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AtVIP1 AND GsNAC2 ENHANCE SALINE-ALKALI STRESS TOLERANCE BY PROMOTING PLANT GROWTH AND INDUCING STRESS RESPONSE GENE DURING THE GERMINATION PERIOD OF SORGHUM

AI Wenxiang, WU Rong, CHAI Guofei, LIU Ange, QIN Yihang, LIU Runhan, HE Xin, JIANG Xuewei, DAI Lingyan*

College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 163319, China

*Corresponding author, Email: dailingyan770416@126.com

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ABSTRACT

The quality and yields of Sorghum bicolor (L.) plants are seriously affected by saline-alkali conditions. VIP, and NAC (NAM, ATAF, and CUC) transcription factors are specific, and have various functions in plant development and response to various stresses. To study the tolerance to saline-alkali stress of transgenic sorghum overexpressed by AtVIP1 and GsNAC2 genes, sorghum seeds were treated with stress solution (NaHCO3: Na2CO3 = 5: 1, 75 mM, pH 9.63). After the stress treatment, the growth morphological indexes, stress-resistant physiological indexes and stress-related gene expression levels of sorghum buds were measured. The results show that the transgenic sorghum with overexpressing AtVIP1 and GsNAC2 genes had a higher bud length, fresh weight, and moisture content. Compared to the control, lower H2O2 and O2- levels, relative permeability of the plasma membrane, and MDA content, with higher POD, CAT, and SOD activities were found in transgenic sorghum. Gene expression analysis revealed that several stress response genes were up-regulated. Furthermore, these results suggest that AtVIP1 and GsNAC2 gene play potentially important roles in response to saline-alkali stress, and may be used in breeding new varieties to improve sorghum yields under adverse environmental conditions.

Keywords: Sorghum bicolor (L.); AtVIP1; GsNAC2; Overexpression; Saline-alkali stress.

1. INTRODUCTION

Sorghum bicolor (L.) is the fifth largest food crop in the world. It has the characteristics of high photosynthesis efficiency, high yield, drought resistance, water logging resistance, barren resistance and saline-alkali resistance (Fu *et al.* 2019; Paterson *et al*. 2009; Yang *et al*. 2017). It can be used as raw materials for grain, feed, making wine, and making paper (Li *et al*. 1995).

Transcription factor VIP1 (VirE2-Interacting Protein 1) is named from its interaction with VirE2 (Type IV Secretion System Single-Stranded DNA Binding Protein VirE2) (Li *et al*. 2005). AtVIP1 belonged to the IX subfamily of arabidopsis bZIP family has two copies of nuclear location sequence, which indicates that this protein can be located in the nucleus (Dingwall 1991). Some researches have shown that VIP1 may affect the transformation efficiency of *agrobacterium tumefaciens* (Tzfira *et al*. 2000), and may also be involved in sulfur utilization, starch accumulation, signaling in osmotic stress, and root movement by touch-induced (Chen *et al*. 2016; Tsugama *et al*. 2014; Tsugama *et al*. 2016). Lapham *et al*. (2018) found that the growth

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of VIP1 mutant plants were changed under salt stress, indicating that VIP1 plays a role in salt stress, but its specific mechanism is unknown.

NAC is a kind of peculiar transcription factor to plants and contains highly conserved NAC domains at the N-terminal of proteins (Mohanta *et al*. 2020). A large number of studies have shown that plant NAC transcription factors can promote the growth of cell wall, lateral root, leaf senescence, and regulate the biosynthesis of secondary metabolites (Zhong *et al*. 2021; Sun *et al*. 2020; Zhu *et al*. 2017; Zhang *et al*. 2018; Mao *et al*. 2020; Oda-Yamamizo *et al*. 2016; Nagahage *et al*. 2020; Ren *et al*. 2018; Kelly *et al*. 2021). Others have indicated that NAC transcription factors possess abundant and important biological functions, and play important roles in plant growth and development, response to different hormones, and resistance to stress. For example, overexpression of *SlNAC35* in tomato improves the tolerance of transgenic tobacco to drought stress, salt stress and bacterial pathogens (Wang *et al*. 2016). *OsNAC6* was overexpressed in rice plants, which enhanced transgenic rice tolerance to water shortage, high salt stress, and rice blast (Singh *et al.* 2021). Overexpression of *OsNAC066* in rice improved the sensitivity of transgenic rice to abscisic acid, while the water loss rate was decreased, the content of proline and soluble sugar was increased, and the accumulation of reactive oxygen species was decreased, all these enhanced the tolerance of rice to drought and oxidative stress (Yuan *et al*. 2019). Overexpression of *SbNAC2* in arabidopsis and rice enhanced their tolerance to abiotic stress (Jin *et al*. 2021).

The wild soybean has strong environmental adaptability, and can grow in waterlogged depression, saline-alkali land, barren soil, and arid soil, and it is an ideal material for cloning tolerance genes to abiotic stress (Cai *et al*. 2011). It is a relative species of cultivated soybean (*Glycine max*), which is rich in genetic diversity, and contains many excellent resistance resources (Wang *e al*. 2019). In this study, to study the effects of overexpression of *AtVIP1* and *GsNAC2* genes under saline-alkali stress, three sorghum strains were selected as materials, some phenotypic indexes, stress-resistant physiological indexes, and expression patterns of several resistance related genes were analyzed. The research results can bring about data support for further understanding the molecular mechanism of plant tolerance to saline-alkali stress, and also provide new genes for molecular breeding of sorghum tolerance to saline-alkali stress.

2.MATERIALS AND METHODS

Experimental Materials

Three sorghum strains, wild type *P898012* lines (WT), receptor lines *AtVIP1* (V1), and transgenic lines with overexpressing *AtVIP1* and *GsNAC2* (OE) used in this study were provided by Plant Gene Function Laboratory of College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Heilongjiang Province. The *AtVIP1* lines (V1) overexpressed the Arabidopsis *VIP1* gene (*AtVIP1*) in sorghum through an Agrobacterium mediated method using wild sorghum resource *P898012* as the receptor. After many transformation experiments, it was proved that *AtVIP1* lines as receptor could greatly improve the transformation efficiency of sorghum. *AtVIP1* and *GsNAC2* gene lines (OE) was overexpressed *Glycine soja NAC2* gene (*GsNAC2*) using *AtVIP1* line as receptor.

Experimental design

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Plant material culture and growth conditions

Seeds were selected according to full grains and uniform size. After washed with distilled water for 2-3 times, the seeds were soaked in 75% alcohol for 2-3 min, washed with distilled water again. then soaked in 3.5 % sodium hypochlorite for 3-4 min, and finally rinsed with distilled water for 5-6 times. The sterilized sorghum seeds were put neatly in petri dishes covered with three layers of filter paper, 10 mL distilled water was added into each petri dish. 50 seeds were put neatly in each petri dish, and carried out three biological repeats per treatment. Seeds germinated for 24 h in an artificial incubator at 25 ℃ and dark environment.

Saline-alkali treatment

Sorghum seeds were treated with solution ($NaHCO_3$: Na_2CO_3 = 5: 1, 75 mM, pH 9.63) for saline-alkali treatment after 24 h of germination, the control group was treated with distilled water. Samples were sampled for determination of morphological and physiological indicators after 72 h stress.

Growth morphology observation and growth index measurements

The growth phenotype of sorghum was recorded with an SLR camera. Bud length were measured with a vernier caliper. After the fresh weight was determined, the sorghum buds were deactivated at 105 ℃ for 10 min, and then dried at 85 ℃ for 12 h until the weight was constant. The dry weight was subsequently measured.

Damage degree of membrane system measurements

The relative permeability of plasma membrane was measured by conductivity method. MDA was measured by thiobarbituric acid colorimetry, and the absorbance was measured at 450, 500, and 600 nm wavelengths.

Active Oxygen measurements

The sorghum buds were immersed in DAB/NBT dye solution and placed in vacuum environment for 30 minutes. After stained for 12 h in 37 ℃ incubator, the samples were decolorized with 95 % ethanol in 80 ℃ water bath, and then the ethanol was changed every 10 min until they were completely decolorized. The stained buds were observed under stereomicroscop.

Resistance enzymes measurements

The total activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in sorghum were determined using the kit from the Suzhou Geruisi Biotechnology Co., Ltd., and the procedures were carried out according to their respective instructions. Three parallel samples were made for each treatment.

RNA extraction and cDNA library preparation

Total RNA was extracted from different groups of sorghum buds by TRIzol reagent, RNA concentration was detected by a Nanodrop 2000 TM microspectrophotometer, and RNA quality was assessed by electrophoresis. Reverse transcription was performed using ReverTra Ace® qPCR RT Master Mix with a gDNA Remover kit (TOYOBO, Shanghai, China). After the

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reaction, cDNA was subpackaged and stored in a refrigerator at -150 ℃. To remove RNAse pollution, all utensils were baked at high temperatures or treated with DEPC-H_2O during processes of RNA extraction and reverse transcription.

Real-time fluorescence quantitative PCR

qRT-PCR was performed on a Bio-Rad CFX96 Real Time PCR System using Thunderbird TM SYBR® qPCR Mix (TOYOBO, Shanghai, China). All primers for qRT-PCR were designed using Primer 3 plus software. The β-actin gene was used as an internal reference gene, and the relative expression level of the gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

Data processing

The data were analyzed by IBM SPSS® Statistics software (Version 20.0). The statistical significance was assessed using independent sample T test at $P < 0.05$. The significance analyses were conducted to compare the effects of the different groups (WT, V1 and OE) on each parameter. All graphics were generated using Prism 8 software (Graph Pad, San Diego, CA).

3.RESULTS AND ANALYSIS

Effects of saline-alkali stress on phenotype in sorghum buds

A large number of studies have shown that under saline-alkali stress, the growth and development of plants are slow, and the root system is underdeveloped, which affects the external morphology of plants. After 72 h stress, the buds of WT were shorter, and the OE lines were the longest. Among the three lines, the OE lines had the best sgrowth status of orghum seeds, and followed by V1 lines under saline-alkali stress (Figure 1).

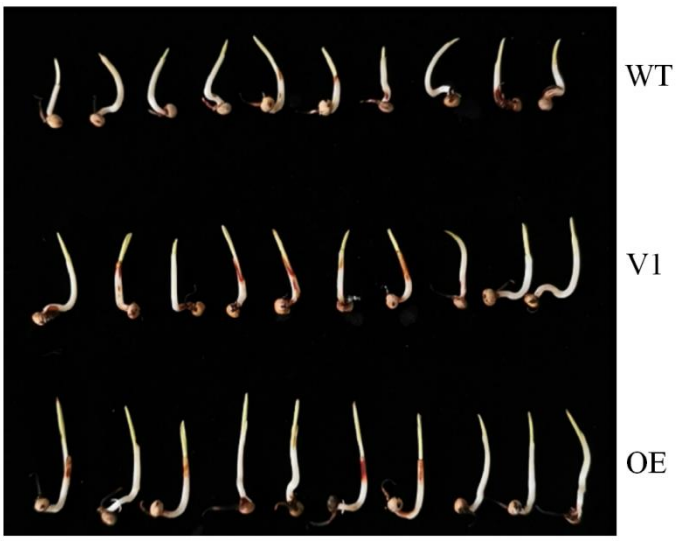

Figure 1 Effects of saline-alkali stress on phenotype of sorghum buds

Effects of saline-alkali stress on growth indexes in sorghum buds

The effects of saline-alkali stress on sorghum growth indexes are shown in Figure 2, saline-alkali solution inhibited the growth of different sorghum lines. Yet, the inhibition degrees of three lines

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were different. Under saline-alkali stress, compared to WT lines, transgenic lines (V1 and OE) had larger values on bud length, fresh weight of bud, and moisture content. Among three lines, the damage degree of OE lines caused by saline-alkali stress was the significantly smallest ($P < 0.05$)

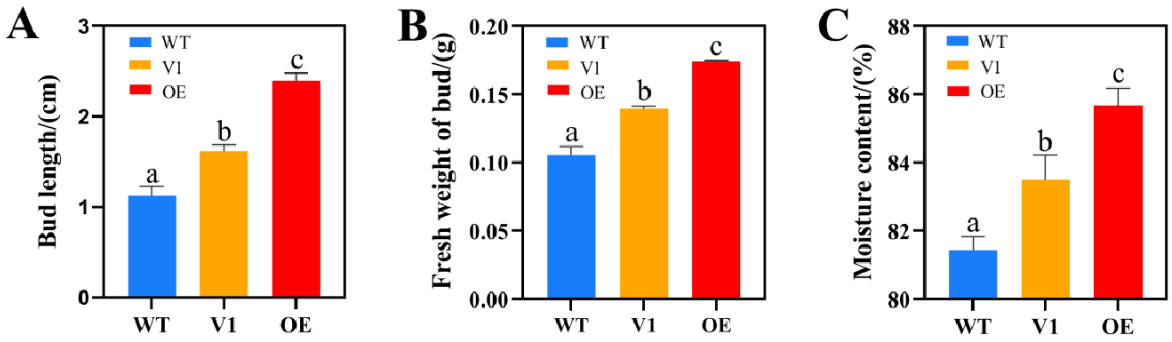

Figure 2 Effects of saline-alkali stress on growth indexes of sorghum buds. (A) Bud length. (B) Fresh weight of bud. (C) Moisture content. Different lowercase letters indicate that there are significant differences between treatments ($P < 0.05$)

Effects of saline-alkali stress on damage degree of the membrane system in sorghum buds

The effects of saline-alkali stress on the relative permeability of the plasma membrane of sorghum buds are shown in Figure 3A. Under normal circumstances, the relative permeability of plasma membrane of different sorghum buds were low. But, under saline-alkali stress, the relative permeability of plasma membrane of sorghum buds were increased. Compared with control, OE lines were increased by 1.25 times, WT lines were increased by 1.53 times, which indicated that the plasma membrane of WT were damaged most seriously, while the OE lines were suffered the least, and there was a significant difference between any two lines ($P < 0.05$). The effects of saline-alkali stress on MDA content in sorghum buds of three lines were consistent with the relative permeability of plasma membrane (Figure 3B).

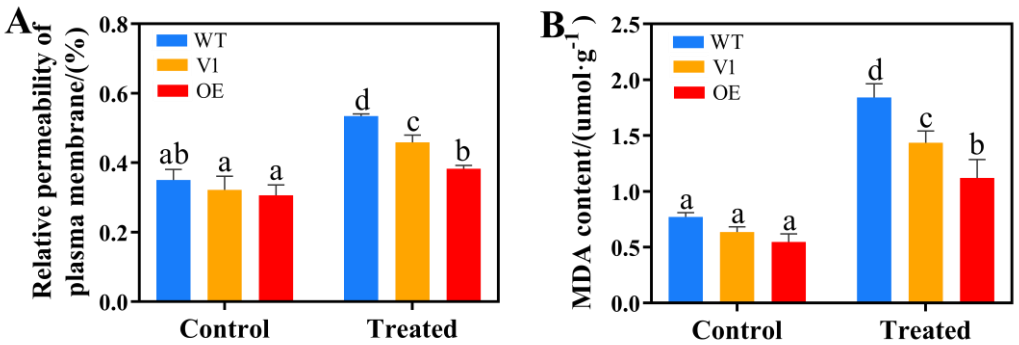

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Figure 3 Effects of saline-alkali stress on damage degree of a membrane system of sorghum buds. (A) Relative permeability of plasma membrane. (B) MDA content. Different lowercase letters indicate that there are significant differences between treatments ($P < 0.05$)

Effects of saline-alkali stress on active oxygen in sorghum buds

To further explore the effects of saline-alkali stress on active oxygen (ROS), sorghum buds were stained with NBT (detection of O_2^-) and DAB (detection of H_2O_2) (Figure 4). The results showed that the balance between the production and removal of ROS in sorghum buds was broken after saline-alkali treatment, and ROS was accumulated in three lines to various degrees. Under saline-alkali stress, more ROS was accumulated in WT buds, while less in OE.

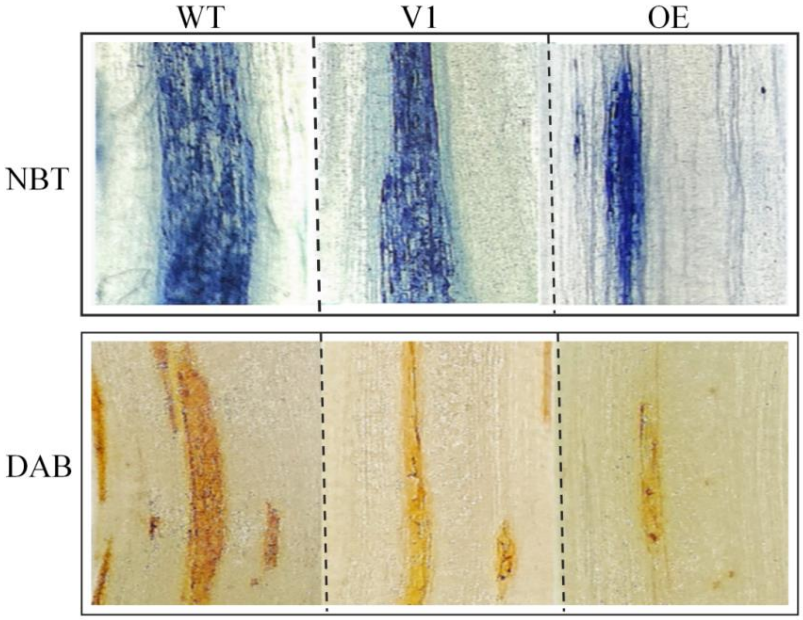

Figure 4 Effects of saline-alkali stress on ROS of sorghum buds

Effects of saline-alkali stress on antioxidant enzyme activities in sorghum buds

Under saline-alkali stress, plants accumulate a large amount of active oxygen, and enzymes such as POD, SOD, and CAT play important roles in scavenging ROS. Saline-alkali stress increased the activities of antioxidant enzymes in sorghum buds, and there were different degree of changes among three sorghum lines. After 72 h stress, the activities of POD, SOD, and CAT increased among all lines, and there were no significant differences in POD activities between V1 and OE (Figure 5A), but there were significant differences in CAT and SOD activities among the three lines (Figure 5B, C). However, SOD activities of WT did not increase but decreased by 14.7 % (Figure 5C).

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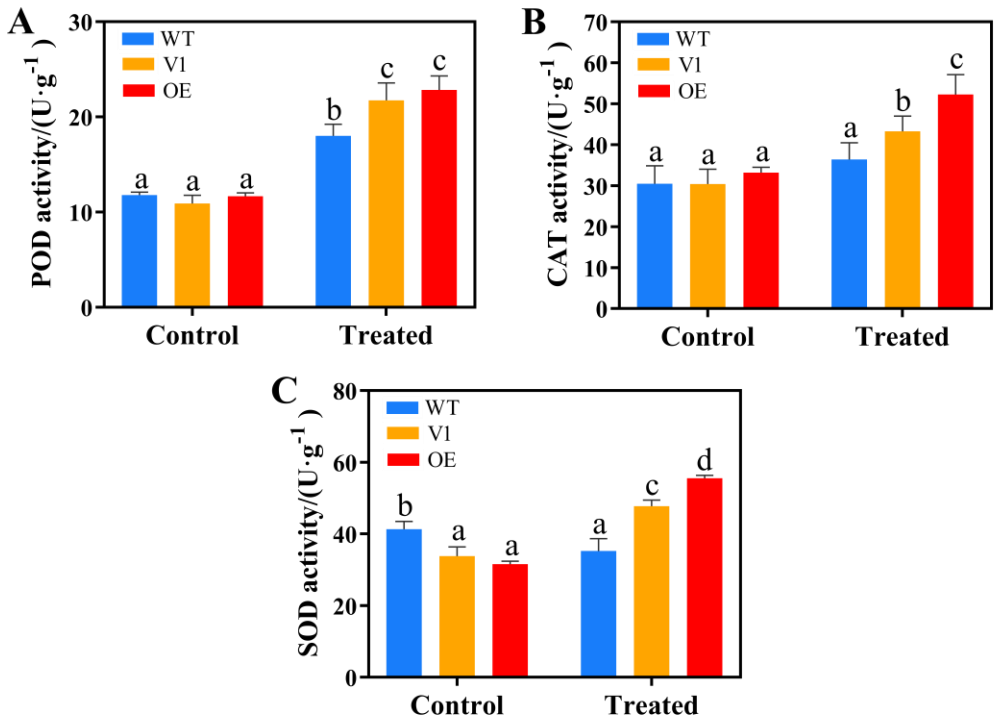

Figure 5 Effects of saline-alkali stress on antioxidant enzyme of sorghum buds. (A) POD activity. (B) CAT activity. (C) SOD activity. Different lowercase letters indicate that there are significant differences between treatments ($P < 0.05$)

Expression pattern of several stress resistance genes in sorghum buds under saline-alkali stress

To further explore the molecular mechanism of saline-alkali tolerance in sorghum, the relative expression levels of some stress resistance genes in three sorghum lines were detected by qRT-PCR, that is, *MYB44*, *VP1*, *MPK3*, *AP37*, *DREB2B*, and *SOS2* (Figure 6). Under normal conditions, there was no significant difference of all genes expression levels among sorghum lines. However, after 72 h saline-alkali stress, the expression levels of *MYB44*, *VP1*, *MPK3*, *AP37*, *DREB2B*, and *SOS2* genes were significantly increased, and there were significant differences among the three lines. The expression levels of all genes was the highest in OE lines, followed by V1 lines, and the lowest in WT lines.

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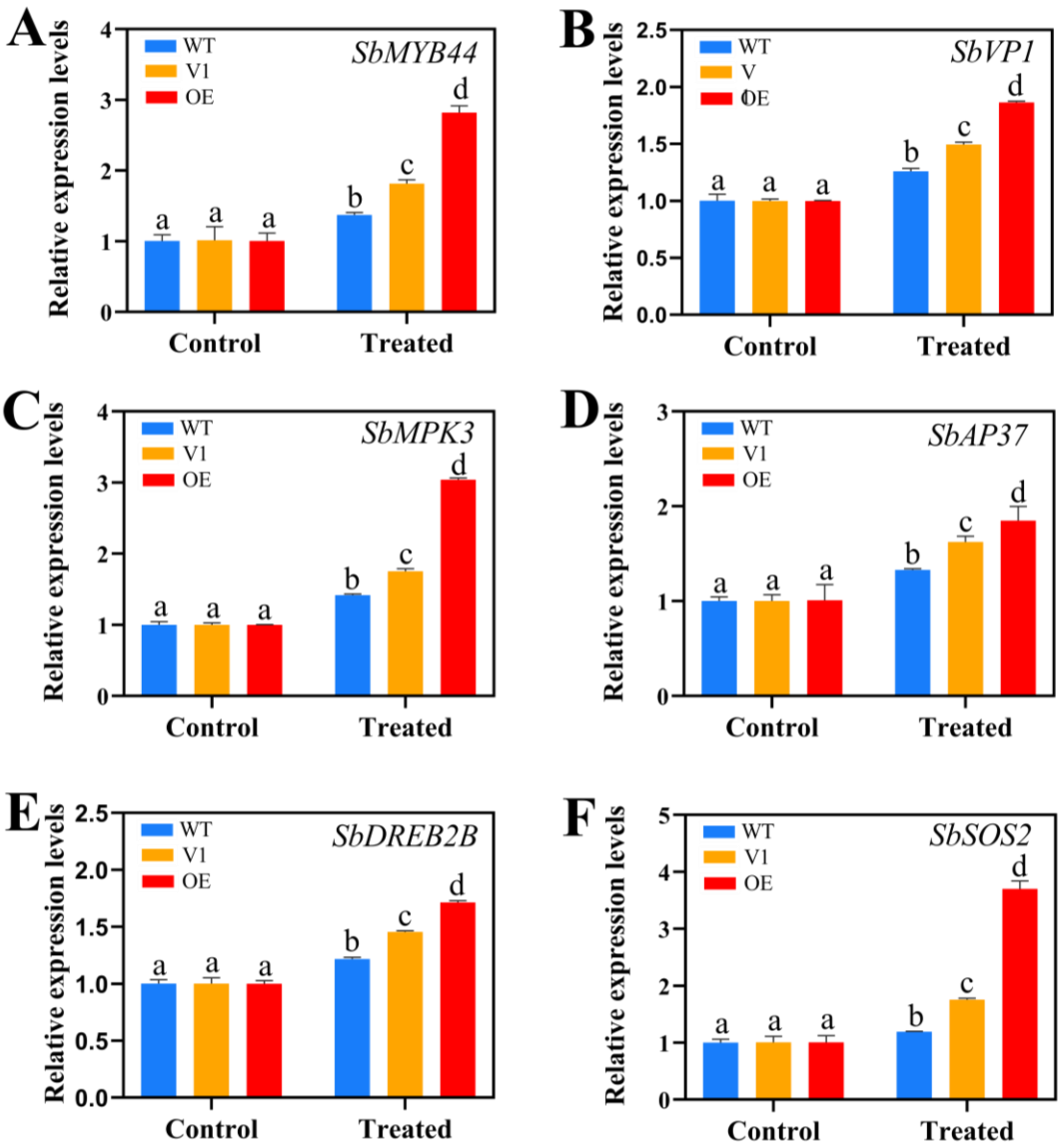

Figure 6 Effects of saline-alkali stress on relative expression levels of stress resistance genes in sorghum buds. Different lowercase letters indicate that there are significant differences between treatments ($P < 0.05$)

4. DISCUSSION

Soil salinization is one of the main factors leading to the reduction of crop yield, which has a significant inhibitory effect on the growth and development of plants. Saline-alkali stress increases the salinity and pH of the environment in which plants grow, which hinders the normal water absorption of plants, and at the same time causes nutrient deficiency in plants due to stress-induced ion toxicity (Munns *et al*. 2002; Rogers *et al*. 2003). In order to adapt to the saline-alkali environment, plants must live in a continuous interaction with environmental fluctuations and

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stress constraints, which means a series of complex environmental stress sensing and signaling mechanisms are involved (Zhu *et al*. 2016).

In this study, compared with WT and V1, in terms of sorghum growth, OE had strong resistance to saline-alkali stress, which was reflected in the growth morphology, including less inhibition in bud length, bud fresh weight, and moisture content inhibition after stress (Figure 1, 2). It can be seen that overexpression of the gene *AtVIP1* and *GsNAC2* can improve the saline-alkali resistance of sorghum buds by reducing the effect of saline-alkali stress on plant growth and development.

A large number of studies have shown that the cell membrane permeability changes are less in the tolerant varieties under salt stress, while changes are bigger in the sensitive varieties. Saline-alkali stress also increases lipid peroxidation in membranes, and MDA, as a product of membrane lipid peroxidation, can cross-link and inactivate enzymes and proteins in membranes, further damaging the structure and function of cell membranes (Dinneny *et al*. 2015; Yao *et al*. 2019; Mohamed *et al*. 2015). In this study, the relative permeability of plasma membrane and MDA content of sorghum buds were increased after stress, and the increased trend of OE lines was the smallest (Figure 3), indicating OE lines can significantly reduce the relative permeability of plasma membrane, and the accumulation of MDA, and protect the stability of the cell membrane, thereby enhancing the resistance of sorghum buds to saline-alkali stress.

ROS stress is one of the common damages of plants under abiotic stresses such as salinity and drought. Saline-alkali stress increases the ROS content in plants, and the ROS content in salt-tolerant cultivars is lower than that in sensitive cultivars (Sairam *et al*. 2002). In our study, the content of two most common ROS, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), was detected by NBT and DAB staining. The results of NBT and DAB staining showed that the OE lines had less ROS accumulation than V1 and WT after saline-alkali stress treatment (Figure 4). The overall results suggested that OE sorghum lines may achieve saline-alkali tolerance by reducing the accumulation of ROS.

Under saline-alkali stress, the accumulation of ROS in plants will disturb the normal physiological functions of cells, lead to metabolic disorder, and seriously affect the growth and development of plants. In order to resist the damage caused by ROS, plants can form a set of ROS scavenging system, such as POD, CAT and SOD, to cope with stress environment, thus improving the tolerance of plants in adversity environments (Guo *et al*. 2019; Qin *et al*. 2021; Abass *et al*. 2018). The activities of protective enzymes in sorghum buds under saline-alkali stress were increased, and the activities of SOD, CAT, and POD were highest in OE lines, which indicated that overexpression of *AtVIP1* and *GsNAC2* genes could significantly enhance the resistance of sorghum to saline-alkali stress.

We further explored the underlying molecular mechanisms of sorghum buds responding to saline-alkali stress. Several resistance-related genes including *SbMYb44*, *SbVP1*, *SbMPK3*, *SbAP37*, *SbDREB2B and SbSOS2 were* screened. *VP1* can directly act on the VREs motif (ACNGCT) in the promoter region of *MYB44* to initiate stress response (Tzfira *et al*. 2001). *VP1* can be phosphorylated by *MPK3* and then triggered a stress defense response in Arabidopsis (Djamei *et al*. 2007). *AP37* belongs to the AP2/ERF class of transcription factors (Sharoni *et al*. 2011), and NAC2 can directly bind to the *SbAP37* motif (TTACGTA) to respond to abiotic stress (Jin *et al*. 2021). *DREB2B* is a member of the DREB2 family, and regulates the expression of several stress-inducible genes in enhancing plant tolerance to drought and salinity (Quan *et al*.

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2017). A. thaliana can specifically activate the expression of *SOS2* genes after receiving salt stress signals (Lin *et al.* 2009). The *SbMYb44*, *SbVP1*, *SbMPK3*, *SbAP37*, and *SbDREB2B* genes had higher expression levels in the OE lines compared with the WT and V1, indicating that the overexpression of *AtVIP1* and *GsNAC2* genes initiates the transcriptional changes of resistance-related genes.

Sorghum overexpressed *AtVIP1* and *GsNAC2* genes showed strong resistance in both metabolic process and growth and development under saline-alkali stress. But, the molecular mechanism of the double genes response to saline-alkali stress remains to be further studied.

5.DECLARATION OF FUNDING

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