Natural variation of an EF-hand Ca\textsuperscript{2+}-binding-protein coding gene confers saline-alkaline tolerance in maize

Yibo Cao\textsuperscript{1}, Ming Zhang\textsuperscript{1}, Xiaoyan Liang\textsuperscript{1}, Fenrong Li\textsuperscript{1}, Yunlu Shi\textsuperscript{2}, Xiaohong Yang\textsuperscript{1,2,3} & Caifu Jiang\textsuperscript{1,2,4}\textasteriskcentered

Sodium (Na\textsuperscript{+}) toxicity is one of the major damages imposed on crops by saline-alkaline stress. Here we show that natural maize inbred lines display substantial variations in shoot Na\textsuperscript{+} contents and saline-alkaline (NaHCO\textsubscript{3}) tolerance, and reveal that ZmNSA1 (Na\textsuperscript{+} Content under Saline-Alkaline Condition) confers shoot Na\textsuperscript{+} variations under NaHCO\textsubscript{3} condition by a genome-wide association study. Lacking of ZmNSA1 promotes shoot Na\textsuperscript{+} homeostasis by increasing root Na\textsuperscript{+} efflux. A naturally occurred 4-bp deletion decreases the translation efficiency of ZmNSA1 mRNA, thus promotes Na\textsuperscript{+} homeostasis. We further show that, under saline-alkaline condition, Ca\textsuperscript{2+} binds to the EF-hand domain of ZmNSA1 then triggers its degradation via 26S proteasome, which in turn increases the transcripts levels of PM-H\textsuperscript{+}-ATPases (MHA2 and MHA4), and consequently enhances SOS1 Na\textsuperscript{+}/H\textsuperscript{+} antiporter-mediated root Na\textsuperscript{+} efflux. Our studies reveal the mechanism of Ca\textsuperscript{2+}-triggered saline-alkaline tolerance and provide an important gene target for breeding saline-alkaline tolerant maize varieties.
Saline-alkaline stress is a widely spread abiotic stress affecting an estimation of 4.15 × 10^8 ha of lands all over the world, which is emerging as a major constraint of global crop production. Saline-alkaline soil is characterized by high salt (salinity) and high pH (above pH 8.0; alkalinity), which causes combined damages of high pH stress, ion toxicity, and osmotic stress. In order to cope with saline-alkaline stress, plants have evolved a range of adaptive strategies. For instance, the H^+ efflux from root to soil acidifies the rhizosphere then promotes adaptation to high pH stress, the Na^+ -preferring transporters (e.g., SOS1 and HKT1) enable the circumvention of Na^+ toxicity, the accumulation of osmoprotectants (e.g., glycinebetaine) attenuates osmotic damage. These adaptive mechanisms act together to enable plant to survive saline-alkaline stress. Up to this day, it remains largely unknown how plants sense saline-alkaline stress and convert it into second signaling messengers (e.g., Ca^2+), and how plants decode the second messengers then activate/inactivate the downstream responses.

The major natural basic salts in saline-alkaline farmlands are sodium hydrogen carbonate (NaHCO₃) and sodium carbonate (Na₂CO₃), and Na^+ is the most abundance soluble salt in the saline-alkaline farmlands. Excessive accumulation of tissue Na^+ is deleterious for most crops, thus the maintenance of Na^+ homeostasis is essential for the crop saline-alkaline tolerance. Previous studies have shown that Na^+ selective transporters substantially confer cellular and whole-plant Na^+ homeostasis. For example, SOS1 Na^+/H^+ antiporters (e.g., AtSOS1) transport Na^+ out of root cells, the HKT family Na^+ transporters (e.g., AtHKT1) regulate long distance Na^+ delivery, and the root-to-shoot Na^+ translocation and Na^+ exclusion from the reproductive organ regulate the increases of rhizosphere and cytosolic pH weaken the function of H^+ -gradient-dependent Na^+ transporters (e.g., SOS1 Na^+/H^+ antiporters), then boosting Na^+ damage. Therefore, maintaining the H^+ gradient across the plasma membrane is essential for Na^+ homeostasis, especially under saline-alkaline conditions.

The optimal cytoplasmic pH for plant cells is neutral pH. When the rhizosphere pH is lower than the pH in the cytosolic of root cells, the H^+ gradient across the plasma membrane (membrane potential) drives uptake of nutrients (e.g., phosphorus, nitrate and iron). Under saline-alkaline condition, plants have to export cellular H^+ to rhizosphere to maintain the membrane potential, and the PM-H^+-ATPase is the major pump mediating root H^+ efflux. Previous studies in Arabidopsis have shown that the activity of the PM-H^+-ATPase AHA2 is inhibited by its C-terminal mediated auto-inhibition and by PKS5 mediated phosphorylation at Ser93. The saline-alkaline stress induces the increase of cytosolic Ca^2+, which binds to the 14-3-3 proteins and triggers its interaction with PKS5, then inhibits PKS5 activity thus activates AHA2. In the meantime, a phosphorylation at Thr947 activates AHA2 via triggering its interaction with the dimeric 14-3-3 proteins. These posttranscriptional mechanisms act together to activate AHA2, then promotes root H^+ efflux, thereby activating SOS1 Na^+/H^+ antiporter and other adaptive responses. Moreover, previous studies have also suggested that the transcript levels of some PM-H^+-ATPase increased under stress conditions, e.g., phosphorus deficiency increases the transcript levels of AHA2 and AHA7, salt stress upregulates the transcript levels of AHA2. These observations indicate that the transcriptional regulation of PM-H^+-ATPase is also important for the regulation of root H^+ efflux under stress conditions, nevertheless, the mechanism remains largely unknown.

Maize (Zea mays ssp. mays) is a glycophytic specie that is sensitive to saline-alkaline stress. Previous studies have shown that natural maize inbred lines show large variations of sensitivity to saline and saline-alkaline stress, and which is substantially attributed to the variations of shoot Na^+ contents. Here, we show that a calcium-binding EF-hand protein ZmNSA1 underlies the natural variations of shoot Na^+ contents under NaHCO₃ condition by a GWAS analysis. Lacking of ZmNSA1 increases root Na^+ efflux, then promotes shoot Na^+ exclusion and saline-alkaline tolerance. The functional variation of ZmNSA1 is ascribed to a 4-bp deletion located in the 3’UTR of ZmNSA1, which decreases the abundance of ZmNSA1 protein by reducing the translation efficiency of ZmNSA1 mRNA. We further show that, under saline-alkaline condition, Ca^2+ binds ZmNSA1 and triggers its degradation via 26S proteasome, then increases the expression of PM-H^+-ATPases, thereby promoting root H^+ efflux and SOS1 Na^+/H^+ antiporter-mediated root Na^+ efflux, ultimately promoting saline-alkaline tolerance. Our study shows how Ca^2+ triggered degradation of a Ca^2+-binding EF-hand protein confers transcriptional upregulation of PM-H^+-ATPases and saline-alkaline tolerance, providing a mechanistic understanding of crop saline-alkaline stress tolerance and an important genetic target for breeding saline-alkaline tolerant maize varieties.

Results

High pH stress disturbs Na^+ homeostasis in maize. In this study, we aimed to identify factors regulating maize shoot Na^+ homeostasis under saline-alkaline conditions. Given sodium hydrogen carbonate (NaHCO₃) is one of the major basic salts in nature environments, we used 100 mM NaHCO₃ to mimic the saline-alkaline stress, and both the Na^+ concentration (100 mM) and pH value (pH 8.8) were agronomic relevance. Firstly, we compared the shoot Na^+ contents in maize seedlings grown under NaHCO₃ and neutral salt (NaCl) conditions. We grew 419 maize inbred lines under conditions with 100 mM NaHCO₃ or 100 mM NaCl (pH 7.0) for two weeks, then measured the shoot Na^+ contents (see Materials and methods), subsequently observed large variations of shoot Na^+ contents ranging from 0.4 to 35 mg g⁻¹ dry mass (Fig. 1a, b; Supplementary Data 1). The overall shoot Na^+ contents of the plants grown under NaHCO₃ condition were significantly greater than that grown under NaCl condition (P = 2.53 × 10⁻⁶⁰; Fig. 1c), with 95% of the inbred lines conferred greater shoot Na^+ contents under NaHCO₃ condition than under NaCl condition (Fig. 1d). In addition, although different inbred lines showed large variations of shoot K^+ contents (ranging from 22 to 83 mg g⁻¹ dry mass) (Supplementary Fig. 1), the overall shoot K^+ contents of the plants grown under NaHCO₃ condition were significantly greater than that grown under NaCl condition (P < 0.05; supplementary Fig. 1c). Since the major feature distinguishing saline-alkaline stress from saline stress is the high pH stress, we suggest that high pH stress boosts maize shoot Na^+ accumulation under high-Na^+ conditions. Such a perspective is further supported by the observation that the increase of soil pH (from 7.0 to 10.0) caused up to 50% reduction of shoot biomass and up to 30% increase in shoot Na^+ contents under condition with 100 mM NaCl (Supplementary Fig. 2).

ZmNSA1 confers natural variations of shoot Na^+ contents. We next thought to identify the genetic variations underlying natural variations of maize shoot Na^+ contents under NaHCO₃ condition. GWAS analyses were performed using a mixed linear model (MLM; TASSEL 3.0) to identify the SNPs that were significantly associated with shoot Na^+ content under either NaHCO₃ or NaCl condition (see Materials and methods). Among the significant SNPs (|log_{10}(P)| > 5.0) (Supplementary Fig. 3), two SNPs next to
Fig. 1 ZmNSA1 confers natural variations of shoot Na\(^+\) contents under NaHCO\(_3\) condition. a, b Distribution of shoot-Na\(^+\) contents among 419 maize inbred lines under conditions with 100 mM NaCl (a) or 100 mM NaHCO\(_3\) (b). c Comparison of the shoot Na\(^+\) contents under NaCl and NaHCO\(_3\) conditions. The box shows the median, lower and upper quartiles, and dots denote outliers. Statistical significance was determined by a two-sided t-test (n = 419). d The relative change in shoot Na\(^+\) contents under NaHCO\(_3\) condition as compared with NaCl condition. The data were expressed as (m – n)/n. m and n referred to the shoot Na\(^+\) contents under NaHCO\(_3\) and NaCl condition respectively. e, f GWAS results of shoot Na\(^+\) contents under NaCl (e) and NaHCO\(_3\) (f) condition. A 2.5 Mb region (Chr2: 10.5-13.0 Mb) was displayed. Two SNPs (Chr2:12130275 and Chr2:12130134) that showed significantly association with shoot Na\(^+\) content under NaHCO\(_3\) condition were highlighted in red, and the gene underlines the association was designated as ZmNSA1 (Na\(^+\) Content under Saline-Alkaline Condition). g The distribution of shoot Na\(^+\) contents. Statistical significance was determined by a two-sided t-test (n = 375 for genotype C; n = 23 for genotype T). The samples were grouped according to the haplotypes of SNP Chr2:12130275. Source data underlying Fig. 1d, g are provided as a Source Data file.

each other (Chr2:12130275 and Chr2:12130134) were significantly associated with shoot Na\(^+\) contents under NaHCO\(_3\) condition but not under NaCl condition (Fig. 1e, f; Supplementary Fig. 3), with Chr2:12130275 showed greater association (−log\(_{10}\)(P) = 5.5). We designated the gene underlies this significant association as Zea mays L. Na\(^+\) Content 1 under Saline-Alkaline Condition (ZmNSA1), which potentially identities an important mechanin regulating Na\(^+\) homeostasis under saline-alkaline condition. The leading SNP Chr2:12130275 was located in the 3’ untranslated region (3’UTR) of GRMZM2G000397 (Supplementary Fig. 4), at which a thymine (T) and a cytosine (C) were associated with a lower and a greater shoot Na\(^+\) content respectively (Fig. 1g). GRMZM2G000397 encodes a putative calcium-binding family protein, which contains a single EF-hand domain, but with no other domains of known function (Supplementary Fig. 5). The orthologues of ZmNSA1 were identified in most plant species (Supplementary Fig. 6), but their function remains unknown. The phylogenetic analysis indicated that ZmNSA1 and its orthologues likely have evolutionary relationship with CML family protein (Supplementary Fig. 7), however, they haven’t been classified as CML family proteins in previous analysis\(^{35}\). Give previous studies have shown that saline-alkaline stress induces the increase of cytosolic Ca\(^{2+}\), which is perceived by Ca\(^{2+}\)-binding proteins (e.g., the EF-hand containing proteins) then triggers downstream adaptive responses\(^{36}\), it is possible that the calcium-binding EF-hand protein encoded by GRMZM2G000397 may perceives the saline-alkaline induced Ca\(^{2+}\) signal, then confers the regulation of Na\(^+\) homeostasis. Therefore, we suggest that GRMZM2G000397 is a likely candidate of ZmNSA1.

To determine if the candidate of ZmNSA1 (GRMZM2G000397) is associate with shoot Na\(^+\) content and saline-alkaline tolerance in maize, we tried to generate ZmNSA1 knockout mutant using previously described CRISPR-Cas9 technology\(^{34,35}\). Nevertheless, with four CRISPR-Cas9 targets and more than 80 independent transgenic plants (Supplementary Fig. 8), we failed to obtain ZmNSA1 knockout line, which might be the consequence of low mutational efficiency\(^{36}\). Fortunately, we identified a mutant line (ZmNSA1\(^{UFMu}\)), which conferred a UniformMu insertion in the second exon of ZmNSA1 (Supplementary Fig. 9; Fig. 2a). ZmNSA1\(^{UFMu}\) transcript and protein were hardly detected in ZmNSA1\(^{UFMu}\) mutant (Fig. 2b, c), indicating that ZmNSA1\(^{UFMu}\) conferred a function null allele of ZmNSA1. ZmNSA1\(^{UFMu}\) and wild type (W22) plants showed undetectable differences under control condition, but ZmNSA1\(^{UFMu}\) plants were significantly larger and conferred lower shoot Na\(^+\) content than that of W22 under NaHCO\(_3\) (100 mM) condition (Fig. 2d–i). Moreover, we generated two independent ZmNSA1-overexpressing lines (ZmNSA1\(^{OE\_1}\) and ZmNSA1\(^{OE\_2}\)), with both lines showed increased transcript and protein levels of ZmNSA1 (Fig. 2g, h).
We observed that, while the growth of wild type and ZmNSA1-overexpressing plants were comparable under control condition, ZmNSA1<sup>OE-1</sup> and ZmNSA1<sup>OE-2</sup> plants were significantly smaller and conferred greater shoot Na<sup>+</sup> contents than wild type under NaHCO<sub>3</sub> condition (Fig. 2i–k). Taken together, these results indicated that ZmNSA1 is associated with shoot Na<sup>+</sup> content and saline-alkaline (NaHCO<sub>3</sub>) tolerance, supporting the perspective of GRMZM2G000397 being the candidate of ZmNSA1.

**InDel1032 reduces the translation efficiency of ZmNSA1 mRNA.** In order to determine the molecular basis of the functional variation of ZmNSA1, we amplified and resequenced ZmNSA1 from 166 maize inbred lines using two pairs of primers (ZmNSA1-G-F1/ZmNSA1-G-R1 and ZmNSA1-G-F2/ZmNSA1-G-R2) (Supplementary Data 2, 3), subsequently identified 50 SNPs and 3 InDels with minor allele frequency (MAF) above 5% (Supplementary Data 4). The association of these variations with shoot Na<sup>+</sup> contents were analyzed using TASSEL (see the Materials and methods), and the results indicated that a SNP (SNP1173) and an InDel (InDel1032) showed the greatest association with shoot Na<sup>+</sup> content (Fig. 3a). SNP1173 and InDel1032 were located in the 3′UTR of ZmNSA1, and they were in complete LD among the 166 inbred lines (Fig. 3a). Based on the haplotypes of SNP1173 and InDel1032, the 166 maize inbred lines were grouped into two haplotype groups (Hap1 and Hap2) (Fig. 3b; Supplementary Data 3). The Hap1 and Hap2 groups were composed of 148 and 18 inbred lines respectively, with Hap1 and Hap2 designated as the high shoot Na<sup>+</sup> (saline-alkaline-sensitive) and low shoot Na<sup>+</sup> (saline-alkaline-tolerant) allele, respectively.

The functional variation of ZmNSA1 could be due to transcriptional or posttranscriptional changes. We showed by qRT-PCR assay that Hap1 and Hap2 inbred lines showed comparable ZmNSA1 transcript levels under both control and NaHCO<sub>3</sub> conditions (Fig. 3c), suggesting unlikely that the functional variation of ZmNSA1 was associated with the transcriptional change. In contrast, we found that Hap1 lines conferred significantly higher shoot Na<sup>+</sup> content than Hap2 group (Fig. 3b; P = 9.21 × 10<sup>−10</sup>). Therefore, the Hap1 and Hap2 ZmNSA1 were designated as the high shoot Na<sup>+</sup> (saline-alkaline-sensitive) and low shoot Na<sup>+</sup> (saline-alkaline-tolerant) allele, respectively.

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**Fig. 3** InDel1032 promotes shoot Na⁺ exclusion by decreasing the translation efficiency of ZmNSA1 mRNA. **a** The association analysis of the genetic variations in ZmNSA1 with shoot Na⁺ contents among 166 maize inbred lines. Upper panel showed the characterization of variations significantly associated with shoot Na⁺ contents. The red dots highlighted InDel1032 and SNP1173 that showed the highest association. Lower panel displayed the pattern of pairwise LD of the variations, and the red dots highlight the complete LD between InDel1032 and SNP1173. **b** Left panel showed the haplotypes of ZmNSA1 grouped according to the significance of the association analysis. Right panel showed the distributions of shoot Na⁺ contents for each haplotype group. Statistical significance was determined by a two-sided t-test (n = 148 for Hap1; n = 18 for Hap2). **c** Comparison of the transcript (c) and protein (d) levels of ZmNSA1 between randomly selected Hap1 and Hap2 inbred lines under control and NaHCO₃ conditions. **e** The transcript (e) and protein (f) levels of ZmNSA1 in ZmNSA1 UTMs protoplasts transformed with pSUPER-ZmNSA1-3’UTR(Hap1) and pSUPER-ZmNSA1-3’UTR(Hap2) (see Materials and methods). The non-transformed protoplasts provided a control. **g** Carotin displayed the wild type and mutant 3’UTR forms. **h** The transcript (h) and protein (i) levels of ZmNSA1 in ZmNSA1 UTMs protoplasts expressing pSUPER-ZmNSA1 with indicated 3’UTRs. In **f**, co-transformation of pSUPER-GFP and subsequent protein blot assay using GFP antibody provided a control of transformation efficiency. Analysis of actin provided loading controls in **d, f, i**. Data in **c, e, h** were means ± s.d. of three independent experiments. Source data underlying Figs. 3c–f, 3h, and 3i are provided as a Source Data file.

determined if SNP1173 and InDel1032 change the translation efficiency of ZmNSA1 messenger RNA. We generated pSUPER-ZmNSA1-3’UTR(Hap1) and pSUPER-ZmNSA1-3’UTR(Hap2), transformed them into ZmNSA1 UTMs protoplasts, and then compared the transcript and protein levels of ZmNSA1 (see Materials and methods). The results indicated that the protoplasts transformed with pSUPER-ZmNSA1-3’UTR(Hap1) and pSUPER-ZmNSA1-3’UTR(Hap2) conferred comparable ZmNSA1 transcript levels, but the former produced significantly more ZmNSA1 protein than the later (Fig. 3e, f), suggesting that the 3’UTR(Hap1) confers greater translation efficiency than 3’UTR(Hap2). Moreover, two mutant 3’UTR(Hap1) isoforms
resembling 3'UTR(Hap2) were generated, with 3'UTR (Hap1)InsDel1032 conferred an 4-bp (TCGG) deletion and 3'UTR (Hap1)SNP1173 conferred a C to T substitution (Fig. 3g). We found that, while ZmNSA1\textsuperscript{UFMu} protoplasts transformed with pSUPER-ZmNSA1-3'UTR(Hap1), pSUPER-ZmNSA1-3'UTR (Hap2), pSUPER-ZmNSA1-3'UTR(Hap1)InsDel1032 and pSUPER- ZmNSA1-3'UTR(Hap1)SNP1173 showed comparable transcript levels of ZmNSA1 (Fig. 3h), the abundance of ZmNSA1 proteins in the pSUPER-ZmNSA1-3'UTR(Hap1)InsDel1032-transformed protoplasts was significantly lower than pSUPER-ZmNSA1-3'UTR(Hap2)-transformed protoplasts (Fig. 3i). Taken together, we suggest that the 4-bp (TCGG) deletion in the 3'UTR of Hap2 ZmNSA1 reduces the translation efficiency of ZmNSA1 mRNA, thus promoting shoot Na\textsuperscript{+} exclusion under saline-alkaline condition.

**Lacking of ZmNSA1 promotes root Na\textsuperscript{+} efflux.** Above result has shown that lacking of ZmNSA1 promotes shoot Na\textsuperscript{+} exclusion under saline-alkaline condition (Fig. 2f), which could be ascribed to a decreased root Na\textsuperscript{+} uptake, or an increased root Na\textsuperscript{+} efflux, or a decreased root-to-shoot Na\textsuperscript{+} delivery. We then compared the Na\textsuperscript{+} contents in the root and xylem sap of ZmNSA1\textsuperscript{UFMu} and W22 (wild type) under NaHCO\textsubscript{3} (100 mM) condition, and observed that ZmNSA1\textsuperscript{UFMu} conferred significantly lower root and xylem sap Na\textsuperscript{+} contents than W22 (Fig. 4a, b), suggesting likely that lacking of ZmNSA1 decreases root Na\textsuperscript{+} content, thereby reducing xylem sap Na\textsuperscript{+} content and root-to-shoot Na\textsuperscript{+} delivery. In agree with this perspective, we observed that ZmNSA1-overexpressing plants conferred higher root and xylem sap Na\textsuperscript{+} contents than wild type under saline-alkaline condition (Fig. 4c, d).

We further determined how ZmNSA1 regulates root Na\textsuperscript{+} content, i.e. by decreasing uptake or by increasing efflux. Firstly, we used Non-invasive Micro-test Technology (NMT) to measure the Na\textsuperscript{+} flux at the root meristem zone of five-days-old seedlings that have been treated with 100 mM NaCl (pH 8.0) for 24 h, and observed that the roots of ZmNSA1\textsuperscript{UFMu} and ZmNSA1-overexpressing plants showed significantly greater and lower root Na\textsuperscript{+} efflux than that of the wild type controls respectively (Fig. 4e, f). Secondly, we measured the root Na\textsuperscript{+} contents of the plants that have been treated with 100 mM NaCl (pH 8.0) for short time (10 min), which to some extent reflects the rate of short-term Na\textsuperscript{+} uptake. The results indicated that the root Na\textsuperscript{+} contents in ZmNSA1\textsuperscript{UFMu} and ZmNSA1-overexpressing plants were comparable with that of their wild type controls (Supplementary Fig. 10). Taken together, these observations indicated that ZmNSA1 involves in the regulation of root Na\textsuperscript{+} efflux, but is unlikely associated with the regulation of Na\textsuperscript{+} uptake.

Previous studies have demonstrated that the root Na\textsuperscript{+} efflux is substantially mediated by SOS1 family Na\textsuperscript{+}/H\textsuperscript{+} antiporters\textsuperscript{2}, we then thought to determine if ZmNSA1 regulates root Na\textsuperscript{+} efflux by a Na\textsuperscript{+}/H\textsuperscript{+} antiporter dependent mechanism. Amiloride is an inhibitor of Na\textsuperscript{+}/H\textsuperscript{+} antiporter\textsuperscript{39}. We found that, while ZmNSA1\textsuperscript{UFMu} conferred increased root Na\textsuperscript{+} efflux and ZmNSA1-overexpressing plants conferred decreased root Na\textsuperscript{+} efflux (Fig. 4e, f), the application of amiloride reduced root Na\textsuperscript{+} efflux of all tested genotypes, but with different degrees of reduction (Fig. 4e–j). As a result, amiloride application substantially reduced the differences of root Na\textsuperscript{+} efflux between ZmNSA1\textsuperscript{UFMu} and W22, and between ZmNSA1-overexpressing plants and wild type (Fig. 4e–j), suggesting that ZmNSA1-mediated regulation of root Na\textsuperscript{+} efflux is dependent upon SOS1 Na\textsuperscript{+}/H\textsuperscript{+} antiporter. Such a perspective was supported by further observations, which showed that the plasma membrane vesicles isolated from the roots of NaHCO\textsubscript{3} treated ZmNSA1\textsuperscript{UFMu} plants showed greater Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity than W22 (Fig. 4k), and the plasma membrane vesicles isolated from ZmNSA1-overexpressing plants conferred lower Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity than wild type (Fig. 4l). Taken together, we suggest that ZmNSA1 mediates the regulation of root Na\textsuperscript{+} efflux, with lacking of ZmNSA1 increases SOS1 Na\textsuperscript{+}/H\textsuperscript{+} antiporter-mediated root Na\textsuperscript{+} efflux, thereby promoting shoot Na\textsuperscript{+} homeostasis and saline-alkaline tolerance.

**Ca\textsuperscript{2+} binds to ZmNSA1 and triggers its degradation.** We next investigated the mechanisms by which ZmNSA1 responds to saline-alkaline stress then regulates SOS1 Na\textsuperscript{+}/H\textsuperscript{+} antiporter-mediated root Na\textsuperscript{+} efflux. Firstly, we determined the subcellular localization of ZmNSA1, and found that ZmNSA1-GFP fusion proteins were predominantly detected in the cytosol of maize protoplast cells (Fig. 5a), but were hardly detected in nucleus (Supplementary Fig. 11). Secondly, the in situ RT-PCR assays showed that the transcripts of ZmNSA1 were detected in all root cell types (Fig. 5b), and the saline-alkaline (100 mM NaHCO\textsubscript{3}) treatment for 3–12 h had insignificant effect on ZmNSA1 transription in the root tissues (Fig. 5c). Thirdly, the protein blot assays with an anti-ZmNSA1 antibody revealed that 100 mM NaHCO\textsubscript{3} treatment for 3–12 h dramatically reduced the abundance of ZmNSA1 protein in the root tissues of wild type and ZmNSA1-overexpressing plants (Fig. 5d, e). Finally, we showed that the application of MG132 substantially inhibited the NaHCO\textsubscript{3}-induced degradation of ZmNSA1 (Fig. 5f). Taken together, these results indicated that NaHCO\textsubscript{3} treatment triggers the degradation of ZmNSA1 protein via the 26S proteasome pathway, but has negligible effect on the transcript levels of ZmNSA1.

Previous studies have shown that Ca\textsuperscript{2+} is an important messenger mediating plant responses to environmental stresses\textsuperscript{40,41}, and saline-alkaline treatment increases Ca\textsuperscript{2+} concentration in cytosol\textsuperscript{6}. We then tested if the saline-alkaline induced degradation of ZmNSA1 is dependent upon the increase of cytosolic Ca\textsuperscript{2+}. LaCl\textsubscript{3} and verapamil are Ca\textsuperscript{2+}-channel blocker\textsuperscript{42,43}, which can block saline-alkaline stress induced increase of cytosolic calcium\textsuperscript{6}. We found that the treatments with either 5 mM LaCl\textsubscript{3} or 100 μM verapamil substantially inhibited the NaHCO\textsubscript{3}-induced degradation of ZmNSA1 (Fig. 5g; Supplementary Fig. 12a), but had undetectable effect on ZmNSA1 expression and root cell variability (Fig. 5h; Supplementary Figs. 12b, 13), suggesting that saline-alkaline stress induced degradation of ZmNSA1 is dependent upon the increase of cytosolic Ca\textsuperscript{2+} concentration. Moreover, while LaCl\textsubscript{3} treatment inhibited saline-alkaline treatment induced degradation of ZmNSA1 (Fig. 5g), the treatment reduced root Na\textsuperscript{+} efflux (Supplementary Fig. 14), which is consistent with above observations that ZmNSA1-overexpressing plants conferred decreased root Na\textsuperscript{+} efflux and SOS1 activity (Fig. 4f, l).

ZmNSA1 encoded a putative calcium-binding EF-hand protein with a single EF-hand domain (Supplementary Fig. 5). Next, we investigated the Ca\textsuperscript{2+}-binding profiles of ZmNSA1. The microscale thermophoresis (MST) assay indicated that ZmNSA1 binds Ca\textsuperscript{2+} directly (Fig. 5i), and a single amino acid change (ZmNSA1\textsuperscript{E97Q}) in the conserved EF-hand domain resulted in a magnitude decrease of the Ca\textsuperscript{2+}-binding activity (Fig. 5i), indicating that ZmNSA1 binds Ca\textsuperscript{2+} via the EF-hand domain. Notably, while previous studies have shown that, in a typical plant cell, free cytoplasmic Ca\textsuperscript{2+} concentrations are in the range of 100–200 nM, and increase to 500–1000 nM following the onset of external stimulations (e.g., salt stress)\textsuperscript{44}, we observed that the Ca\textsuperscript{2+}-ZmNSA1 binding increase linearly as Ca\textsuperscript{2+} concentrations...
for recording buffer with 50 overexpressing plants and their wild type controls. Five-day-old plants were treated with 100 mM NaCl (pH 8.0) for 24-h, incubate in recording buffer (ZmNSA1 Materials and methods).

Taken together, we conclude that saline-alkaline stress (NaHCO₃) and pSUPER-ZmNSA1E97Q and pSUPER-ZmNSA1 degradation of ZmNSA1. We transformed results indicated that ZmNSA1E97Q mutation had undetectable examined the transcript and protein levels of ZmNSA1. The increase from 100 to 1,000 nM (Fig.5i), indicating that the Ca²⁺-ZmNSA1 binding is physiologically relevant. We further tested if the binding of Ca²⁺ is essential for the NaHCO₃-induced degradation of ZmNSA1. We transformed pSUPER-ZmNSA1 and pSUPER-ZmNSA1E97Q into ZmNSA1UFPMu protoplasts, then examined the transcript and protein levels of ZmNSA1. The results indicated that ZmNSA1E97Q mutation had undetectable effect on the expression of ZmNSA1 (Fig. 5i), but significantly reduced the NaHCO₃-induced degradation of ZmNSA1 (Fig. 5k). Taken together, we conclude that saline-alkaline stress (NaHCO₃) increases cytosolic Ca²⁺, which binds to the EF-hand domain of ZmNSA1 then triggers its degradation, thereby promoting root Na⁺ efflux and promoting saline-alkaline adaptation.

ZmNSA1 negatively regulates the activity of PM-H⁺-ATPase. Previous studies have shown that the PM-H⁺-ATPase-mediated root H⁺ efflux is a major determinant of the membrane potential[22,23], which activates SOS1 Na⁺/H⁺ antiporters then promotes root Na⁺ efflux and saline-alkaline tolerance[18]. The above results have shown that ZmNSA1 regulates SOS1 Na⁺/H⁺ antiporter-mediated root Na⁺ efflux (Fig. 4). We then determined if

Fig. 4 ZmNSA1 regulates root Na⁺ efflux under saline-alkaline conditions. a–d Na⁺ contents in the roots (a, c) and xylem sap (b, d) of ZmNSA1UFMu, ZmNSA1-overexpressing plants and their wild type controls (treatments as indicated). e–j Na⁺ flux at the root meristem zone of ZmNSA1UFMu, ZmNSA1-overexpressing plants and their wild type controls. Five-day-old plants were treated with 100 mM NaCl (pH 8.0) for 24-h, incubate in recording buffer (e, f) or recording buffer with 50 µM amiloride (g, i) for 30 mins, then the Na⁺ flux were measured using Non-invasive Micro-test Technology (NMT) (see Materials and methods). k–l The activity of Na⁺/H⁺ antiporter in the plasma membrane vesicles isolated from the roots of NaHCO₃ treated plants (genotypes as indicated). Data in a–d, k–l were means ± s.d. of three independent experiments. Data in e–j were means ± s.d. n = 5. Statistical significances were determined by a two-sided t-test. Source data are provided as a Source Data file.
ZmNSA1 regulates root Na\(^+\) efflux by an H\(^+\) efflux dependent manner. Firstly, we grown ZmNSA1\(^{UFMu}\), ZmNSA1-overexpression plants and wild type plants under alkaline (pH 8.0) and saline-alkaline (50 mM NaCl, pH 8.0) mediums with the pH indicator bromocresol purple, and observed clear acidification of the mediums by all genotypes under both conditions. However, ZmNSA1\(^{UFMu}\) showed greater activity of medium acidification than W22, and ZmNSA1-overexpressing plants showed lower activity of medium acidification than wild type (Fig. 6a, b), suggesting likely that the function of ZmNSA1 is negatively associated with root H\(^+\) efflux. To confirm this perspective, we measured the net H\(^+\) flux at the meristem zone of 5-days-old plants that have been treated with alkaline stress (pH 8.0) or saline-alkaline stress (100 mM NaCl, pH 8.0) for 24-h (see Materials and methods), and found that ZmNSA1\(^{UFMu}\) conferred greater H\(^+\) efflux than W22 (Fig. 6c, d), and ZmNSA1-overexpression plants conferred lower H\(^+\) efflux than wild type (Fig. 6e, f), confirming that ZmNSA1 negatively regulates root H\(^+\) efflux.

The root H\(^+\) efflux is substantially mediated by PM-H\(^+\)-ATPase\(^{45}\). We then isolated plasma membrane vesicles from the roots of NaHCO\(_3\) treated plants, and then measured the activity of PM-H\(^+\)-ATPase (Fig. 6g–j). The results indicated that ZmNSA1\(^{UFMu}\) conferred greater PM-H\(^+\)-ATPase activities than W22 (Fig. 6g, h), and ZmNSA1-overexpressing plants conferred lower PM-H\(^+\)-ATPase activities than wild type (Fig. 6i, j). These results support the notion that ZmNSA1 influences root Na\(^+\) efflux by regulating PM-H\(^+\)-ATPase-mediated H\(^+\) efflux. In addition, while previous studies have suggested that the V-H\(^+\)-ATPase and V-H\(^+\)-PPase in tonoplast also affect Na\(^+\) homeostasis\(^{46}\), we observed that the tonoplast vesicles isolated from the roots of NaHCO\(_3\) treated ZmNSA1\(^{UFMu}\) and W22 plants showed comparable V-H\(^+\)-ATPase and V-H\(^+\)-PPase activities (Supplementary Fig. 15), indicating that ZmNSA1 has minimal effect on the activities of V-H\(^+\)-ATPase and V-H\(^+\)-PPase.

ZmNSA1 mediates transcriptional upregulation of MHAs. We next investigated the mechanism by which ZmNSA1 regulates the activity of PM-H\(^+\)-ATPase. We showed that there were 13 Maize PM-H\(^+\)-ATPases (MHAs-13) (Fig. 7a; Supplementary Table 1), and the phylogenetic analysis using MEGA6\(^{47}\) showed that the
PM-H\textsuperscript{+}-ATPases from maize and Arabidopsis were grouped into three classes (Fig. 7a). The data from Maize Gene Expression Atlas showed that MHA2, MHA3, MHA4, and MHA12 were predominantly detected in root tissues (Fig. 7b), which is in consistent with our qRT-PCR results (Fig. 7c). While previous studies have shown that the posttranscriptional activation of AHA2 increases root H\textsuperscript{+} efflux, and the Ca\textsuperscript{2+}-binding 14-3-3 proteins directly binds and activates AHA2\textsuperscript{b}, we didn’t observe the direct interaction between ZmNSA1 and MHA2 in yeast two-hybrid and BiFC assays (Supplementary Fig. 16). Intriguingly, we observed that NaHCO\textsubscript{3} treatment significantly increased the transcript levels of MHA2 and MHA4 (the two most expressed MHAs in maize root tissues) (Fig. 7c), and such increases were enhanced in ZmNSA1\textsuperscript{UFMu} and attenuated in ZmNSA1\textsuperscript{–overexpressing plants (Fig. 7d–g), suggesting that ZmNSA1 mediates the transcriptional upregulation of MHAs under saline-alkaline condition. Moreover, while above studies have shown that LaCl\textsubscript{3} treatment attenuated the NaHCO\textsubscript{3}-induced degradation of ZmNSA1 (Fig. 5g), the treatment also inhibited the NaHCO\textsubscript{3}-induced transcriptional upregulation of MHA2 and MHA4 (Fig. 7h). Taken together, we conclude that, under saline-alkaline condition, Ca\textsuperscript{2+}-triggered degradation of ZmNSA1 increases the transcript levels of MHA2 and MHA4, then promotes root H\textsuperscript{+} efflux, thereby enhancing SOS1 Na\textsuperscript{+}/H\textsuperscript{+} exchange.

**Fig. 6** ZmNSA1 regulates PM-H\textsuperscript{+}-ATPase-mediated H\textsuperscript{+} efflux. a, b Rhizosphere acidification assays of ZmNSA1\textsuperscript{UFMu} (a), ZmNSA1-overexpressing plants (b) and their wild type controls (W22 for ZmNSA1\textsuperscript{UFMu}, and Wild type for ZmNSA1-overexpressing plants). Yellow color indicated the acidification of the medium. c–f NMT assays of H\textsuperscript{+} flux at root meristem zone of five-days-old seedlings that have been treated with alkaline (pH 8.0) (c, e) or saline-alkaline stress (100 mM NaCl, pH 8.0) (d, f) for 24 hours. The H\textsuperscript{+} flux were measured using NMT (see Materials and methods). Data were means ± s.d. n = 5. g–j The activity of PM-H\textsuperscript{+}-ATPase in plasma membrane vesicles isolated from the roots of NaHCO\textsubscript{3} treated plants (genotypes as indicated). The assays were performed as described in Materials and Methods. The data showed the timely varying curves of quinacrine fluorescent intensity (g, i) and the calculated activity of PM-H\textsuperscript{+}-ATPase (h, j). Data were means ± s.d. of three independent experiments. Statistical significance was determined by a two-sided t-test. Source data underlying Fig. 6c–j are provided as a Source Data file.
antiporter-mediated Na\(^+\) homeostasis and the adaptation to saline-alkaline environments.

**Discussion**

Maize is a saline-alkaline sensitive crop, and global maize production is increasingly affected by the saline-alkalization of farmlands\(^{39}\). Therefore, there is an urgent need for understanding of maize saline-alkaline-tolerant mechanisms, understanding which can potentially be used to increase the saline-alkaline tolerance of maize. The sodium carbonates (NaHCO\(_3\) and Na\(_2\)CO\(_3\)) are the major basic salt existed in the saline-alkaline farmlands, which causes combined damages of high pH stress, Na\(^+\) toxicity and osmotic stress on maize\(^{1}\). Here, we have shown that natural maize inbred lines confer widely genetic variations of shoot Na\(^+\) exclusion under saline-alkaline (NaHCO\(_3\)) condition (Fig. 3), suggesting that the identification and application of the favorable variations might provide a route for improving maize Na\(^+\) homeostasis and saline-alkaline tolerance. In addition, we have discovered that ZmNSA1 underlies the natural variations of shoot Na\(^+\) contents under NaHCO\(_3\) condition (Fig. 1f). A naturally occurred 4-bp deletion decreases the transcription efficiency of ZmNSA1 mRNA then promotes shoot Na\(^+\) exclusion (Fig. 3), accordingly, lacking of ZmNSA1 promotes shoot Na\(^+\) exclusion and saline-alkaline tolerance (Fig. 2d–f). Our identification of ZmNSA1 provides a gene target for improving maize saline-alkaline tolerance either by marker assisted selection or by CRISPR-Cas9 gene editing.

The major feature distinguishing saline-alkaline stress from saline stress is the high pH stress, which disturbs the H\(^+\)/H\(^+\) exclusion (Fig. 3), and that the posttranscriptional activation of PM-H\(^+/\)H\(^+\) antiporter\(^{6,18,45}\). Previous studies have indicated that the PM-H\(^+/\)H\(^+\) ATPase is the major H\(^+\) pump responsible for root H\(^+\) efflux\(^{45}\), and that the posttranscriptional activation of PM-H\(^+/\)H\(^+\) ATPase (e.g., AHA2) confers Na\(^+\) homeostasis and saline-alkaline tolerance\(^{6,45,48,49}\). Here, we have shown that MHA2 and MHA4 were predominantly detected in root tissues and were significantly upregulated by saline-alkaline stress (Fig. 7b, c). These results together with previous studies suggest
that the transcriptional and post-transcriptional activation of PM-H\(^{+}\)-ATPase act together to ensure the establishment of the membrane potential under saline-alkaline conditions.

Existing knowledge have shown that Ca\(^{2+}\) is an important signaling molecule of saline-alkaline response\(^6\). Following the onset of saline-alkaline stress, the concentration of cytosolic-free Ca\(^{2+}\) increase, which then acts as a messenger to activate/inactivate the downstream signaling components\(^6\). The PM-H\(^{+}\)-ATPase is one of the important downstream targets of Ca\(^{2+}\) signal, e.g., previous studies have shown that the Ca\(^{2+}\) binds to 14-3-3 proteins then mediates the posttranscriptional activation of AHA2\(^18\). We here show that the Ca\(^{2+}\)-binding EF-hand family protein ZmNSA1 confers transcriptional regulation of maize PM-H\(^{+}\)-ATPase (Fig. 7), which acts at the steps downstream of Ca\(^{2+}\) signal. ZmNSA1 negatively regulates the transcript levels of MHAs. Under saline-alkaline treatment, the concentration of cytosolic Ca\(^{2+}\) increase, then Ca\(^{2+}\) binds to the EF-hand domain of ZmNSA1 and triggers its degradation via 26S proteasome pathway (Fig. 5d–k), in turn promotes the transcription of MHA2 and MHA4 (Fig. 7). Given ZmNSA1 has no transcription activation domain and is barely detected in nucleus (Supplemental Fig. S5, 11), the ZmNSA1-mediated upregulation of MHAs transcript levels is likely to be an indirect response. SOS1 Na\(^{+}/H^{+}\) antiporter is the major transporter responsible for root Na\(^{+}\) efflux\(^7\). We show that ZmNSA1 involves in the regulation of Na\(^{+}/H^{+}\) antiporter-mediated root Na\(^{+}\) efflux, with lacking of ZmNSA1 increases the activity of Na\(^{+}/H^{+}\) antiporter (Fig. 4). As previous studies have demonstrated that PM-H\(^{+}\)-ATPase-mediated root H\(^{+}\) efflux is essential for the activation of SOS1 Na\(^{+}/H^{+}\) antiporter,\(^18\) we suggest that ZmNSA1-mediated regulation of Na\(^{+}/H^{+}\) antiporter activity is ascribed to its regulatory roles on the transcription of PM-H\(^{+}\)-ATPases. In conclusion, we have discovered ZmNSA1, an important QTL conferring natural variations of shoot Na\(^{+}\) contents under saline-alkaline (NaHCO\(_3\)) condition, with which we have discovered a saline-alkaline tolerance mechanism (Fig. 8), i.e., under saline-alkaline treatment, the concentration of cytosolic Ca\(^{2+}\) increase, Ca\(^{2+}\) binds to ZmNSA1 and triggers its degradation via 26S proteasome pathway, then increases the transcript levels of maize PM-H\(^{+}\)-ATPases (MHA2 & MHA4) and promotes root H\(^{+}\) efflux, thereby enhancing SOS1 Na\(^{+}/H^{+}\) antiporter-mediated root Na\(^{+}\) efflux, ultimately promoting saline-alkaline tolerance. Our study provides a mechanistic understanding of Ca\(^{2+}\)-mediated plant saline-alkaline tolerance and an important gene target for breeding saline-alkaline tolerant maize varieties.

**Methods**

**Plant growth and treatments.** The natural maize population used in this study was the same population used in previously study\(^6\). In order to measure the shoot Na\(^{+}\) and K\(^{+}\) contents of the 419 maize inbred lines, pots (diameter of 30 cm and height of 35 cm) filled with uniformly mixed substrate (www.pindstrup.com) were watered to soil saturation with 100 mM NaCl or 100 mM NaHCO\(_3\) solutions. Eight inbred lines (six plants for each) were planted in each pot, grown in a glasshouse for 2 weeks, and then the shoot tissues were collected for measuring Na\(^{+}\) and K\(^{+}\) contents.

**Measurement of Na\(^{+}\) and K\(^{+}\) contents.** The samples were dried at 80 °C for 24 h, weighed, then incinerated in a muffle furnace at 300 °C for 3 h and 575 °C for 6 h. The ashes were dissolved in 10 mL 1% hydrochloric acid, appropriately diluted with 1% hydrochloric acid, and then Na\(^{+}\) and K\(^{+}\) contents were analyzed. In order to measure the Na\(^{+}\) and K\(^{+}\) contents in xylem sap, 2-weeks-old seedlings grown under control or 100 mM NaHCO\(_3\) conditions were de-topped with blade, the xylem sap exuding at the cut surface of the de-topped root system was collected by a micropipette every 15 min for 1 h. The contents of Na\(^{+}\) and K\(^{+}\) were analyzed using the 4100- MP AES device (Agilent, Santa Clara, CA, USA).

**Genome-wide association study.** The genotype used in this study was generated by Maize SNP50 array (containing 56,110 SNPs), RNA-seq or by joint application of IBID (identity by descent) based projection and KNN (the k-nearest neighbor) algorithm, and in total 556,809 high quality SNPs (MAF ≥ 0.05) were selected to perform the GWAS analysis\(^2\). Association analysis for shoot Na\(^{+}\) content under NaCl and NaHCO\(_3\) conditions were conducted by the mixed linear model (MLM; TASSLE3)\(^3\). Both kinship (K) and population structure (Q) were taken into account to avoid spurious associations\(^3\). We used the P < 1.0 × 10\(^{-8}\) as the final significance cutoff in the association analysis.

**Characterization of ZmNSA1UniformMu.** We ordered the UniformMu line (mu1089781) from Maize Genetics COOP Stock Center. The mutant line has been suggested to confer a UniformMu insertion in the second extron of ZmNSA1. In order to confirm the UniformMu insertion, we designed three primers (UFMu-F, UFMu-R and UFMu-S-F) (Supplementary Fig. 9; Supplementary Data 2), with which we can obtain PCR products from W22 when using UFMu-F and UFMu-R as primers, and can obtain PCR products from mu1089781 when using UFMu-S-F and UFMu-R as primers. Subsequently, we confirmed that mu1089781 conferred a UniformMu insertion in the second extron of ZmNSA1 (Supplementary Fig. 9), and the mutant was designated as ZmNSA1UniformMu.

**Generation of ZmNSA1 overexpressing lines.** We generated the transgenic lines overexpressing ZmNSA1 at Center for Crop Functional Genomics and Molecular Breeding, China Agricultural University, Beijing. The coding sequence of ZmNSA1 was cloned to PBBCXUN vector, transformed into Agrobacterium strain EHA105 to infecting immature embryo of inbred line 32990709 (a maize inbred line with increased transformation efficiency), then the regenerate seedlings were obtained from the infected embryo. The homozygous overexpression lines were obtained by anti-herbicide selection of the self-pollinated T1, T2 and T3 generation plants.

**qRT-PCR assay.** Total RNA was extracted using RNA prep pure plant kit (Tiangen, Beijing, China), then 1.5 μg RNA was used to synthesize first-strand cDNA using M\(_S\) Super qPCR RT kit with gDNA remover (Mei5 biotechnology, Beijing, China), and then qRT-PCR analysis was conducted using the 2 × Real time PCR Super mix (SYBRgreen) (Mei5 biotechnology, Beijing, China) on the ABI 7500 thermocycler (Applied Biosystems). Ubi2 gene (UnilProtKb/TfEMBL, Q4241S) provided a control, and the 2\(^{-ΔΔCt}\) method was used to calculate the expression.

**ZmNSA1 association mapping and linkage analysis.** To identify the genetic variation responsible for the functional variation of ZmNSA1, we used two pairs of primers (ZmNSA1-G-F1/ZmNSA1-G-R1 and ZmNSA1-G-F2/ZmNSA1-G-R2) to amplify and sequence ZmNSA1 from 166 inbred lines randomly selected from the population. A genomic region including the 5\’ to 3\’ UTR of ZmNSA1 was analyzed. Multiple sequence alignments were performed using BIOEDIT (+7.2.9.2; North Carolina State University, Raleigh, NC, USA), and the polymorphic sites (SNPs and InDels) (MAF ≥ 0.05) were extracted. The associations between the genetic
variations and Na⁺ contents were analyzed using TASSEL 3.0, under the standard MLM.

Protoplast-based assay. In order to determine the subcellular localization of ZmNSA1, we isolated maize mesophyll protoplasts, generated pSUPER-ZmNSA1-GFP vector, and then co-transformed pSUPER-ZmNSA1-GFP with pGPTVII-AcBCL-Off56 or 35S:AtWryko40-mCherry56 into the mesophyll protoplasts. AtCBL1 is a plasma membrane localized protein55, and AtWryko40 is located in nucleus56. Fluorescent signals were captured using a confocal laser scanning microscope (Carl Zeiss LSM710). The excitation was at 488 nm and the detection was between 515 and 530 nm for GFP, and the excitation was at 543 nm and the detection was over 570 nm for mCherry. In order to analyze the transcription and protein levels of ZmNSA1 in ZmNSA1::pGPTVII protoplasts, the indicated constructs were transformed into the protoplasts, cultured for 16 h, then the constructs were used for analyzing ZmNSA1 transcript and protein levels (Fig. 3e–i), or used for saline-alkaline treatments and follow up analysis of ZmNSA1 transcript and protein levels (Figs. 5i, k).

Immunoblot assay. Total proteins were extracted using extraction buffer (10 mM Tris, pH 7.5, 2.5 mM EDTA, 2.5 mM EGTA, 150 mM NaCl, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, Roche), then the target proteins were analyzed by immunoblot analysis. The antibodies used in this study include anti-ZmNSA1 generated by Beijing Protein Innovation, anti-β-Actin (CWIBIO 10265/60205, 1/5000) and anti-GFP (Abclonal AE01133345, 1/5000).

Non-invasive micro-test technology. The net Na⁺ and H⁺ fluxes were measured using non-invasive micro-test technology (NMT) (Younger USA, LLC). In order to measure the net Na⁺ and H⁺ fluxes, five-day-old seedlings were treated with 100 mM NaCl solution (pH 8.0) for 24 h, then Na⁺ and H⁺ eﬄuxes (Figs. 4c–g, h and 6d, f) were measured at primary root meristem zone (~500 μm from the root tip). In addition, the H⁺ eﬄuxes at meristem zone of five-day-old seedlings treated with water (pH 8.0) for 24 h were analyzed (Fig. 6c, e). The NMT measurement procedures as follows: The backﬁlling solution (250 mM NaCl for Na⁺ measurement; 15 mM NaCl plus 40 mM KH₂PO₄ for H⁺ measurement) were ﬁlled into the pre-pulled and salinized micro sensor (ø45 μm, XY- CGQ –01) to a length of 1 cm, and then 50–60 μm LDX (XY-Si-Na for Na⁺ measurement; XY-Si-H for H⁺ measurement) were ﬁlled into the tip of the micro sensor. The micro sensor was calibrated in the calibration liquid (0.1 mM CaCl₂, 0.1 mM KCl, 0.3 mM MES, and 0.5 mM or 5.0 mM NaCl, pH 6.0 for Na⁺ measurement; 0.1 mM CaCl₂, 0.1 mM KCl, 0.3 mM MES, pH5.5 or pH 6.5 for H⁺ measurement). The roots were incubated in the measuring solutions (0.1 mM CaCl₂, 0.1 mM KCl, 0.3 mM MES, and 0.5 mM NaCl, pH 6.0 for Na⁺ measurement) for 10 min, then the Na⁺ or H⁺ net ﬂuxes were measured and calculated using iCal V3.3 (Younger, USA)18.

Assay of rhizosphere acidification. The kernels were sterilized in 75% ethanol for 5 min, wash with sterilized water for three times, then enclosed with seed coating agent for later use. The culture MS media contained 30 g L⁻¹ sucrose, 8 g L⁻¹ agar, and 0.5% (w/v) sucrose, poured into 50 mm diameter Petri dishes. The pretreated kernels were then labeled by dye (NT-647-NHS) using a Pierce BCA Protein Assay Kit (Thermo Fischer Scientific). The labeled proteins were incubated with indoxyl acetate (ligands) for 10 min, then the samples were analyzed by Monolish NT-115 (NanoTemper Technologies) at 25°C, 20% MST power and 20% LED power using hydrophobic capillaries (Polymermultech). The displayed results in Fig. 5i were based on three biological replicates and analyzed by MO. Affinity Analysis software (V2.2.4)57.

Measurement activities of H⁺-ATPase and Na⁺/H⁺ antiporter. The isolation of plasma membrane vesicles, and the measurement of the activities of H⁺-ATPase and Na⁺/H⁺ antiporter were as described below. Plasma membrane vesicles were isolated from the roots of 2-week-old plants that have been treated with 200 mM NaHCO₃ for 2 days. Fifty μg of plasma membrane proteins were used to determine the activity of H⁺-ATPase and Na⁺/H⁺ antiporter. The quenching in the ﬂuorescence of a pH-sensitive ﬂuorescent probe (a pH-sensitive fluorescent probe) provides a measure of H⁺-ATPase activity58. Na⁺/H⁺ reverse exchange activity was calculated based on Na⁺-induced dissipation59. The quinine fluorescence was measured using a Hitachi F-7500 imager.

Measurement of the V-H⁺-ATPase and V-H⁺-PPase activities. Two-week-old plants were treated with 200 mM NaHCO₃ for 2 days, and then collected the roots to isolated tonoplast vesicles using differential centrifugation (25/33/50% (w/v) sucrose gradients). The vesicles that sedimented at the interface between 25% and 33% sucrose were collected. Fifty micrograms of tonoplast proteins were used to measure the proton transporting activity of V-H⁺-ATPase and V-H⁺-PPase. The reaction substrate for V-H⁺-ATPase is ATP (3 mM), and for V-H⁺-PPase is PP₁ (1 mM). The quenching of the fluorescence detected by Hitachi F-7500 imager was regarded as the H⁺-transport activity60.

Data availability

Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. The source data underlying Figs. 1d, 2b, c–e, h, j, k, 3c–f, h, i, 4, 5c–k, 6c–f, and 7c–h, as well as Supplementary Figures 2c–f, 9b, 10, 12, 14, 15, and 16c–f are provided as a Source Data file.

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Author contributions

Y.C., M.Z., X.L., F.L., X.Y., and C.J. planned and designed the research. Y.C., X.L., M.Z., X.Y., and C.J. planned and designed the research. Y.C., X.L., M.Z., X.Y., and C.J. planned and designed the research. Y.C., X.L., M.Z., X.Y., and C.J. planned and designed the research. Y.C., X.L., M.Z., X.Y., and C.J. planned and designed the research.
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Correspondence and requests for materials should be addressed to C.J.

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