance. Furthermore, transcriptome analysis revealed that the acquisition of alkaline stress

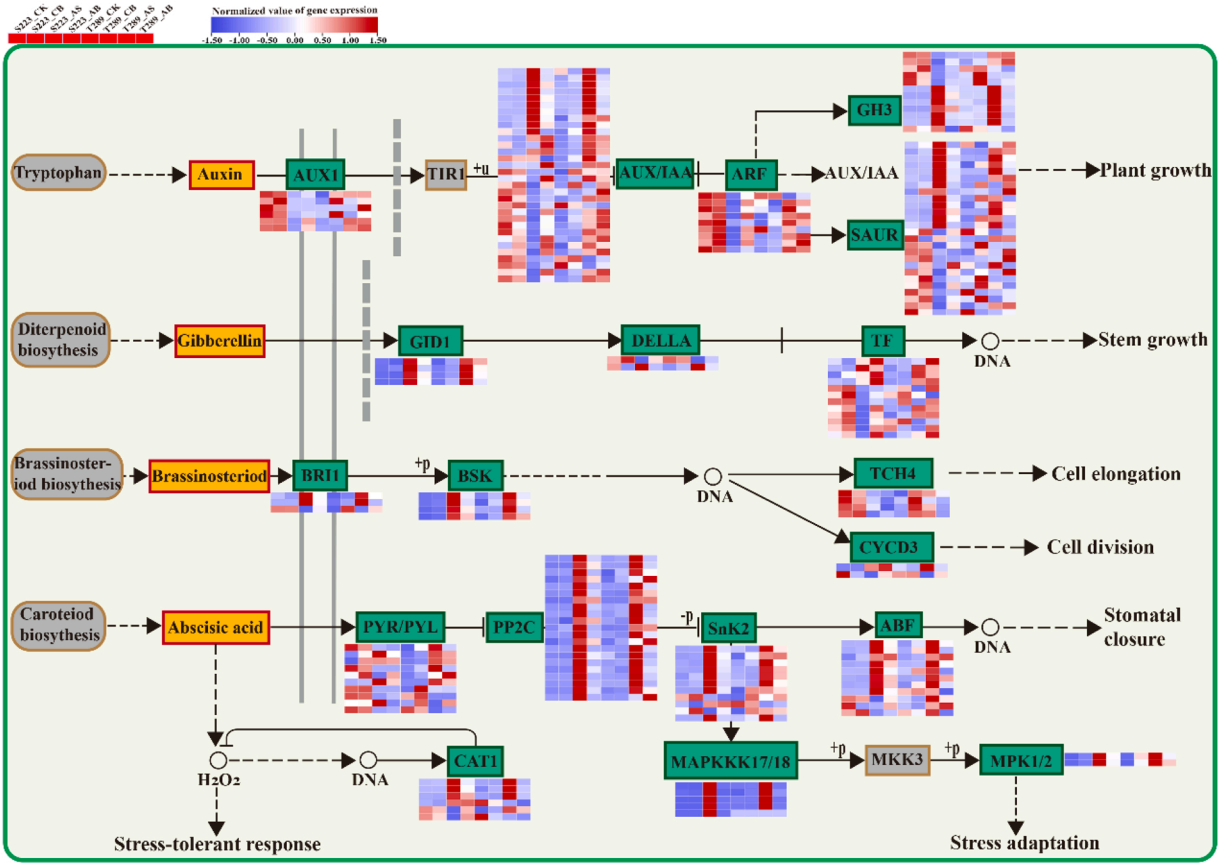

Fig. 6. Plant hormone signal transduction and MAPK signaling pathway - plant of S223 and T289 under different treatments. The color block represents the normalized value of gene expression. Purple and Red indicate significantly downregulated and upregulated genes, respectively. For enzyme reactions, the arrows between two metabolites represented the directions of catalytic reactions. The transporter proteins and transcriptional factors are represented in green rectangles, whereas metabolites are in orange ellipses. AUX1: auxin influx carrier. TIR1, transport inhibitor response 1; AUX/IAA: auxin-responsive protein IAA; ARF, auxin response factor; GH3, auxin responsive GH3 gene family; SAUR, SAUR family protein; GID1, gibberellin receptor GID1; DELLA, DELLA protein; TF, phytochrome-interacting factor 3; BRI1, protein brassinosteroid insensitive 1; BSK, BR-signaling kinase; TCH4, xyloglucan:xyloglucosyl transferase TCH4; CYCD3, cyclin D3, plant; PYR/PYL, abscisic acid receptor PYR/PYL family; PP2C, protein phosphatase 2 C; SnK2, serine/threonine-protein kinase SRK2; ABF, ABA responsive element binding factor; CAT1, catalase; MAPKKK17/18, mitogen-activated protein kinase kinase kinase 17/18; MKK3, mitogen-activated protein kinase kinase 3; MPK1/2, mitogen-activated protein kinase 1/2.

tolerance is orchestrated by an extensive transcriptional reprogramming of genes involved in multiple pathways, including the synthesis of alkaloids, amino acid and sugar metabolism, plant hormone signal transduction, and MAPK signaling pathway. Moreover, 24-episbrassinolide enhanced the adaptability of broomcorn millet to alkaline stress. These results provide novel insights into the physiological and molecular mechanisms of alkaline-tolerant plants.

4.1. Phenotypic and physiological responses to alkaline stress

Cultivar S223 was more sensitive to alkaline stress than T289, as evidenced by physiomorphological development and dry matter accumulation (Fig. 1), indicating that alkaline tolerance in broomcorn millet is related to genetic characteristics of the cultivar (Ma et al., 2021). MDA accumulation and electrolyte leakage rate reflect the extent of oxidative damage caused by stress in plant cells (Zou et al., 2012; Tian et al., 2021), both of which are enhanced in alkaline stress (Ma et al., 2021). In the present study, MDA levels in S223 and T289 increased significantly. The electrolyte leakage rate was similar to that of MDA, demonstrating that alkaline stress caused membrane lipid peroxidation damage in broomcorn millet. In contrast, 24-episbrassinolide reduced the oxidative damage of broomcorn millet caused by alkaline stress. Soluble sugars are important osmotic regulators in cells (Theocharis et al., 2012). Additionally, reducing sugars play a vital role in ROS scavenging in plant cells (Dahro et al., 2016). In the present study, glucose, fructose, maltose, and sucrose levels were increased by alkaline stress, suggesting stress perturbed osmotic balance and ROS homeostasis in broomcorn millet (Mostofa et al., 2020; Zhao et al., 2020; Xiao et al., 2020). Similar conclusions were reported by Yuan et al. (2021b). Generally, plants maintain water by regulating the stomatal closure (Ma et al., 2021). Our study showed the damage in stomata caused by alkaline stress, including stomatal closure, shrinkage, apoptosis, and disintegration. These results are similar to those in a previous report investigating NaCl stress (Yuan et al., 2021b). Under alkaline stress, 24-epibrassinolide provided moderate protection to stomatal morphology. Our results physiologically revealed the cultivar specificity of alkaline tolerance in broomcorn millet and the alleviation of alkaline stress by 24-epibrassinolide, especially for an alkaline-sensitive cultivar.

4.2. Transcriptional responses to alkaline stress

When plants encounter abiotic stress, a series of programs, such as molecular function or biological processes, are initiated to regulate signal transduction and growth to adapt to adversity (Tian et al., 2021). Transcriptome profiling indicate that alkaline stress stimulated various transcriptional differences in the leaves of the two broomcorn millet cultivars, which explained the diverse physiological responses of the two cultivars to alkaline stress. Under alkaline stress, more BR-induced DEGs

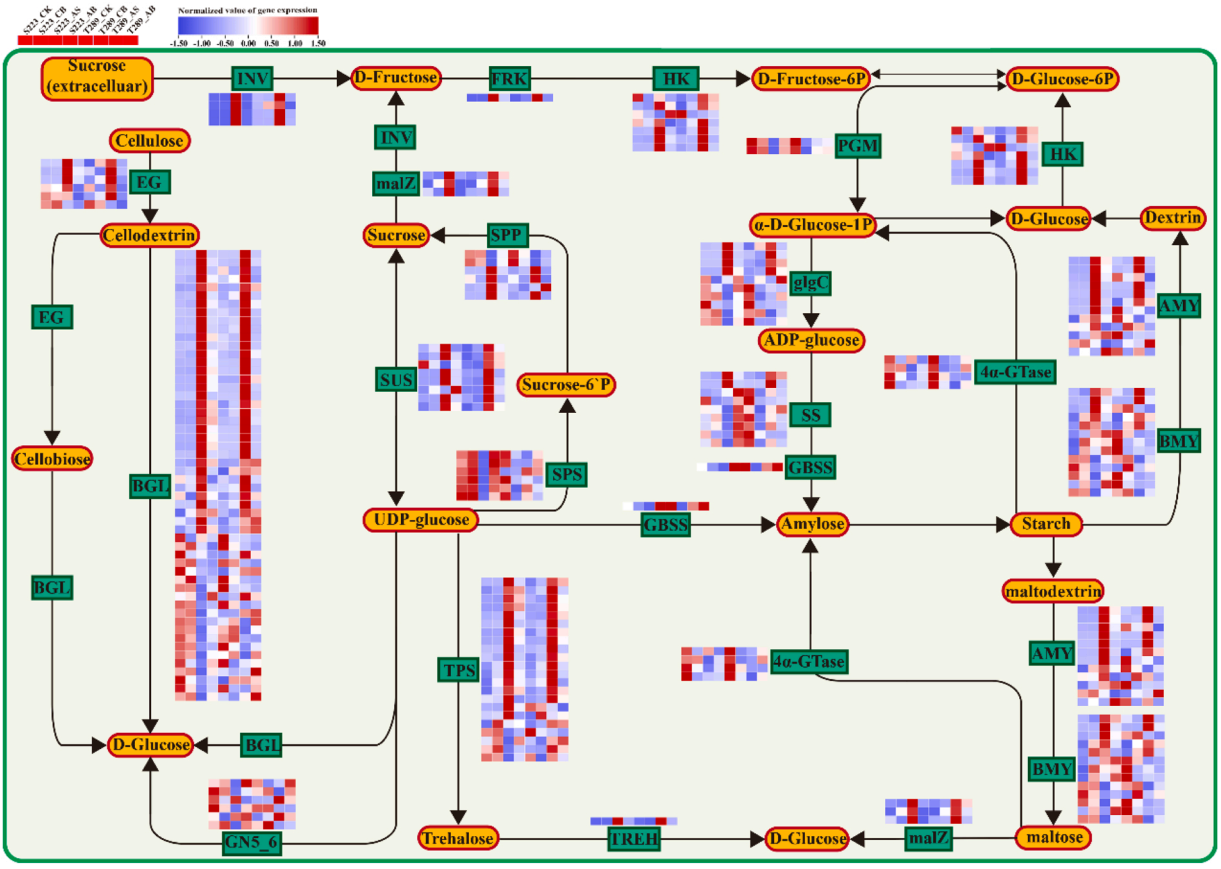

Fig. 7. Starch and sucrose metabolism of S223 and T289 under different treatments. The color block represents the normalized value of gene expression. Purple and Red indicate significantly downregulated and upregulated genes, respectively. For enzyme reactions, the arrows between two metabolites represented the directions of catalytic reactions. The transporter proteins and transcriptional factors are represented in green rectangles, whereas metabolites are in orange ellipses. INV, beta-fructofuranosidase; malZ, alpha-glucosidase; BGL, beta-glucosidase; EG, endoglucanase; glgC, glucose-1-phosphate adenylyltransferase; SS, starch synthase; GBE1, 1,4-alpha-glucan branching enzyme; AMY, alpha-amylase; BMY, beta-amylase; TPS, trehalose 6-phosphate synthase/phosphatase; PGM, phosphoglucomutase; GBSS, granule-bound starch synthase; HK, hexokinase; SPP, sucrose-6-phosphatase; SUS, sucrose synthase; SPS, sucrose-phosphate synthase; GN5_6, glucan endo-1,3-beta-glucosidase 5/6; FRK, fructokinase; 4α-GTase, 4-alpha-glucanotransferase; TREH, alpha,alpha-trehalase.

were observed in S223 than in T289, indicating that BR induced specific transcriptional expression of different alkaline-tolerant cultivars under this stress. In addition, cluster and PCA analysis of DEGs showed separation of S223_AS and S223_AB, but not T289_AS and T289_AB (S. Figs. 1A and 1B). This indicated that the alleviating effect of BR on alkaline-sensitive cultivars was stronger than that on alkaline-tolerant cultivars.

4.3. Steroid and brassinolide responses to alkaline stress

Steroid signaling has received increasing attention in the study of plant responses to abiotic stress. In the present study, under the combined stress of Na_2CO_3 and $NaHCO_3$, most steroid hormone biosynthesis genes were upregulated in T289, but they were mostly downregulated in S223. Genes related to brassinolide biosynthesis are specifically upregulated under Na_2CO_3 stress (Zhang et al., 2013). However, the expression of multiple genes in steroid signaling pathways is significantly downregulated by $NaHCO_3$ stress (Chen et al., 2021). The inconsistent findings probably are due to the different species and stress conditions. Furthermore, the reduction in brassinosteroid-related gene expression may be due to growth inhibition rather than stress. Therefore, in addition to alkaline stress, it is possible that the difference in brassinolide-related gene expression between T289 and S223 is due to the optimized growth state of T289 compared to that of S223.

4.4. Plant hormone and MAPK signaling responses to alkaline stress

Plant hormone signal transduction is known to contribute to crop resistance to environmental stress (Bürger and Chory, 2019; García-García et al., 2020; Li et al., 2021). In this study, the gene expression of AUX1, ARF, AUX/IAA, and SAUR was either mostly or completely suppressed by alkaline stress in S223, resulting in growth inhibition of S223. In contrast, these genes were downregulated to a lesser extent in T289, thus providing better growth. Moreover, the expression of TF genes for gibberellin signaling was downregulated under alkaline stress. Genes encoding PP2C, SnK2, and ABF in the abscisic acid signaling pathway were activated under alkaline stress, thus promoting stomatal closure to maintain osmotic balance by retaining water. These results are consistent with a previous study that discussed the broomcorn millet response to drought stress (Yuan et al., 2022). Moreover, Li et al. (2021) reported that Nano-selenium application modulated plant hormone signal transduction and improved cadmium tolerance in peppers. Our data showed that 24-epibrassinolide attenuated the transcriptional response and enhanced alkaline tolerance in broomcorn millet exposed to alkaline stress. The MAPK signaling pathway is a crucial defense pathway in plants against stress (Jiang et al., 2015; Mahmood et al., 2019). In this work, genes for MAPKKK17/18 and MPK1/2 involved in the MAPK signaling pathway were uniformly upregulated by alkaline stress. Therefore, we speculate that broomcorn millet enhanced adaptability to alkaline stress by activating the MAPK cascade. Most of the CAT1-related genes mediated by gibberellic acid were specifically

upregulated under alkaline stress, inhibiting the accumulation of hydrogen peroxide. Likewise, 24-epibrassinolide attenuated these transcriptional responses.

4.5. Starch and sucrose metabolism responses to alkaline stress

Carbon metabolism is a direct effect of stomatal closure initiated by stressed plants to reduce water loss and protect osmotic balance (Thalmann et al., 2016; Rodrigues et al., 2019). In this study, INV and malZ were activated under alkaline stress to boost sucrose degradation. A previous study also showed that stress stimulates upregulation of INV activity (Roitsch et al., 2003). Concurrently, the key enzymes in the decomposition pathway to produce D-glucose, TPS and TREH were highly expressed by alkaline stress. Most AMY and BMY genes were also shown to be upregulated under alkaline stress. Kesten reported that plant cell walls adapt to stress by reshaping growth (Kesten et al., 2017). Starch and sucrose are transported to sinks to promote the elongation and expansion of roots and stems (Song et al., 2020). In the present study, broomcorn millet promoted cellulose degradation under alkaline stress by inducing a high expression of many EG and BGL genes, suggesting that broomcorn millet resisted alkaline stress by sacrificing growth. 24-epibrassinolide suppressed the gene expression of EG and BGL in the AB group and maintained growth under alkaline stress.

5. Conclusion

In this study, alkaline stress significantly inhibited growth and biomass. Under alkaline stress, the influence of steroid hormone biosynthesis, plant hormone and MAPK signaling pathways, and starch and sucrose metabolism were identified by transcriptional analysis. The use of 24-epibrassinolide significantly ameliorated alkaline-induced damage. This study provides a comprehensive perspective on the effect of exogenous brassinolide on both the adaptability and molecular process response of broomcorn millet to alkaline stress, as well as the mitigation of brassinolide to this stress. This report provides innovative ideas for sustainable agricultural development in alkaline soil regions. The direct planting of an alkaline-tolerant cultivar, and the combination of an alkaline-sensitive cultivar with 24-epibrassinolide will protect natural ecosystems and enhance the sustainable development of agriculture in alkaline regions. However, this work has two main limitations: First, the effects of alkaline stress and 24-epibrassinolide on the growth and yield formation in the whole growth period of broomcorn millet are unknown; b): further studies on the mechanisms of alkaline stress and brassinolide on the quality of broomcorn millet are required. In conclusion, this study is the first to use omics technology to study the physiological mechanism and stress mitigation of broomcorn millet seedlings under alkaline stress. This research provides an important theoretical basis for improving agricultural development of alkalized land using broomcorn millet.

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CRediT authorship contribution statement

Qian Ma: Methodology, Formal analysis, Visualization, Writing – review & editing. **Enguo Wu and Honglu Wang**: Investigation, Data curation, Writing – original draft. **Yuhao Yuan, Yu Feng and Jiajia Liu**: Conceptualization, Resources. **Lin Zhao**: Writing – review & editing. **Baili Feng**: Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The authors do not have permission to share data.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114298.

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