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Synergistic responses of NHX, AKT1, and SOS1 in the control of Na^+ homeostasis in sweet sorghum mutants induced by $^{12}C^{6+}$-ion irradiation

Wen-Ting Gu[1] · Li-Bin Zhou[1] · Rui-Yuan Liu[1] · Wen-Jie Jin[1] · Ying Qu[1] · Xi-Cun Dong[1] · Wen-Jian Li[1]

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Abstract Sweet sorghum mutants induced by $^{12}C^{6+}$-ion irradiation were planted under different soil salinity conditions to investigate the mechanisms maintaining the transport and spatial distribution of Na^+. The functions of the synergistic responses of NHX, AKT1, and SOS1 related to Na^+ accumulation were investigated in control (KFJT-CK) sorghum and KF1210-3 and KF1210-4 mutants. The results indicated that the NHX, AKT1, and SOS1 proteins in sweet sorghum are mainly involved in the transport, exclusion, and spatial distribution of Na^+, respectively. In addition to physiological parameters, we also measured the expression levels of *NHX*, *AKT1*, and *SOS1* genes. The experimental results indicated that 150 mM NaCl induced marked increases in the transcripts of NHX and SOS1 after 8 and 12 h in the KF1210-3, KF1210-4, and KFJT-CK cultivars. In contrast, however, a decrease in AKT1 was observed. On the basis of our results, we propose a model in which cooperation among NHX, AKT1, and SOS1 facilitates Na^+ homeostasis in sweet sorghum in response to an increase in salt concentration. Accordingly, study of the regulatory mechanisms in sweet sorghum generated by carbon ion irradiation is essential for the selection of salt-tolerant cultivars.

Keywords $^{12}C^{6+}$-ion irradiation · Sweet sorghum · Salt stress · NHX · AKT1 · SOS1

1 Introduction

As one of the major abiotic stresses, salinity constrains the expansion of agriculture on uncultivated land and limits the productivity of crops worldwide [1–3]. It has been reported that approximately one-third of farmland has suffered from the adverse effects of salinity. Most plants are very sensitive to salt stress. On the one hand, salt stress interferes with the ion homeostasis of cells, which results in the dysfunction of cell membranes, whereas on the other hand, it attenuates metabolic activity, which inhibits plant growth [4, 5]. However, in order to adapt to environments and maintain homeostasis between Na^+ and K^+, halophytes have developed various protective mechanisms [6–9]. The key mechanisms underlying resistance to salinity in plants involve a reduction in the accumulation of Na^+ and maintenance of K^+ stabilization [10, 11], including the extrusion of Na^+ from plant roots, compartmentalization of Na^+ into the cell vacuole, and reduction of the Na^+ flux into plant roots. A considerable amount of interest has therefore been focused on the physiological and molecular mechanisms underlying the responses of salt-tolerant plant to salt stress.

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Wen-Ting Gu, Rui-Yuan Liu and Li-Bin Zhou have contributed equally to this research.

✉ Xi-Cun Dong
dongxicun@impcas.ac.cn

✉ Wen-Jian Li
wjli@impcas.ac.cn

[1] Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, China

Among salt-tolerant plants, sweet sorghum is characterized by its excellent adaptability to adverse environments. Our previous studies showed that sweet sorghum can accumulate larger quantities of Na^+ for osmotic adjustment [12–14]. It is imperative to screen novel varieties and increase the amount of superior raw materials of sweet sorghum. However, given that traditional hybridization-based breeding is associated with a lack of genetic resources, $^{12}C^{6+}$-ion irradiation, which is an effective method for generating sweet sorghum mutants, has been widely applied to induce mutations in plant breeding [15–17]. The high linear energy transfer (LET) of $^{12}C^{6+}$-ion irradiation can be applied to increase the mutation frequency and spectrum, and this technique can accordingly be used to generate increased amounts of DNA fragments within irradiated plants [18, 19]. Such nuclear techniques can therefore provide us with valuable resources for screening and identifying sweet sorghum lines that are tolerant to salt stress.

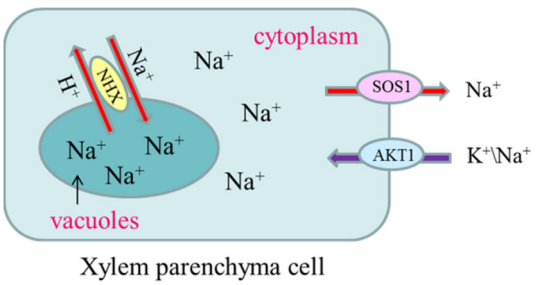
It has also been revealed that the response of sweet sorghum to salt is related to the accumulation of Na^+ [15]. It is well known that high concentrations of Na^+ disturb the absorption of K^+, which leads to a lack of K^+, thereby inhibiting plant growth [5]. However, although the concentration of Na^+ in sweet sorghum can increase significantly under salt stress, the concentration of K^+ decreases only slightly or even remains unchanged. This indicates that sweet sorghum has a powerful ability to regulate the homeostasis of Na^+, which can reduce the osmotic potential of cells and maintain plant growth. NHX (a tonoplast Na^+/H^+ antiporter) plays a key role in the sequestration of Na^+ into cell vacuoles to induce the concentration of Na^+ in the cytoplasmic [20–23]. Studies on various plant species have shown that NHX can improve salt tolerance via the mechanisms of osmotic adjustment and reduction in the toxicity of Na^+ in the cytosol under salinity stress [24]. It has been determined that Na^+ can be transported into cells by Na^+/K^+ carriers, which are associated with AKT1 (an affinity K^+ transporter), which has a high affinity for K^+ and a low affinity for Na^+ [25]. As one of the family of K^+ transporters, AKT1 is linked to the permeation of Na^+ and acts as a Na^+-selective uniporter to control the absorption of Na^+ [26, 27]. In addition, the extrusion of Na^+ from cells has been shown to be mediated by SOS1 (a plasma membrane Na^+/H^+ antiporter) [28], which is an important Na^+ transporter located in the plasma membrane of xylem parenchyma cells. Although SOS1 has the same expression location as AKT1, the physiological function of SOS1 contrasts with that of AKT1. Shi et al. [29] reported that the activity of SOS1 can be sharply induced by salt stress, and that SOS1 can improve the efflux of Na^+ into the apoplastic spaces by reducing the accumulation of Na^+. However, to date, it has remained unclear how NHX, AKT1, and SOS1 function synergistically in regulating the homeostasis between Na^+ and K^+ in sweet sorghum. A more thorough knowledge of these transport systems and regulatory mechanisms is crucial for a better understanding of the salt tolerance mechanisms of sweet sorghum.

In the present study, sweet sorghum mutants were screened using a heavy ion radiation mutation breeding technique. The functions of the synergistic responses of NHX, AKT1, and SOS1 to Na^+ accumulation were investigated in control sorghum and the mutant sorghum lines KF1210-3 and KF1210-4. Under conditions of salt stress, we evaluated the roles of the *NHX*, *AKT1*, and *SOS1* genes in regulating the homeostasis of Na^+. The results indicated that the NHX, AKT1, and SOS1 proteins in sweet sorghum are mainly involved in the transport, exclusion, and spatial distribution of Na^+, respectively. NHX, AKT1, and SOS1 play vital roles in controlling the absorption of ions, which maintains Na^+ homeostasis and regulates the growth of sweet sorghum. Studies of the relative expressions of *NHX*, *AKT1*, and *SOS1* and the associated regulatory mechanisms in sweet sorghum cultivars generated by carbon ion irradiation are essential for the selection of salt-tolerant cultivars (Scheme 1).

2 Experimental methods

2.1 Plant materials and growth conditions

Seeds of sweet sorghum, *Sorghum bicolor* (L.) Moench "KFJT-CK," were provided by the Institute of Modern Physics, Chinese Academy of Sciences (IMPCAS). Dry seeds of equal size without mold or lesions were selected. The carbon ions used for mutagenesis were generated by the Heavy Ion Research Facility in Lanzhou (HIRFL), IMPCAS. The energy of $^{12}C^{6+}$-ions used was 100 MeV/u, with a $^{12}C^{6+}$-ion irradiation dose rate of approximately 20 Gy/min. The dry seeds of sweet sorghum were irradiated by $^{12}C^{6+}$-ion at doses of 0, 20, 40, 60, and 80 Gy. The



Scheme 1 (Color online) A simplified model of the functions of NHX, AKT1, and SOS1 in regulating the Na^+ transport system in xylem parenchyma cells of sweet sorghum under saline conditions

KF1210-3 and KF1210-4 mutants were screened using an $^{12}C^{6+}$-ion irradiation dose of 80 Gy provided by the accelerators at HIRFL and bred for further experiments.

Seeds of KF1210-3, KF1210-4, and KFJT-CK were grown in growth chambers at 22 °C and 70% relative humidity under continuous illumination at 5000 lx for 7 days. Three replicates of five seeds each were utilized for each treatment. NaCl concentrations of 0, 50, 100, 150, 200, 250, and 300 mM were used as salinity treatments. After 7 days, plant heights were measured. The plantlets were rinsed in deionized water to remove surface salts, and then, the fresh tissue weight was measured. Thereafter, tissues were immediately dried at 108 °C for 1 h and 65 °C for 24 h, and the dry weight was measured.

2.2 Catalase analysis

Following the treatments with 0, 50, 100, 150, 200, 250, and 300 mM NaCl, root samples of sweet sorghum (100 mg each) were ground into a fine powder using liquid nitrogen in a pre-chilled mortar and pestle. Further grinding was performed in a solution of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 2% (w/v) polyvinyl polypyrrolidone (PVPP) for catalase (CAT) assays. The homogenates were centrifuged twice at 14,000×*g* for 15 min at 4 °C. The final supernatants were used immediately for enzyme activity assays or stored at − 20 °C for later use. Total CAT activity was determined using CAT kits (Keming Biotech Co., Ltd, Suzhou, China).

2.3 Real-time quantitative PCR analysis

The roots of sweet sorghum plants treated with 150 mM NaCl were harvested at 0, 2, 4, 8, 12, and 24 h. The expression levels of *NHX*, *AKT1*, and *SOS1* genes were analyzed by real-time quantitative PCR. The roots were treated as follows. (1) Total RNA was extracted using an RNAprep pure plant kit (TianGen, Biotech Co., Ltd, Beijing, China). (2) RNA samples were quantified according to the absorbance at 260 nm, with the purity being evaluated according to the 260/280 nm ratio. (3) First-strand cDNA was synthesized using MMLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). (4) The reverse-transcribed cDNA samples were used for real-time quantitative PCR, which was performed using a thermal cycler (QIAGEN, Dusseldorf, Germany). A specific fragment (158 bp) of *SOS1* was amplified using the primer pair P1 and P2; the *AKT1*-specific fragment (111 bp) was amplified using primers P3 and P4; and the *NHX*-specific fragment (140 bp) was amplified using primers P5 and P6 (primer sequences are shown in Table 1). Experiments were performed at least three times. The *Actin* gene was also amplified for the purpose of RNA normalization. The specific primers for *Actin* (A1 and A2; Table 1) amplified a 170-bp fragment. SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for 20 μL PCR, with amplification being performed under the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. Each sample was amplified three times. The relative expression levels (RELs) of all the samples were calculated and analyzed (ABI PRISM 7500 sequence detection system). The REL of each sample was calculated using the following equation: $\text{REL} = 2^{-\text{ddCt}}$, where the Ct value of target genes and *Actin* in different samples was obtained after the quantitative real-time PCR. In brief, the normalized *Actin* Ct value was subtracted from the Ct value of the gene of interest (target gene) to give the dCt value of the sample. The dCt value of the calibrator (the sample with the 0 h dCt value in our experiment) was subtracted from that of every other sample to yield the respective ddCt values. The two to the power − ddCt ($2^{-\text{ddCt}}$) values for each sample were used as the relative expression levels [30].

Table 1 Primer sequences used in the real-time quantitative PCR analysis

Primer	Sequence (5′–3′)
P1	ggtgccagcaaaaagctaag
P2	aaacccaagccacaaaacag
P3	agaacaggtggttggagtgg
P4	cacctaccattacgccgtct
P5	gctcgcatcttttggttttc
P6	ttgcaagtaagtcccccaac
A1	aggagcttgagaaggagccca
A2	tccacgctcttgatgactcca

2.4 Statistical analysis

The results are presented as means with standard error of the means. Data analysis was performed with one-way analysis of variance (ANOVA) using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was applied to distinguish the difference between means at a significance level of $P < 0.05$.

3 Results and discussion

3.1 Biomass production of KFJT-CK, KF1210-3, and KF1210-4

The effects of salt on the biomass production of sweet sorghum are shown in Fig. 1. Under control conditions, the KF1210-3 mutant had the highest plant height, fresh

weight, and dry weight. These results revealed that the plant height and biomass of sweet sorghum can be affected by carbon beam irradiation. Moreover, the plant heights and fresh weights of KFJT-CK, KF1210-3, and KF1210-4 were reduced with an increase in salt concentrations, whereas the dry weights showed little variation with an increase in salinity. Among the KFJT-CK, KF1210-3, and KF1210-4 cultivars subjected to increasing salt concentrations in culture rooms, the KF1210-3 cultivar was characterized by the strongest resistance to salinity. These results indicate that carbon ion irradiation significantly altered the growth of sweet sorghum under both normal and saline conditions.

3.2 Catalase activity

The effects of NaCl on the CAT activities of KFJT-CK, KF1210-3, and KF1210-4 are shown in Fig. 2. The CAT activity of the sweet sorghums was observed to increase with an increase in the concentrations of NaCl from 0 to 150 mM ($P < 0.05$). At a concentration of 150 mM NaCl, the CAT activity in KFJT-CK was the highest. However, whereas at a concentration of 200 mM NaCl, the CAT activity in KF1210-3 continued to increase, that in KFJT-CK and KF1210-4 showed a decrease. An increase in the antioxidant activities of sweet sorghum could be a response

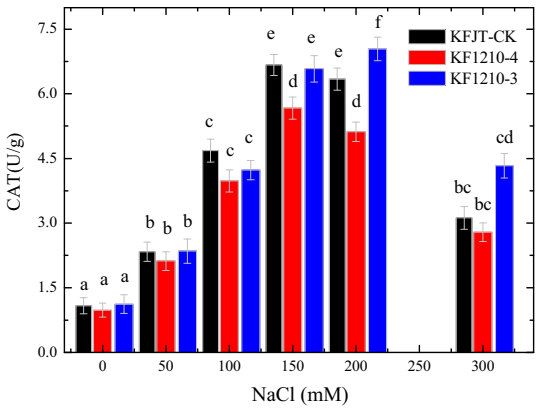


Fig. 2 (Color online) Catalase (CAT) activities of sorghum cultivars KFJT-CK, KFJT-1210-3, and KFJT-1210-4 under stress induced by salt different concentrations. Experiments were performed at least three times. Values are the mean ± SE ($n = 3$), and bars indicate the SE. Means with the same letter for each cultivar are not significantly different according to Duncan's test at $P < 0.05$

to cellular damage induced by salinity and may thus represent a defense mechanism against the generation of NaCl-induced O_2^- and H_2O_2. CAT can eliminate H_2O_2 via the direct formation of H_2O and O_2 [31]. Our results indicate that among the three sorghum cultivars examined, KF1210-3 has the highest dismutation capacity in response to an increase in salt concentration. It is notable that the

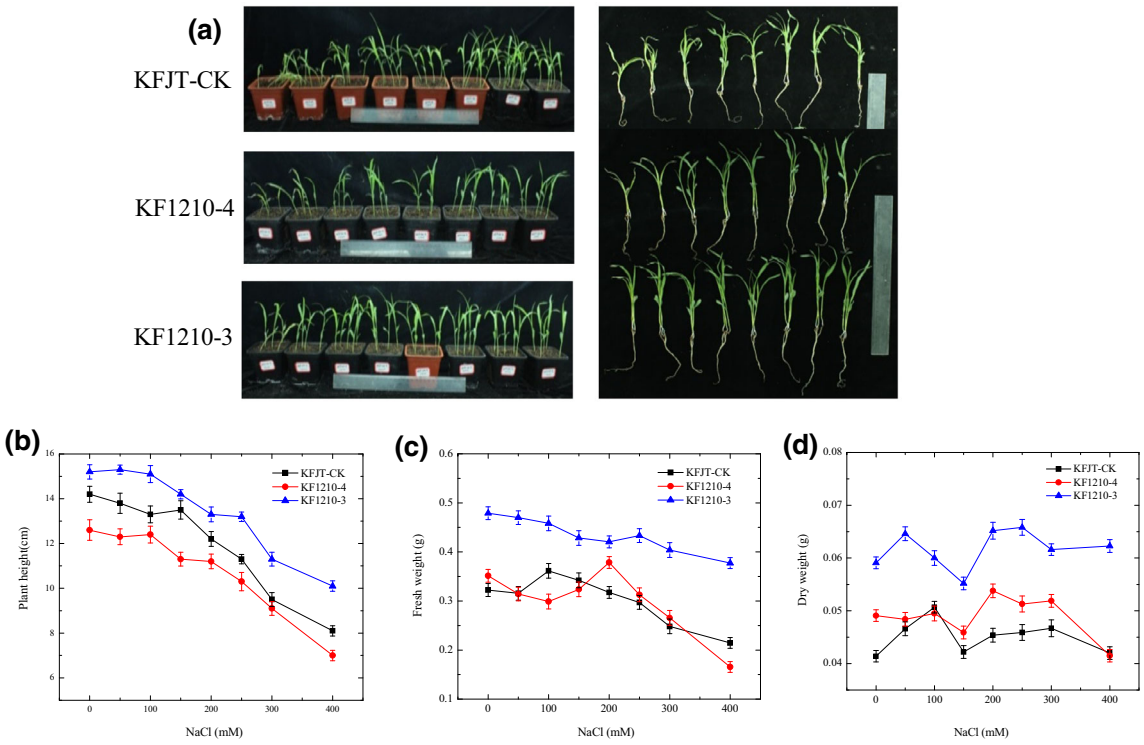


Fig. 1 (Color online) Morphology (**a**), plant height (**b**), fresh weight (**c**), and dry weight (**d**) of 1-week-old sweet sorghum KFJT-CK, KF1210-3, and KF1210-4 treated with NaCl solutions of different concentrations for 7 days. Experiments were performed at least three times. Values are the mean ± SE ($n = 3$), and bars indicate SE

CAT is a relatively effective antioxidant enzyme in preventing cellular damage, which is related to an enhanced tolerance to salt stress [32]. Accordingly, CAT analysis represents an effective approach for selecting salt-tolerant cultivars of sweet sorghum generated by $^{12}C^{6+}$-ion irradiation.

3.3 Expressions of NHX, AKT1, and SOS1 in the roots of sweet sorghum under saline conditions

NHX, which is located in the tonoplast, plays a critical role in minimizing the concentration of cytoplasmic Na^+ through sequestration of Na^+ into the vacuoles of many plants [20]. Previous results have shown that overexpression of NHX enhances salt tolerance by increasing the accumulation of Na^+ in plants [22]. However, none of the previous studies have addressed the role of the NHX protein in relation to the regulation of Na^+ concentration. In the present study, the expression levels of *NHX* in the root of KFJT-CK, KF1210-3, and KF1210-4 sorghum were determined by real-time quantitative PCR at 0, 2, 4, 8, 12, and 24 h after the samples had been treated with 150 mM NaCl. At 8 h after treatment, the expression level of *NHX* in the roots KF1210-4 and KF1210-3 was 7 and 73% higher, respectively, than in the roots of the control (KFJT-CK) (Fig. 3a). In response to treatment with 150 mM NaCl, the expression of *NHX* in the roots of KF1210-3 was significantly higher than that in KFJT-CK roots. These results indicate that expression levels of *NHX* in the roots of the mutant KF1210-3 were upregulated under saline conditions. The experimental results also demonstrated that NHX is crucial for the control of cytoplasmic Na^+ homeostasis and maintenance of salt accumulation in the vacuoles of sweet sorghum under salt stress.

It is well known that AKT1 plays a crucial role in controlling the homeostasis between Na^+ and K^+ in plants. AKT1 can unload Na^+ from the xylem to the xylem parenchyma cells (XPCs) [33]. In the present study, we demonstrated that 150 mM NaCl significantly decreased the expression level of *AKT1* in the roots of KFJT-CK, KF1210-3, and KF1210-4 (Fig. 3b). In this regard, Zhang et al. found that a reduction of AKT1 in roots could limit the entry of Na^+ into plants [5, 6], which implies that the main role of AKT1 is the mediation of K^+ and Na^+ under salt stress.

SOS1 has been proposed to be an important transporter for the spatial distribution of Na^+ and could therefore be a plasma membrane Na^+/H^+ antiporter [34]. It has been suggested that SOS1 plays a necessary role in protecting K^+ uptake mediated by AKT1. In the present study, we found that NaCl at a concentration of 150 mM significantly increased the expression level of *SOS1* by 423% in KF1210-3, but only by 338% in the roots of KFJT-CK at 12 h after treatment (Fig. 3c). These results indicate that SOS1 may trigger a significant regulation in the roots of sweet sorghum under saline conditions. SOS1 could load Na^+ into the xylem, thereby controlling delivery to the shoot and storage in leaf mesophyll cells. We found that downregulated expression of *AKT1* in roots may also be involved in a decrease in K^+ accumulation in root cells. Furthermore, elevated cytoplasmic Na^+ levels could influence K^+ uptake under conditions of salt stress.

Under salt stress, both AKT1 and SOS1 play important roles in the unloading and exclusion of Na^+ to maintain a low level of Na^+ in plants. AKT1 retrieves Na^+ from the xylem under conditions where absorption and accumulation of Na^+ could induce damage in plants. Furthermore, NHX could minimize the concentration of cytoplasmic Na^+ by the sequestration of Na^+ in vacuoles, thereby enhancing the salt tolerance of sweet sorghum. This process would not only contribute to limiting the rapid accumulation of Na^+ in the root, but also alleviate the damage caused by high concentrations of Na^+. On the basis of our results, we propose a model in which the cooperation of NHX, AKT1, and SOS1 facilitates Na^+ homeostasis in sweet sorghum under conditions of saline stress.

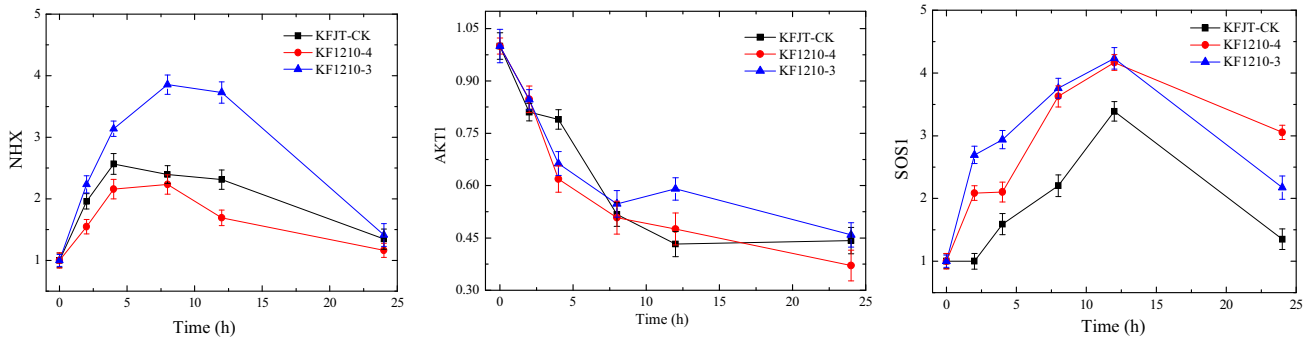


Fig. 3 (Color online) Time courses of *NHK*, *AKT1*, and *SOS1* expressions in the roots of sweet sorghum treated with 150 mM NaCl. Experiments were performed at least three times. Values are the mean ± SE ($n = 3$), and bars indicate the SE

4 Conclusion

In this study, we investigated the functions of synergistic responses of NHX, AKT1, and SOS1 related to the accumulation of Na^+ in the sweet sorghum mutants KF1210-3 and KF1210-4, which were obtained using a heavy ion radiation mutation breeding technique. The NHX, AKT1, and SOS1 proteins in the sweet sorghum play vital roles in the control of ion absorption, which may contribute to Na^+ homeostasis maintenance and growth regulation in sweet sorghum. On the basis of these results, we propose a model in which cooperation among NHX, AKT1, and SOS1 facilitates Na^+ homeostasis in sweet sorghum when exposed to saline stress. Studies on the relative expressions of *NHX*, *AKT1*, and *SOS1* and the associated regulatory mechanisms in sweet sorghum lines generated by irradiation with carbon ions are essential for the selection of salt-tolerant cultivars.

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