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Endogenous bioactive gibberellin/abscisic acids and enzyme activity synergistically promote the phytoremediation of alkaline soil by broomcorn millet (*Panicum miliaceum* L.)

Qian Ma [a], Yuhao Yuan [a], Enguo Wu [a], Honglu Wang [a], Ke Dang [a], Yu Feng [a], Aliaksandr Ivanistau [b], Baili Feng [a,*]

[a] College of Agronomy, State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest A & F University, Yangling, 712100, Shaanxi, PR China
[b] Belarusian State Agricultural Academy, 213407, Gorki, Mogilev Region, Belarus

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ABSTRACT

Broomcorn millet (*Panicum miliaceum* L.), an important food crop, grows in arid and semi-arid areas that face soil saline-alkalization. To date, no studies have investigated the mechanisms by which broomcorn millet seeds respond to and tolerate alkali stress. In this study, six broomcorn millet genotypes (B102, B220, B269, B279, B289, and B297) were selected to explore the physiological and molecular mechanisms of alkali stress at the germination stage. The results showed that alkali stress delayed the germination of broomcorn millet, and α-amylase activity was positively correlated with the germination rate. After alkali stress, the genotypes with lower alkali damage rates exhibited stronger antioxidant defenses. Real-time polymerase chain reaction analysis showed that alkali stress downregulated gibberellic acid (GA) synthesis genes but upregulated GA inactivation and abscisic acid (ABA) synthesis genes. Similarly, seeds displayed lower GA concentrations and higher ABA concentrations after alkali stress. Therefore, the ratios of various GAs/ABA decreased within the range of 35.77% to approximately 96.45%. Additionally, genotypes associated with lower alkali damage rates had higher GA/ABA ratios. These findings indicate that the alkali tolerance of broomcorn millet at the germination stage may be attributed to higher GA/ABA ratios, higher α-amylase activity, and stronger antioxidant defense, which synergistically resist alkali stress. This study will contribute to molecular breeding aiming to enhance alkali-tolerance and restoration of alkaline soils.

1. Introduction

Soil alkalization is an abiotic stress caused by accumulation of alkali salts ($NaHCO_3$ and Na_2CO_3), which severely limit sustainable agricultural production and ecological balance on global scale (Wu et al., 2014). In Northeast China, more than 70% of grasslands are alkaline (Kawanabe and Zhu, 1991). However, many studies have focused on salt tolerance. The inhibitory effect of neutral salt stress on crop growth is mainly due to Na^+ poisoning and osmotic stress (Ismail et al., 2014; Ibrahim, 2016; Shu et al., 2017; Rasheed et al., 2019; de Zelicourt et al., 2016). However, alkali stress is more complicated than neutral salt stress because it disturbs cellular pH homeostasis during crop growth (Romero, 2004; Liu et al., 2021). Therefore, suitable technology for restoration of alkalized land is urgently needed. Phytoremediation is an economical and sustainable remediation measure. Alkali-loving plants are uncommon, and researches on the selection of alkali-tolerant plant resources have been performed. If the alkali-tolerance mechanism is completely understood and applied to enhance alkali-tolerance in other plants via molecular breeding, it would contribute to the restoration of alkalized lands. Under alkali stress conditions, successful seed germination is a requirement for crop alkali tolerance.

Plants have developed specific defense mechanisms to sense and respond to environmental changes (de Zelicourt et al., 2016). Gibberellic acid (GA) and abscisic acid (ABA) are two important hormones that regulate seed germination under abiotic stress such as drought and salt stress (Rabbani et al., 2003). The former promotes seed germination, and the latter induces seed dormancy to delay germination (Shu et al., 2017). GA enhances crop resistance, but a common response of abiotic stress in plants is to reduce the level of GA, thereby inhibiting plant growth (Colebrook et al., 2014). Stress induces the production of

* Corresponding author.
E-mail address: fengbaili@nwafu.edu.cn (B. Feng).

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reactive oxygen species (ROS) in plant cells, which are a class of harmful molecules (Israr et al., 2011; Colebrook et al., 2014), causing irreversible damage to plant cells and tissues (including photosynthetic pigments, nucleic acids, proteins, lipid destruction), resulting in stunted growth, low fertility, and premature aging (Gill and Tuteja, 2010; de Zelicourt et al., 2016). Therefore, plants have developed an antioxidant defense system to protect plant cells and sub-cells from oxidative damage (Li et al., 2013; Karmous et al., 2014; Nanda and Agrawal, 2016). Various studies have shown that reasonable application of exogenous growth regulators (e.g., salicylic acid and abscisic acid) can dramatically promote the tolerance of plants to cold, drought, and high salinity (Larkindale and Huang, 2004). However, few studies focused on endogenous hormones and antioxidant systems to explore the resistance of plants to alkali stress.

Broomcorn millet (*Panicum miliaceum* L.), an ancient cereal crop that originated from arid and semi-arid regions of China, is one of the earliest domesticated cereal crops in the world (Hunt et al., 2014; Ma et al., 2021), and long-term edge planting has improved its resistance to harsh environments by natural selection. In recent years, broomcorn millet has been considered as a pioneer crop for stress resistance because of its high nutrient and water use efficiency compared to other cereal crops (Yue et al., 2016). However, various studies have focused on its genetic diversity, physical and chemical properties and its response to drought and salt stress (Yang et al., 2018; Zhang et al., 2019; Yuan et al., 2021). There is still a gap in the research on the effect of alkali stress on broomcorn millet growth. Especially, the mechanisms by which hormones and enzymes regulate the germination under alkali stress are still unclear. The exploration of the defense mechanism of millet against alkali stress could provide useful information for improving the alkali tolerance of other plant species.

This work aims to (1) determine the influence of alkali stress on germination of broomcorn millet by measuring the germination rate, alkali damage rate, germination state, and the activity of α-amylase; (2) study the effects of alkali stress on osmotic adjustment and antioxidant system of broomcorn millet by determining osmotic adjustment indicators such as soluble sugar, soluble protein, and malondialdehyde (MDA), as well as antioxidant system such as activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT); (3) clarify the internal hormone regulation mechanism under alkaline stress at the germination stage by measuring the concentration of GA and ABA and via RT-PCR analysis; (4) explore the practical application of broomcorn millet in phytoremediation of alkalized lands. This is the first study to experimentally examine the intraspecific variation in alkali tolerance of broomcorn millet at the germination stage. These findings will provide valuable information for understanding the mechanism of germination of broomcorn millet seeds in response to alkali stress and performing molecular breeding of plant alkali-tolerant plants. Hence, this research will contribute to the phytoremediation of alkalized soil and world food security.

2. Materials and methods

2.1. Broomcorn millet genotypes, growth conditions, and treatments

Broomcorn millet genotypes (B102, B220, B269, B279, B289, and B297), provided by Northwest A & F University (Yangling, Shaanxi, China), were selected for this study. The seeds were disinfected with 0.1% $HgCl_2$ for 5 min, rinsed five times with sterile water, followed by absorption of surface water was absorbed and drying. In each Petri dish, 50 seeds were arranged evenly and cultivated in 8 mL of distilled water (control) or 80 $mmolL^{-1}$ mixed alkali solution (molar ratio $NaHCO_3$: Na_2CO_3 = 9:1). The seeds were germinated in a controlled greenhouse incubator (30 °C day/18 °C night, 14 h light/10 h dark cycle, and 60% relative humidity). Distilled water and mixed alkali solution were replaced every 24 h.

The seeds, exposed to two different treatments named above, were sown, and left to grow for 7 days under the above-mentioned culture conditions. Subsequently, the plants were harvested on the 7th day for growth measurement, and the germinated seeds were harvested for the determination of endogenous hormones, and various physiological and biochemical parameters. The experiments were conducted in three replicates of treatments for each genotype, and the measurements were taken in triplicates for each parameter measured (averaged to obtain a single mean) under the same experimental conditions.

2.2. Determination of plant growth

The seed germination rate and root length or sprout length (using a vernier caliper) were measured on the 7th day. Further, root fresh weight and sprout fresh weight were measured, respectively, using an electronic balance scale (METTLER TOLEDO Instruments Co., Ltd., Shanghai, China). Additionally, relative alkali damage was calculated as shown in Eqs. (1) and (2).

$$\text{Germination rate (GR)} = \left(\frac{\text{number of germinated seeds on the } 7^{\text{th}} \text{ day}}{\text{total number of tested seeds}}\right) \times 100 \quad (1)$$

$$\text{Relative alkali damage rate (RAD)} = 1 - \left(\frac{\text{germination rate in alkali}}{\text{germination rate of the control}}\right) \times 100 \quad (2)$$

2.3. Determination of total soluble sugar and soluble protein content

Fresh germinated seeds were collected and stored at −80 °C. The total soluble sugar and soluble protein content were determined spectrophotometrically according to the description by Mostofa et al. (2020).

2.4. Determination of malondialdehyde content and α - amylase activity

The malondialdehyde (MDA) content and α-amylase activity were determined spectrophotometrically according to the method described by Yuan et al. (2021) and Li et al. (2019), respectively. MDA content was quantified using an extinction coefficient of 155 mM^{-1} cm^{-1} after reading the absorbance differences at 532 and 600 nm (UV2600A UV–Visible Spectrophotometer, Liberty Tech Instruments Co., Ltd., Beijing, China). The α-amylase activity was calculated from the absorbance measured at 570 nm.

2.5. Determination of antioxidant enzyme activities

The supernatant was used to determine the antioxidant enzyme activity, and the nitrotetrazolium blue chloride (NBT) reduction method was used to determine the SOD activity of seeds. POD and CAT were also measured according to the published methods (Mostofa et al., 2020).

2.6. Quantification of endogenous gibberellic acid

Endogenous gibberellic acid (GA) content was determined according to a previously described method (Wang et al., 2017). The seeds were frozen in liquid nitrogen, ground into a fine powder, and extracted with 80% (v/v) methanol. The GA d2 isotope standard was regarded as a standard product. After the crude extract was purified by reverse-phase solid-phase extraction, ether extraction, and derivatization, the content of endogenous gibberellin in the mixture was quantitatively analyzed by high performance liquid chromatography (Vanquish, Thermo, USA)-mass spectrometry (Q exactive, Thermo, USA) (HPLC-MS). The total GA was calculated by summing the content of GA1, GA3, GA3, and GA4.

2.7. Quantification of endogenous abscisic acid

To analyze the abscisic acid (ABA) content in broomcorn millet seeds, this study adopted the method described by Shu et al. (2017). First, the sample was extracted in methanol for 24 h, during which D6-ABA (OIChemIm Co. Ltd.) was selected as the internal standard. Subsequently, purification was performed with an Oasis Max solid-phase extract cartridge (Waters, Milford, MA, USA) and eluted with 5% formic acid in methanol. Finally, the dried and reconstituted eluent was injected into a liquid chromatography tandem mass spectrometry system to quantify the ABA content.

2.8. Analysis of gene expression

Ribonucleic acid (RNA) was extracted using the plant sample RNA extraction kit provided by Tiangen Biotechnology (Beijing, China; Cat. No. DP432), according to manufacturer instructions. The PrimeScript™ RT reagent Kit (TaKaRa Bio, No. RR047B) with g-deoxyribonucleic acid (gDNA) Eraser was used for cDNA reverse transcription. Real-time PCR in an optical 96-well plate equipped with Stepone TM real-time PCR system (Applied Biosystems, Waltham, MA, USA) proceeded as follows: 95 °C for 30 s; 40 PCR cycles (95 °C, 5 s; 60 °C, 40 s, to collect fluorescence). To establish the melting curve of PCR products, the amplification reaction was completed at (95 °C, 10 s; 60 °C, 60 s; 95 °C, 15 s), and slowly heated from 60 °C to 99 °C (the instrument automatically performs this process, ramp rate is 0.05 °C/s). The following primers were used: GA20ox2,-5'-CTACTGCGAGGCGATGAA-3' and 5' -GTCGGCGAAGAAGTCCCT-3'; GA20oX3: 5' -GTCGCTCACCTTCTTCCT-3' and 5' -CACTTCTCCTGTCCGCTA-3'; GA2ox3: 5' -TCCGTCCTCCGCTCCAAC-3'- and 5'-TTTCAGCCTCCAAGGAAGT-3'; and NCED1: 5'-TTTCAGCTTCCAAGGAAGT-3' and 5' -CGGACTCGTT-GAAGATGG-3'. The target gene and internal control of each sample were subjected to real-time PCR, and each sample was tested in three replicates. The relative expression levels were analyzed using the comparative $2^{-\triangle\triangle CT}$ method (Yuan et al., 2021).

2.9. Statistical analysis

The obtained experimental data were statistically analyzed using the SPSS statistics 23 (United States, IBM Software). One-way analysis of variance (ANOVA) was used to determine significant differences between the treatments. Duncan's test was used to determine which treatment means differed significantly among themselves and/or against the control. An error rate (P = 5% or 0.05) was used as the threshold for rejecting the null hypothesis. Data were expressed as mean ± standard deviation to indicate sample variability. All figures were drawn using the Origin 2021 (United States, OriginLab software, USA).

3. Results

3.1. Effect of alkali application on germination of broomcorn millet seeds

Germination analysis showed that alkali treatment delayed the germination of all six genotypes. Broomcorn millet seeds exposed to alkali stress for 7 d showed clear phenotypic disturbance, which was manifested as a lowered germination rate and severe inhibition of root and sprout growth (Fig. 1). During the germination process, the germination rates of broomcorn millet in the control groups were 1.38–20.88 fold greater than those of alkali stress groups, which indicated that alkali stress significantly reduced ($F = 1023.78$, $P < 0.001$) the germination rate of the six genotypes. The germination rate of B102, B220, B269, B279, B289, and B297 genotypes decreased by 95.21%, 27.27%, 80.50%, 64.58%, 45.45%, and 94.78%, respectively (Fig. 1 A). Genotype B102 exhibited the highest alkali damage rate, implying that it is the most sensitive to alkali stress; in contrast, B220 exhibited the lowest alkali damage rate (Fig. 1 B). Post-germination growth parameters

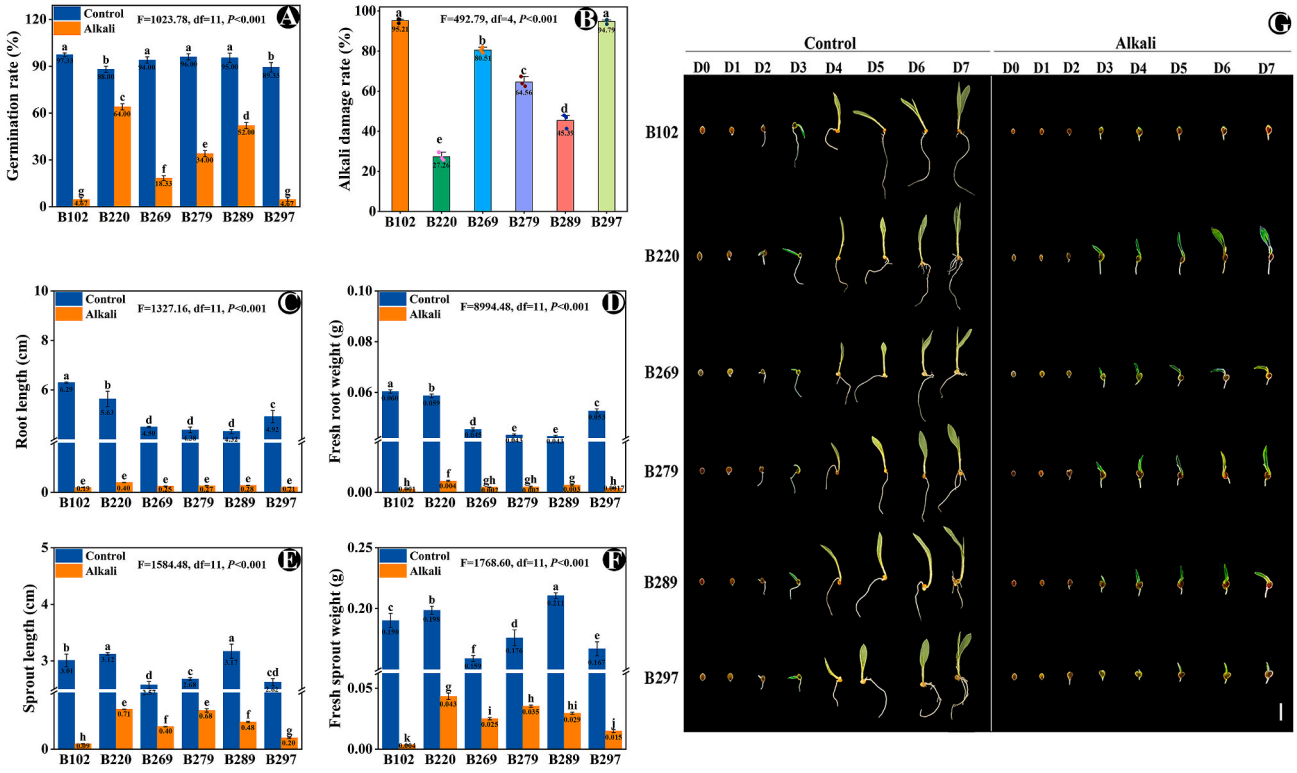

Fig. 1. The growth characteristics of the six broomcorn millet genotypes at germination stage. (A): germination rate, (B): alkali damage rate, (C): root length, (D): fresh root weight, (E): sprout Length, (F): fresh sprout weight, and (G): germination state. The data represent the mean ± SD (n = 3). Different lowercase letters indicate significant differences according to Duncan's test. D0-D7 represent the 0–7th day of germination. Bar = 1 cm.

including root length (Fig. 1 C), root fresh weight (Fig. 1 D), shoot length (Fig. 1 E), and shoot fresh weight (Fig. 1 F), were inhibited upon exposure to alkali stress. Results depicted in Fig. 1 G confirm the inhibitory effect of alkali stress on the germination of broomcorn millet seeds, which were obtained upon a follow-up observation on the germination of six genotypes. These results demonstrated that exogenous alkali treatment posed significant inhibitory effects on the germination process.

3.2. Response of α-amylase activity to the application of exogenous alkali

We further compared the difference in α-amylase activity of the six genotypes under control and exogenous alkali application. The α-amylase activity of the six genotypes was higher in the control groups than that in the experimental groups (Fig. 2 A). In the alkali-treated groups, the α-amylase activity of all six genotypes was significantly reduced ($F = 168.21$, $P < 0.001$), but the highest activity was maintained in genotype B220 seeds (Fig. 2 A).

3.3. Response of lipid peroxidation to the application of exogenous alkali

We detected the MDA content on the 7th day of germination of the six genotypes in the control and alkali stress groups. The results of MDA analysis conducted on day 7 of germination showed that the application of exogenous alkali significantly increased MDA content in broomcorn millet seeds. In the control groups, the MDA contents of the six genotypes were not significantly different, but after exogenous alkali application, the MDA content in genotypes B102 and B297 was significantly higher ($F = 18.81$, $P < 0.001$) than that in the other four genotypes (Fig. 2 B).

3.4. Response of organic osmolytes to the application of exogenous alkali

In the present study, we observed that after 7 d of alkali application, the total soluble sugar content in the seeds of genotypes B102, B220, B269, B279, B289, and B297 decreased significantly ($F = 262.08$, $P < 0.001$) by 39.98%, 44.91%, 34.84%, 41.82%, 51.58%, and 45.79%, respectively (Fig. 2 C). In contrast, total soluble protein content increased significantly ($F = 2026.04$, $P < 0.001$) by 38.91%, 103.71%, 45.72%, 63.55%, 49.54%, and 69.47%, respectively (Fig. 2 D).

3.5. Response of antioxidative defense to the application of exogenous alkali

The activity of SOD in the control group was lower than that in the alkali treatment group for all genotypes (Fig. 2). The activity of SOD increased differently with the genotypes (Fig. 2 E). Similar to the trend of SOD, the POD activity of each group was increased significantly ($F = 799.19$, $P < 0.001$) under the application of exogenous alkali. The POD activities of genotypes B102, B220, B269, B279, B289, and B297 under alkali treatment conditions were 3.57-, 4.14-, 2.99-, 3.97-, and 4.22-fold greater than that of the control group, respectively (Fig. 2 F). In comparison with SOD and POD, the absolute value of CAT activity was significantly ($F = 37.56$, $P < 0.001$) lower. Similar to SOD and POD, the results showed that alkali treatment increased CAT activity of the seeds (Fig. 2 G), indicating that CAT has a good regulatory effect under alkali stress.

3.6. Effect of alkali application on expression levels of gibberellin, abscisic acid and the related genes

The content of active GA and ABA was detected in the broomcorn millet seeds on day 7 of germination (Fig. 3). Compared with the control, alkali stress groups exhibited different degrees of inhibitory effects on the active GA content in the seeds of the six genotypes. In particular, GA4 showed significant ($F = 118.56$, $P < 0.001$) reduction, with genotypes B102, B220, B269, B279, B289, and B297 exhibiting a decrease of 88.15%, 76.87%, 77.75%, 86.10%, 67.61%, and 81.08%, respectively (Fig. 3 C). In addition, the calculated total gibberellin (TGA) including four GAs (GA1, GA3, GA4, and GA7) indicated that the exogenous alkali significantly ($F = 268.71$, $P < 0.001$) reduced the GA content in the seeds, but the inhibitory effects on the six genotypes varied (Fig. 3 E). Among the six genotypes, B220 maintained the largest TGA value after exogenous alkali treatment, while B120 had the smallest. In contrast to GA, exogenous alkali treatment promoted the accumulation of ABA inside the seeds (Fig. 3 F). Compared with the control, the ABA content in genotypes B220 and B289 was lower but it was higher in genotypes B102 and B297. In the present study, the GA1/ABA ($F = 472.63$, $P < 0.001$), GA3/ABA ($F = 349.25$, $P < 0.001$), GA4/ABA ($F = 363.38$, $P < 0.001$), GA7/ABA ($F = 518.17$, $P < 0.001$), and TGA/ABA ($F = 243.70$, $P < 0.001$) of the six genotypes were significantly reduced by exogenous alkali application with a reduction of 45.73% ~ 96.45%, 60.74% ~ 90.95%, 76.52% ~ 94.92%, 35.77% ~ 74.00%, and 37.68%–92.60%, respectively (Fig. 3G–K). After 7 d of exogenous alkali treatment, the largest and smallest GA/ABA values were observed in genotypes B220 and B102, respectively.

To explore the response mechanism of GA and ABA in different genotypes under exogenous alkali stress, we monitored the expression changes of *PmGA20ox*, *PmGA2ox*, and *PmNCED*, which are the key genes involved in the regulation of the biosynthesis and catabolism of broomcorn millet GA and ABA. As shown in Fig. 4 and Fig. 5, the control of genotype B102 was used as the control sample of relative gene expression. Alkali stress had a significant effect on the down-regulation of *PmGA20ox2* ($F = 14.20$, $P < 0.001$) and *PmGA20ox3* ($F = 171.85$, $P < 0.001$) expression in the seeds of all six genotypes (Fig. 4 A, B). On the contrary, alkali stress promoted the expression levels of *PmGA2ox3* ($F = 63.22$, $P < 0.001$) and *PmNCED1* ($F = 57.48$, $P < 0.001$) (Fig. 4 C, D). It can be seen that the expression levels of different genotypes also responded differently (Fig. 5). Among the six genotypes and after 7 days of alkali stress, the expression of *PmGA20ox2* in genotype B220 was the highest, the expressions in genotypes B289, B279, B269 and B220 had no significant difference, while the expression of *PmGA20ox2* in genotype B102 was the lowest (Fig. 4). Alkali stress after 7 days induced significantly higher expression level of *PmGA20ox2* in B220 seeds than that of genotypes B102 and B297. Similar to *PmGA20ox2*, alkali stress significantly downregulated the expression of *PmGA20ox3* in six genotypes, especially genotypes B102 and B297. As shown in Fig. 4 A and B, it can be concluded that the downregulation of *PmGA20ox3* by alkali stress was stronger than that of *PmGA20ox2*. However, among the six genotypes, genotype B220 still maintained the maximum gene expression level of *PmGA20ox2* and *PmGA20ox3* after 7 days of alkali stress. As shown in Fig. 4 C, contrary to *PmGA20ox2* and *PmGA20ox3*, exogenous alkali stress induced the expression of *PmGA2ox3* in the seeds of broomcorn millet. Especially in genotype B102, the effect of alkali stress on the upregulation of *PmGA2ox3* was significantly higher than that of other genotypes. The effect of alkali stress on *PmNCED1* in the seeds of broomcorn millet was similar to that on *PmGA2ox3*. Compared with the control group, alkali stress promoted the expression of *PmNCED1*, and its expression in genotype B102 was significantly higher than that of other genotypes, except for genotype B297.

3.7. Hierarchical cluster analysis and Pearson's correlation analysis

The heat map paired with the dendrogram obtained by hierarchical clustering demonstrated that the treatments were divided into two significantly different clusters: control and exogenous alkali application (Fig. 6). In addition, within the clusters subjected to exogenous alkali application, the six genotypes were divided into three sub-clusters, for which there were differences in antioxidant enzyme activity, membrane lipid peroxidation, hormone synthesis, and soluble protein.

A Pearson's correlation heat map further showed the relationship between different parameters (Fig. 7). There were significant positive

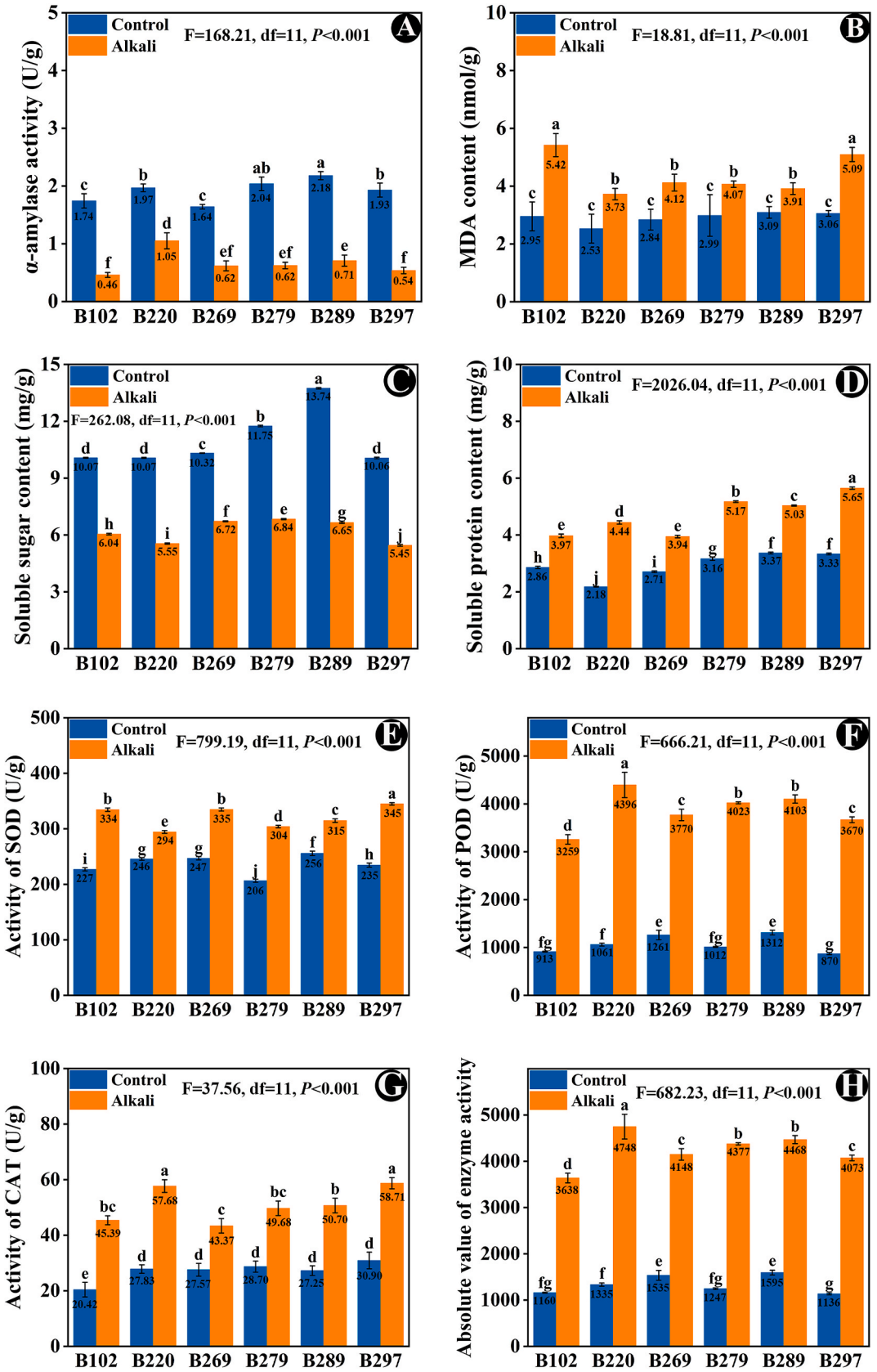

Fig. 2. Effects of alkali stress on α-amylase activity, MDA content, soluble sugar and protein, and antioxidant enzyme activities. (A): α-amylase activity, (B): MDA content, (C): soluble sugar content, (D): soluble protein content, (E): SOD activity, (F): POD activity, (G): CAT activity, and (H): absolute value of antioxidant enzyme activity. The data represent the mean ± SD (n = 3). Different lowercase letters indicate significant differences according to Duncan's test.

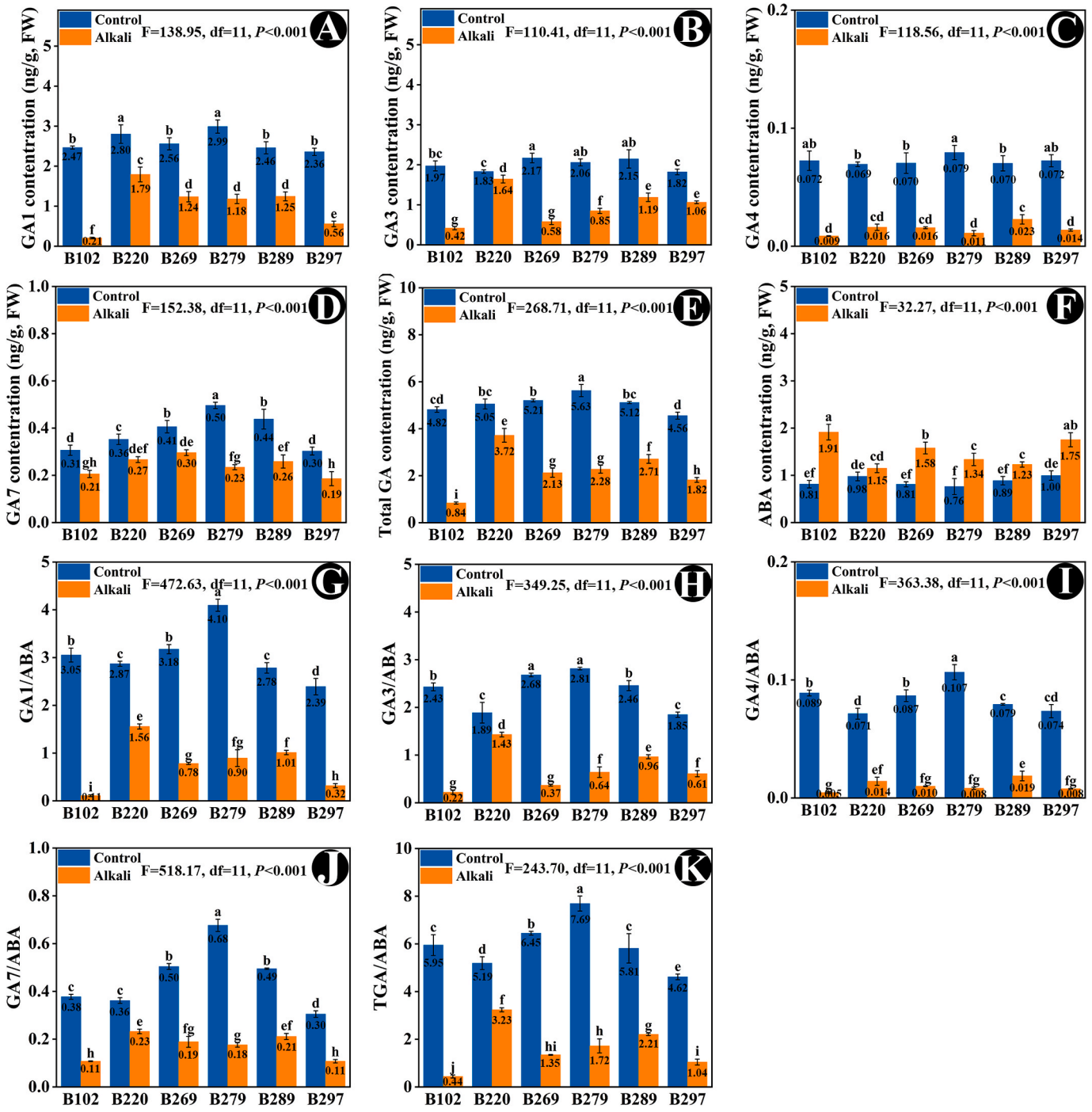

Fig. 3. Effects of alkali stress on GAs content, ABA content, and ratios of GAs/ABA. The data represent the mean ± SD (n = 3). TGA represents the sum of GA1, GA3, GA4, and GA7. Different lowercase letters indicate significant differences according to Duncan's test.

correlations between the growth indicators that directly reflect the germination, and soluble sugar content, α-amylase activity, *PmGA20ox2*, and *PmGA20ox3*, but were significantly negatively correlated with soluble protein content, antioxidant enzyme activity, *PmGA2ox3*, and *PmNCED1*.

4. Discussion

Extensive studies have been carried out to clarify the mechanism of plant response and adaptation to salt-alkali stress, many of which have shown a delay in seed germination and inhibition of plant growth and development under saline conditions (Liu et al., 2018; Yuan et al., 2021). However, to date, no study has evaluated the mechanism and influence of mixed alkalis (Na_2CO_3 and $NaHCO_3$) on seed germination. In the present study, six broomcorn millet genotypes were used to investigate the effect of alkali stress on seed germination. The results showed that the seed germination rate, root length, fresh root weight, sprout length, and fresh sprout weight were significantly reduced under alkali stress, which indicated that alkali stress exerted an inhibitory effect on seed germination and root and sprout growth. Genotype B220 exhibited the lowest alkali damage rate, suggesting that it had the highest tolerance to alkali stress and adaptability to the alkali environment (Fig. 1 B).

One of the important reasons for salt-alkali stress in damaged plants

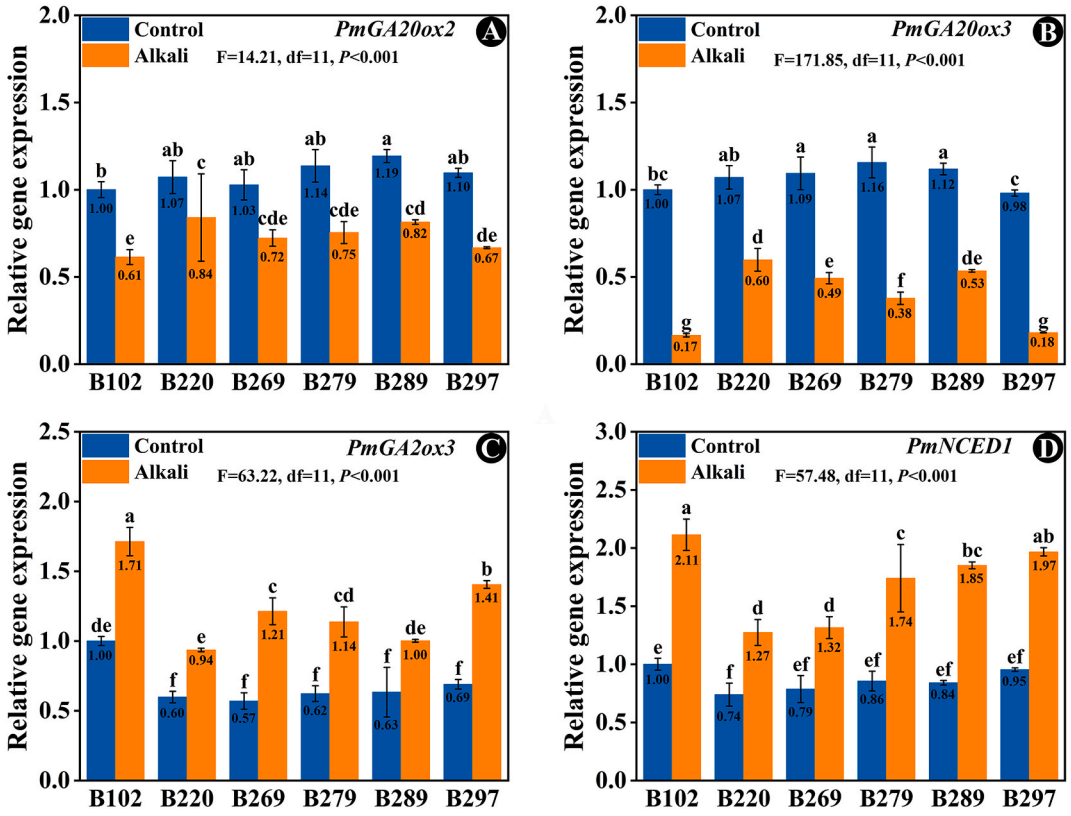

Fig. 4. Effects of alkali stress on expression of GA and ABA related genes. The data represent the mean ± SD (n = 3). Different lowercase letters indicate significant differences according to Duncan's test.

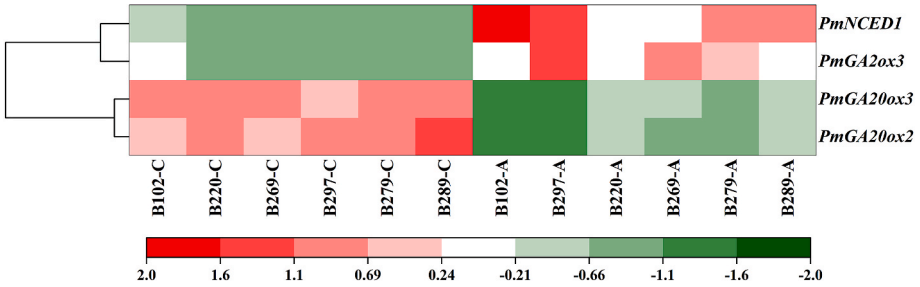

Fig. 5. Expression responses of GA and ABA related genes in six broomcorn millet genotypes to alkali stress. As indicated by the color bar, from brightest green equals most down-regulated to brightest red equals most up-regulated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

is osmotic stress. It is generally believed that the tolerance of crops to osmotic stress is manifested in the accumulation of organic and inorganic osmotic substances to maintain normal water absorption capacity and prevent tissue cells from apoptosis due to physiological water shortages (Yuan et al., 2021). In the present study, six genotypes were investigated, and the total soluble sugar and total soluble protein content were estimated.

Unexpectedly, the total soluble sugar content decreased significantly within the seeds under application of exogenous alkali stress for 7 days. This is contrary to the conclusion that salt-alkali stress increased the soluble sugar content in the leaves of broomcorn millet. Carbohydrates are an important source of energy for plant growth and development. A large amount of nutrients stored in the seed itself need to be consumed to produce energy for embryo development and utilization, especially in the process of seed germination. Seeds that encounter alkali stress must not only maintain growth during the germination process, but also resist and adapt to stress, which increases energy consumption of energy (Liu et al., 2018). We believe that this may be the reason why the total soluble sugar content of the seeds decreased during the germination process under alkali stress. In addition, α-amylase is the main enzyme that hydrolyzes starch into soluble sugars and acts as an important factor in the energy supply during seed germination. Therefore, the activity of α-amylase is also considered an important factor controlling seed germination (Ashraf et al., 2002; Kim et al., 2008). It has been reported that the α-amylase activity of seeds exposed to salt solution is lower than that of the control solution (Bialecka and Kepczynski, 2009; Liu et al., 2018). Therefore, to investigate whether alkali application inhibits the germination of broomcorn millet seeds by impairing the activity of α-amylase, we further compared the differences in α-amylase activity of the six genotypes under the control and exogenous alkali treatments. The results showed that alkalinity significantly inhibited the α-amylase activity of the broomcorn millet seed, with variations between the genotypes. These results indicate that the inhibition of germination of broomcorn millet may be related to the impairment of

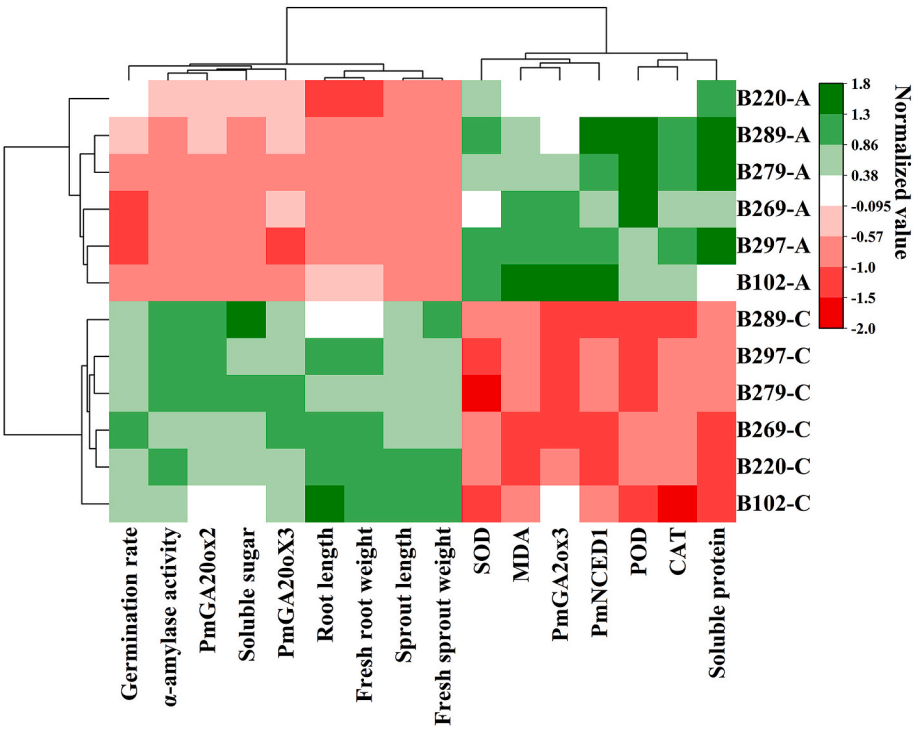

Fig. 6. Hierarchical clustering and heat map of biological traits of 6 broomcorn millet genotypes in control or alkali stress. Each column represents a trait and each row represents a genotype exposed in control/alkali stress. Colors indicate the normalized values (Z-score) of biological parameters after 7 days of alkali exposure. As indicated by the color bar, from deepest green equals the maximum to deepest red equals the minimum. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

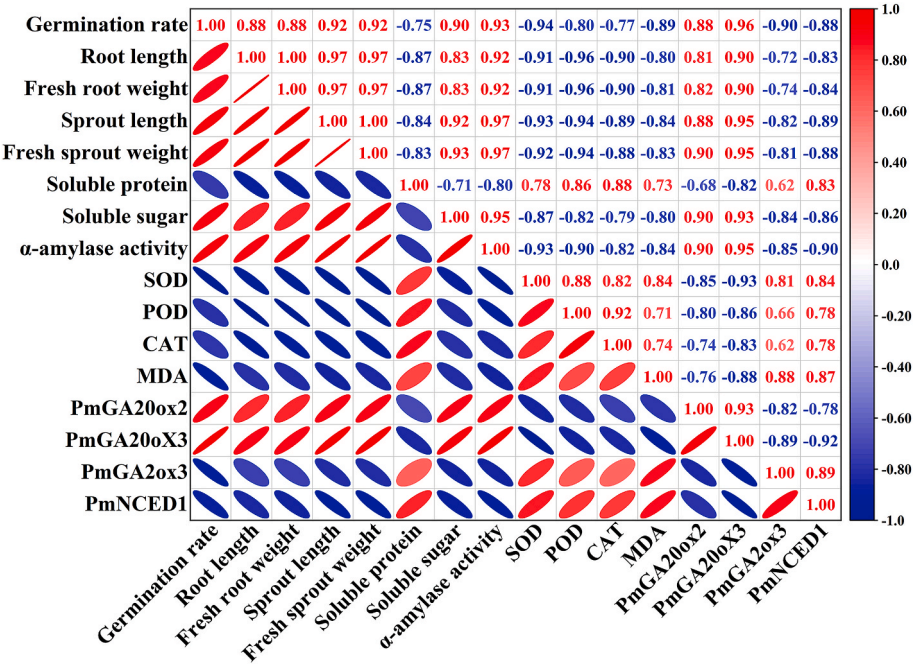

Fig. 7. Pearson's correlation coefficients of all biological traits of the six broomcorn millet genotypes under control and alkali stress. The thinner the ellipse, the stronger the correlation. As shown by the color bar, from the deepest red equals to the strongest positive correlation to the deepest blue equal to the strongest negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alkalinity-induced α-amylase activity (Liu et al., 2018). Interestingly, the soluble protein content increased under alkali stress. We believe that there are three explanations for this phenomenon. One is that increased protein content, which is an important osmolyte, helps in relieving the osmotic stress caused by the application of exogenous alkali. Another explanation could be that seed germination is a complex process of morphogenesis. It is not only affected by the growth potential of hypocotyls, but also by the ability of the seed covering (endosperm, seed coat, and pericarp) to overcome inhibition. The weakening and rupture of these tissues requires the action of cell wall proteins, such as expansion proteins and multiple hydrolases (Causin et al., 2020). In addition, the results of antioxidant defense revealed that the application of exogenous alkali significantly induced the activities of SOD, POD, and CAT, indicating that a considerable number of enzymes were activated under alkali stress.

Appropriate amount of ROS is a positive signal to promote

germination and break dormancy. Seed germination begins with the production and accumulation of ROS, which may be related to metabolic activities, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, lipid catabolism, lipid β-oxidation, and mitochondrial respiration (Mhamdi and Van Breusegem, 2018). In a previous study, a low concentration of H_2O_2 upregulated tobacco antioxidants to suppress the symptoms of necrotizing diseases, which also implied a positive effect of ROS on antioxidant formation (Hafez et al., 2012). However, other studies have shown that environmental stress leads to the excessive accumulation of ROS in plant cells. Excessive ROS is highly toxic to cells, leading to cell structure destruction and metabolic disorders, and ultimately to abnormal growth and even apoptosis (Mostofa et al., 2020; Patel and Parida, 2021). To combat the negative effects of excessive ROS, many antioxidant enzymes (SOD, POD, CAT, etc.) accumulate in cells to form an oxidative stress defense system to scavenge excess ROS and maintain normal physiological metabolism of cells (Mostofa et al., 2020). In the present study, the application of exogenous alkali stress significantly increased the activities of SOD, POD, and CAT in broomcorn millet seeds, which indicated that the oxidative stress caused by alkali stress led to lipid peroxidation and membrane damage. The increase in MDA content supported this. The activity of SOD increased differently in different genotypes, indicating that SOD plays a regulatory role in maintaining cell stability. In comparison with SOD and POD, the absolute value of CAT activity was lower, which is consistent with the results of previous studies. CAT activity was higher in the alkali stress groups than in the control groups on the 7th day, which was consistent with previous studies, showing that CAT mainly contributed to the later stages of stress response regulation (Hafez et al., 2012). This phenomenon has also been reported in other studies involving environmental stress (Nanda and Agrawal, 2016; Mostofa et al., 2020). The increase in MDA content also shows that, in the case of exogenous alkali exposure to broomcorn millet seeds, the anti-oxidative stress defense system cannot balance the accumulation of excessive alkali-induced ROS, which leads to inevitable damage to the cell membrane. The final effect was the inhibition of seed germination (the germination rate was reduced and growth during the budding period was inhibited).

The antagonism of GA and ABA has important regulatory effects on seed germination (Li et al., 2016; Nanda and Agrawal, 2016; Liu et al., 2018). Active GA/ABA is an important regulatory factor for seed germination. The germination of seeds subjected to abiotic stress, or that of GA-deficient mutants, is often inhibited. It has been reported that salt stress downregulates GA production but upregulates the biosynthesis of ABA, resulting in a decrease in the ratio of GA/ABA, impairing the germination of soybean seeds (Shu et al., 2017). In addition, studies have found that exogenous application of GA3 improved the germination rate of crops under salt and low temperature stress (Liu et al., 2018; Wang et al., 2018). Liu et al. (2018) detected higher concentrations of GA1 and GA4 in rice seeds with stronger salt tolerance. However, the effect of exogenous alkali application on GAs and ABA on the germination of broomcorn millet seeds is still unknown. Therefore, we studied the biosynthesis of GAs and ABA in the seeds of broomcorn millet germination (with/without alkali application) for one week. Our findings demonstrated that the application of exogenous alkali inhibited the germination of broomcorn millet seeds and the concentrations of GA1, GA3, GA4, and GA7 were all impaired under alkali stress. The ratio of GA to ABA during germination of broomcorn millet seeds was determined by the biosynthesis and inactivation of GA and ABA. To further explore the regulatory mechanism of exogenous alkali application on GA and ABA biosynthesis in germinated seeds of broomcorn millet, the effect of exogenous alkali application on the expression of *GA20ox*, *GA2ox*, and *NCED* was investigated in the present study. *GA20ox* and *GA2ox* are the key enzymes that encode GA synthesis and inactivation, respectively (Liu et al., 2018). In addition, *NCED* is a key enzyme in ABA biosynthesis (Shu et al., 2017). Our results showed that the application of exogenous alkali significantly downregulated the expression of *GA20ox2* and *GA20ox3* in the seeds of broomcorn millet, but upregulated the expression of the GA inactivation-related gene *GA2ox3* and the ABA synthesis-related gene *NCED1*. Furthermore, higher *GA20ox* but lower *GA2ox* and *NCED* expression levels in genotypes with low alkali damage rates were observed. We believe that the higher expression levels of GA biosynthesis-related genes, and lower expression levels of GA inactivation-related genes and ABA synthesis-related genes in alkali-stressed plants may be the main contributors to the high germination rate of broomcorn millet seeds to alkali stress. In addition, the Pearson's correlation heat map further showed the interaction between different parameters (Fig. 7). In cluster analysis, the six genotypes were divided into three sub-clusters in the cluster where exogenous alkali was applied, and their antioxidant enzyme activity, membrane lipid peroxidation, hormone synthesis and soluble protein were found to be different. These results indicate that there are natural differences in alkali tolerance between different genotypes.

Large tracts of saline-alkaline land can limit the sustainable development of world agriculture, but after reclamation and improvement, saline-alkaline land can be used as a reserve resource to ensure global food security. The restoration and improvement of saline-alkaline land, however, is expensive because it requires a lot of money and time. Vegetation restoration is an efficient measurement of ecological and economic benefits (Zhu et al., 2020). Therefore, the breeding and cultivation of salt-alkali-tolerant crops are of pivotal importance for the improvement of saline-alkaline land. In the present study, we explored the mechanism through which alkali stress represses the germination of broomcorn millet seeds, and found that this effect is controlled by the synergistic effect of hormones and enzyme activities.

5. Conclusion

Growing alkali-tolerant plants in alkalized soil can greatly increase the arable land area and improve and restore soil conditions and food security. Thus, understanding the regulation mechanism of alkali-tolerant plants at germination stage would be useful to improve the ability of other crops to resist alkali damage. This study is the first to experimentally examine the intraspecific variation in alkali tolerance of broomcorn millet at the germination stage. Broomcorn millet seeds maintained a strong antioxidant defense ability, high GA/ABA, and high α-amylase activity as common mechanisms that contribute to alkali resistance at the germination stage. More specifically, our results showed that higher concentrations of endogenous bioactive GA and lower concentrations of endogenous bioactive ABA enhanced the tolerance to alkali stress of broomcorn millet seeds at the germination stage. In addition, the activation of α-amylase and antioxidant enzymes (SOD, POD, and CAT) conferred resistance against exogenous alkali stress to broomcorn millet. These findings provide valuable information for understanding the mechanism of germination of broomcorn millet seeds in response to alkali stress and molecular breeding of alkali-tolerant plants. Therefore, this study will contribute to the phytoremediation of alkalized soil and world food security. In addition, plant resistance and adaptation to alkali stress is a complex trait involving multiple levels and multiple periods. Future research should integrate multi-omics analysis to determine the alkali tolerance mechanism of crops from germination to maturity. This research mainly focused on the osmotic regulation, antioxidant regulation, and hormone regulation mechanisms that occur at the germination stage. However, the effect of alkali stress on other growth stages is still unclear. Therefore, the endogenous hormone regulation pathway during the germination process and physiological and molecular mechanisms during the entire growth cycle should be studied in the future research on alkali tolerance of broomcorn millet.

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Author statement

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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.

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