SiPLATZ12 transcript factor regulates multiple yield traits and salt tolerance in foxtail millet (*Setaria italica*)

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Grain yield and salt tolerance are critical for crop production. However, the genetic and biochemical basis underlying the trade-off of these characters remain poorly described in crops. We show here that SiPLATZ12 transcription factor positively regulates multiple elite yield traits at the expense of salt tolerance in foxtail millet. SiPLATZ12 overexpression increases seed size, panicle length, and stem diameter, while reduces plant height and salt tolerance of foxtail millet. A 9-bp insertion in the SiPLATZ12 promoter has significant effects on the different expression of SiPLATZ12, multiple yield traits, and salt tolerance between foxtail millet and its wild ancestor, green foxtail. Moreover, SiPLATZ12 upregulates the expression of genes involved in seed development, but repressing the transcription of most NHX, SOS, and CBL genes to regulate Na+, K+ and pH homeostasis. Therefore, our results uncover a domesticated site that could be used to improve grain yield and salt tolerance in foxtail millet.

**Keywords** foxtail millet; PLATZ transcription factor; natural variation; grain yield; salt tolerance

**Introduction**

Foxtail millet (*Setaria italica*), one of the most ancient domesticated crops in the world, is mainly cultivated in arid and semi-arid regions in Asia, Eurasia, and Africa for food, feed, fuel, and bioenergy (Brutnell *et al*., 2010; Jia *et al*., 2013; Yang *et al*., 2020; Zhang *et al*., 2012). There is a demand for new foxtail millet cultivars with high yield and strong abiotic stress tolerance. However, the yield and salt tolerance of foxtail millet are inherited quantitatively, so increasing yield and salt tolerance through traditional genetic improvement is difficult and time consuming. Once the key genes controlling important agronomic traits and their interrelationship are identified, improving foxtail millet through biotechnology will be very effective.

To achieve high yield, plants require not only high yield component traits, but also an optimal architecture that, to a large extent, is determined by plant height. Moreover, heading date (or flowering time) is a critical determinant enabling crops to adapt to seasonal changes and make maximum use of the temperature and sunlight. Yield per plant in foxtail millet is determined by two component traits: number of grains per panicle (panicle size) and grain weight (grain size). Multiple genetic factors controlled seed size have been revealed in Arabidopsis. These include the VQ domain containing protein HAIKU1 (IKU1), the leucine rich repeat receptor kinase IKU2, MINI- seed3 (MINI3), short Hypocotyl under Blue 1 (SHB1), the KLUH (KLU), fertilization independent seed 2 (FLS2), and cytokinin independent 1 (CKI1) coding genes (Kesavan *et al*., 2013; Li *et al*., 2016; Luo *et al*., 2005; Wang *et al*., 2010; Zhou *et al*., 2009). In rice, serine carboxypeptidase (GS5) (Li *et al*., 2011), gibberellic acid-stimulated regulator (GASR7)/grain weight (GW6) (Shi *et al*., 2020; Tang *et al*., 2021), and OsALMT7 (Heng *et al*., 2018) have been identified to function in seed size control. Besides,
dwarf/semidwarf (D/SD) and stem diameter also contribute to high grain yield mainly by increasing the lodging-resistance of crops. Molecular cloning and functional analyses of several genes associated with plant height in rice and wheat have shown that these genes are mostly related to the synthesis and regulation of the phytohormone gibberellin (Peng et al., 1999; Spielmeyer et al., 2002). Widespread utilization of such mutant gene, sd1, in worldwide breeding programs of staple crops, such as rice and wheat, results in the development of modern semidwarf lodging resistant cultivars (Monna et al., 2002; Spielmeyer et al., 2002). Extensive studies in Arabidopsis thaliana have established that flowering time is controlled by multiple pathways (Siplson et al., 2002), of which the photoperiod pathway acts as a major one. A key component functioning in this pathway is the CONSTANS (CO) TF. One of the downstream targets of CO is flowering locus T (FT), which controls flowering time by integrating input from various pathway (Santiago et al., 2021). Although the function of most of these genes has not been characterized, Loose Panicle 1, a group I WRKY transcription factor (TF), has been revealed to control plant height, panicle and seed size in foxtail millet (Xiang et al., 2017). Besides, SiMADS34 has been recently characterized to regulate panicle architecture, grain weight, and seed shape in foxtail millet (Hussin et al., 2021).

Except for plant architecture, external abiotic stresses seriously threat the food security worldwide. Salt stress is one of the main abiotic stresses. The salt tolerance of plants has been proved to be largely associated with cytoplasm Na⁺ homeostasis which is maintained by membrane localized Na⁺/H⁺ antiporters (NHXs) and their regulatory proteins, such as Salt Overly Sensitives (SOSs) and Calcineurin B-Like proteins (CBLs) (Halfter et al., 2000; Liu and Zhu, 1998; Quintero et al., 2011; Zhou et al., 2022). The transporter activities of NHX7/SOS1 are activated by SOS2-SOS3 (CBL4)/SCaBP8(CBL10) complex (Halfter et al., 2000; Qiu et al., 2002; Quan et al., 2007). Studies also showed that the NHX proteins are important for compartmentalization of K⁺ into vacuoles, cellular pH homeostasis (Barragán et al., 2012; Bassil et al., 2011a; Bassil et al., 2011b), and protein trafficking (Reguera et al., 2015). Thus, NHX operations play multiple roles in stomatal regulation (Barragán et al., 2012), plant growth (Bassil et al., 2012; Bassil et al., 2011a; Bassil et al., 2011b), and silique and seed development (Wu et al., 2016). However, the function of most of these genes has been poorly investigated in foxtail millet.

PLATZ TFs are a class of plant-specific zinc-dependent and A/T-rich sequence binding proteins (Nagano et al., 2001). In Arabidopsis, PLATZ1 positively regulates drought tolerance in vegetative tissues (Gonzalez-Morales et al., 2016). AtPLATZ2 negatively regulates plant salt tolerance (Liu et al., 2020). ORESARA15, as known as AtPLATZ3, promotes leaf cell proliferation during earlier stages of development and suppresses leaf senescence during later stages (Kim et al., 2018). In crops, PLATZs from maize (Zea mays) (Li et al., 2017), rice (Oryza sativa) and wheat (Wang et al., 2019; Zhou...
and Xue, 2020; Guo et al., 2022) regulate endosperm development and seed filling, grain length and number, respectively. PLATZs therefore play important roles in plant growth, development and abiotic stress responses. However, the function of PLATZs has not been characterized in foxtail millet.

Here, we identified SiPLATZ12 transcription factor as a positive regulator of multiple yield traits but a negative regulator of salt tolerance in foxtail millet. A 9-bp insertion in the SiPLATZ12 promoter significantly associated with the function of SiPLATZ12. Moreover, SiPLATZ12 regulates the expression of IKU1, IKU2, MINI3, SHB1, KLU, FLS2, and CKI1 genes for seed development and SiNHX, SiSOS, and SiCBLs genes for both development and salt tolerance. In particular, SiPLATZ12 directly binds to the A/T rich sequences in SiNHX2, SiCBL4 and SiCBL7 promoters. This study provides genetic resources for breeding high yield cultivars of foxtail millet by increasing SiPLATZ12 expression or salt tolerant cultivars by editing SiPLATZ12.

Results

SiPLATZ12 affects multiple yield traits and salt tolerance in foxtail millet

To identify PLATZ genes in foxtail millet, we scanned the ‘Yugu1’ genome assembly for open reading frames (ORFs) containing the PLATZ domain sequences from Arabidopsis thaliana. This identified 16 PLATZ genes in foxtail millet, which were named SiPLATZ1 through SiPLATZ16 on the basis of chromosomal localization (Table S1). Neighbor-joining analysis classified SiPLATZs and PLATZs from maize, rice, and Arabidopsis into three subfamilies (Appendix Fig S1A). The subfamily homologous to maize FL3, including SiPLATZ7-13, was selected for further analysis.

To quantify the expression patterns of SiPLATZ-13, we carried out quantitative reverse transcription-PCR (RT-qPCR) and found that except for SiPLATZ10, other SiPLATZ genes of this subfamily exhibited higher transcript levels in roots, followed by in panicles and shoots, than that in leaves (Appendix Fig S1B). SiPLATZ8 and SiPLATZ12 showed the highest transcript levels in roots of 14-day-old seedlings. Moreover, these SiPLATZ genes were also induced at 3 and 24 h after salt, alkaline, temperature and ABA treatments (Appendix Fig S1C).

To characterize the function of SiPLATZ12, we generated transgenic foxtail millet lines in the ‘Ci846’ background. Transcript levels of SiPLATZ12 in 14 independent 35S::SiPLATZ12 transgenic lines were verified by RT-qPCR (Appendix Fig S2A), and three lines (#22, #24, #28) were selected for further investigation. The transgenic plants exhibited significantly larger seeds with up to 29.7% longer and 36.5% wider seeds, resulting in 40.3% higher TGW than those of ‘Ci846’ (Fig 1A-D). The transgenic plants also exhibited 32.2% longer and 36.7% wider panicles (Fig 1E-G), 17.7% lower grain number per panicle, and 32.2% higher grain weight per panicle (Fig 1H and I) than those of ‘Ci846’.

Moreover, compared with ‘Ci846’, the three transgenic plants also showed increased main stem diameter (Fig 1J and K),
reduced plant height and leaf number (Fig 1L-N), as well as delayed heading time (Fig 1L). The higher transcript levels of \textit{SiPLATZ12} in panicles, especially young panicles than other indicated tissues, such as flag leaves, roots, stems and leaves of young seedlings confirmed the role of \textit{SiPLATZ12} in regulating the corresponding organ development (Appendix Fig S2B). These results indicated the function of \textit{SiPLATZ12} in positively regulating multiple elite yield traits in foxtail millet.

Moreover, when 3-day-old seedlings were transferred into Hoagland medium containing series of concentrations of NaCl, growth of transgenic seedlings was seriously inhibited, with 40%–50% shorter roots and 30% lower fresh weight than ‘Ci846’ under 250 mM NaCl (Fig 2). These results illustrated that \textit{SiPLATZ12} negatively regulate salt tolerance in foxtail millet. However, the longer roots and larger shoots of \textit{SiPLATZ12} transgenic foxtail millet than ‘Ci846’ under normal conditions and low concentrations NaCl (< 100 mM) (Fig 2A) might be due to their large seeds.

In addition, the function of \textit{SiPLATZ12} was verified by heterologously expressing in Col-0 \textit{Arabidopsis}. The transgenic Arabidopsis lines (#1, #4 and #6) were confirmed by RT-qPCR (Appendix Fig S3A). Consistent with the results in foxtail millet, the \textit{SiPLATZ12} transgenic \textit{Arabidopsis} also showed larger seeds and higher TGW than those of the WT (Appendix Fig S3B and C). Besides, these transgenic \textit{Arabidopsis} seedlings also displayed slower growth and lower fresh weight than the WT (Appendix Fig S3E and F). The salt tolerance of these transgenic \textit{Arabidopsis} seedlings were reduced obviously with lower germination rate, growth, fresh weight and SUR compared with the WT (Appendix Fig S3D-G). These results confirmed the positive role of \textit{SiPLATZ12} in regulating TGW but negative role in regulating salt tolerance in plants.

\textbf{A 9-bp insertion in the \textit{SiPLATZ12} promoter improves its expression and yield traits in millets}

To verify the function of \textit{SiPLATZ12} genetically, we compared the nucleotide differences of DNA regions including 3 Kb upstream and 2 Kb downstream and gene body of \textit{SiPLATZ12} among 1893 millet accessions. Total of 18 variations, representing 158 haplotypes, exist within this region (Fig 3A). Five major haplotypes with high frequency (more than 40 millet accessions each) were selected for further analysis (Fig 3B and C). Hap1 was the predominant haplotype, with 1099 foxtail millet (cultivated) accessions. Hap18 was the second major haplotype, with 264 green foxtail accessions (wild varieties). Hap2 and Hap14 were represented by 77 and 64 accessions, with only one wild and cultivated accession, respectively. Hap3 was assigned to 44 accessions: 11 cultivated and 33 wild accessions. These distributions indicated that selection of \textit{SiPLATZ12} likely occurred during domestication and breeding.

To confirm the domestication and selection of \textit{SiPLATZ12}, we analyzed the TGW among the five major haplotypes (Supplementary dataset 1). Hap1, Hap2, Hap3, and Hap14 millet accessions showed higher TGW (2.683, 2.605, 1.847,
2.441 g, respectively) than that of Hap18 accessions (0.834 g) (Fig 3B). The survival rate (SUR) of 801 millet accessions under salt stress were identified (Dataset EV1), and found lower SUR of Hap1, Hap2, and Hap3 (31.411%, 28.671%, 44.583%, respectively) than that of Hap18 accessions (56.667%) (Fig 3B). Obviously, the cultivated foxtail millet accessions have higher TGW but lower SUR than the wild ones, confirming the selection of SiPLATZ12 during domestication and breeding.

To know whether and how SiPLATZ12 is regulate in different millet varieties, we analyzed cis-element in the SiPLATZ12 promoter and found a 9-bp insertion in a heat stress response element (HSE) (AGAANNTTCT) in Hap18 promoter resulted in Hap1, Hap2, Hap3, and Hap14 SiPLATZ12 promoters (Fig 3B and D). Thus two haplotypes were generated at this site: HapHSE (AGAANNTTCT) and HapHSEIn9 (AGCTCGGGATGAANNTTCT). The distribution analysis showed that total of 1227 S.italica and 253 S.viridis accessions belong to HapHSEIn9 type, while 413 S.viridis accessions belong to HapHSE type (Dataset EV1). This distribution of the two haplotypes indicated the selection of HSEIn9 by breeders in domestication of Setaria.

To validate the regulation of SiPLATZ12 by the 9-bp insertion in the HSE element, we detected the transcripts of SiPLATZ12 in two haplotypic accessions, and found that SiPLATZ12HapHSEIn9 had higher transcripts than that of SiPLATZ12HapHSE under normal condition but lower transcripts than that of SiPLATZ12HapHSE under salt stress (Fig 3E, Appendix Fig S4A). These results indicated greater but lower salinity-inducible activity of the HapHSEIn9 promoter than the HapHSE promoter. Consistently, the HapHSEIn9 promoter drove higher GUS activity than that of the HapHSE promoter in tobacco (Nicotiana tabacum) epidermal cells (Fig 3F). And higher GUS transcript and protein levels driven by the HapHSEIn9 promoter were detected in tobacco epidermal cells than those driven by the HapHSE promoter (Fig 3G and H). Importantly, SiPLATZ12HapHSEIn9 was positively associated with TGW, panicle length, main stem diameter, and heading time, while SiPLATZ12HapHSE was positively correlated with SUR (Fig 3I-M, Dataset EV1). The changes in TGW and SUR of some represent HapHSEIn9 and HapHSE accessions, and expression levels of SiPLATZ12 were illustrated in Appendix Fig S4B-F. These results indicated that variation in cis-regulation of SiPLATZ12 contribute to its differential expression, and thus diverse yield traits and salt tolerance in millet.

SiPLATZ12 regulates the expression of seed development and salt tolerance related genes

To explore the mechanism of SiPLATZ12 in regulating seed development and salt stress response in foxtail millet, the expression of the related genes was detected. The transcript levels of IKU1, IKU2, MINI3, SHB1, KLU, FLS2, and CKII were obviously up-regulated by SiPLATZ12 overexpression in foxtail millet (Fig 4A). In contrast, transcript levels of most SiNHX, SiSOS, and SiCBL genes were significantly reduced by SiPLATZ12 overexpression in foxtail millet under...
both normal and salt-stress conditions (Fig 4B and C). Similar expression patterns for \textit{AtNHX}, \textit{AtSOS}, and \textit{AtCBL} genes were obtained in \textit{SiPLATZ12} transgenic \textit{Arabidopsis} seedlings when compared with Col-0, except for \textit{AtCBL9} (Appendix Fig S5 and 6). Therefore, \textit{SiPLATZ12} increases the expression of genes controlling seed size, but inhibits that of \textit{NHX}, \textit{SOS}, and \textit{CBL} genes in both foxtail millet and \textit{Arabidopsis}.

Since NHXs act in cell expansion by regulating Na\textsuperscript{+}, K\textsuperscript{+} and vacuolar pH homeostasis (Bassil \textit{et al}., 2011a; Bassil \textit{et al}., 2011b), we measured the Na\textsuperscript{+}, K\textsuperscript{+} and pH changes in ‘Ci846’ and \textit{SiPLATZ12}-overexpressing foxtail millet seedlings. We observed higher Na\textsuperscript{+} contents in the transgenic foxtail seedlings than in ‘Ci846’ under both normal and salinity conditions (Fig 4D). In contrast, K\textsuperscript{+} contents in \textit{SiPLATZ12}-overexpressing foxtail millet were lower than those in ‘Ci846’ under both normal and salinity conditions (Fig 4E). Although no significant difference in vacuolar pH between root tip cells of ‘Ci846’ and \textit{SiPLATZ12}-overexpressing seedlings, vacuolar pH in cells of the mature root zone was nearly 0.25 units lower in \textit{SiPLATZ12}-overexpressing seedlings compared with ‘Ci846’ seedlings (Fig 4F, Appendix Fig S7). These data suggested that \textit{SiPLATZ12} disturb Na\textsuperscript{+}, K\textsuperscript{+} and vacuolar pH homeostasis through inhibiting the expression of \textit{NHX} and related genes in foxtail millet.

To establish the relationship of \textit{SiNHX1}, \textit{SiNHX2}, \textit{SiNHX3}, and \textit{SiNHX7} with seed size and salt tolerance in foxtail millet, we detected the expression of them in HapHSE and HapHSE\textit{In9} millet accessions, respectively. The results exhibited that transcript levels of \textit{SiNHX1}, \textit{SiNHX2}, \textit{SiNHX3}, and \textit{SiNHX7} in seedlings of HapHSE millet accessions was significantly higher than that in HapHSE\textit{In9} millet accessions under both normal and salinity conditions (Fig 4G). Then, we performed correlation analysis and found that the TGW of these accessions showed negative and weak correlations (0.3<\textit{R}^2<0.5), while the SUR of these accessions showed positive and strong correlations (\textit{R}^2>0.7) with the expression levels of the four genes (Fig 4H), indicating negative and partial contribution of \textit{SiNHX1}, \textit{SiNHX2}, \textit{SiNHX3} and \textit{SiNHX7} to TGW while positive and main contribution of them to SUR in millet.

\textbf{\textit{SiPLATZ12} binds directly to the promoters of \textit{SiNHX2}, \textit{SiCBL4} and \textit{SiCBL7}}

To test whether \textit{SiPLATZ12} acts as a direct transcription regulator of genes mentioned above, we verified the direct binding of \textit{SiPLATZ12} to the promoters of \textit{SiNHX2}, \textit{SiCBL4}, and \textit{SiCBL7} by yeast one hybrid (Y1H) experiment (Fig 5A). After the confirmation of the nuclear localization of \textit{SiPLATZ12} in foxtail millet hairy-roots and transgenic \textit{Arabidopsis} roots (Appendix Fig S8A and B), we performed chromatin immunoprecipitation (ChIP)-qPCR analysis using the \textit{SiPLATZ12}-GFP transgenic hairy roots of foxtail millet using specific primers for cloning A/T rich DNA fragments within the promoters of \textit{SiNHX2}, \textit{SiCBL4}, and \textit{SiCBL7} (Table S1, Appendix Fig S9A). The results showed that greater enrichment of P4 and P5 DNA fragments within the \textit{SiNHX2} promoter, P7 and P9 DNA fragments within the \textit{SiCBL4}
promoter, and P10 and P12 within the SiCBL7 promoter were obtained in pPLATZ12::PLATZ12-GFP transgenic samples than in pPLATZ12::GFP transgenic samples, while other indicated fragments showed no difference between them (Fig 5B-D), indicating the binding of SiPLATZ12 to SiNHX2, SiCBL4, and SiCBL7 promoters in vivo. The results of microscale thermophoresis (MST) assays between SiPLATZ12 and P5, P9, and P10 DNA fragments within SiNHX2, SiCBL4, and SiCBL7 promoters confirmed the binding of SiPLATZ12 to SiNHX2, SiCBL4, and SiCBL7 promoters in vitro (Fig 5E, Appendix Fig S9B). Finally, we performed electrophoretic mobility shift assays (EMSAs) and found that SiPLATZ12-His shifted all of the labeled SiNHX2, SiCBL4, and SiCBL7 probes (Fig 6F). These shifted probes were gradually weakened by increasing concentrations of unlabeled SiNHX2, SiCBL4, and SiCBL7 DNA fragments until they were no longer detectable. Meanwhile, no shifted bands were observed when SiNHX2, SiCBL4, or SiCBL7 probes were mutated to C/G DNA probes. These data proved direct binding of SiPLATZ12 to some A/T rich sequences in the promoters of SiNHX2, SiCBL4, and SiCBL7 in vitro.

To determine the genetic relationship of SiPLATZ12 and SiNHX2 and SiCBL4, SiNHX2 and SiCBL4 were overexpressed in hairy-roots of foxtail millet overexpressing SiPLATZ12 (#28) and ‘Ci846’. As expected, all ‘Ci846’ and #28 seedlings with 35S::SiNHX2 and 35S::SiCBL4 transgenic hairy roots grew better than with empty vector transgenic hairy roots (Fig 5G-I) under normal conditions. Meanwhile, SURs of seedlings with SiNHX2 and SiCBL4 transgenic hairy roots were 55% and 66.7% higher than that with the empty vector transgenic hairy roots under salt stress (Fig. 5G and J). These observations support that SiPLATZ12 functions upstream of SiNHX2 and SiCBL4.

Discussion

Seed size is a major factor controlling grain yield, and is strongly affected by various genetic factors, such as the IKU pathway, the ubiquitin-proteasome pathway, G-protein signaling, the mitogen-activated protein kinase signaling pathway, phytohormones, and transcriptional regulatory factors in Arabidopsis (Kesavan et al., 2013; Li and Li, 2016). We revealed that SiPLATZ12 play important roles in regulating seed size in foxtail millet (Fig 1A-D; Fig 3B and I) by upregulating the expression of genes involved in the IKU pathway, including IKU1, IKU2, MINI3, and SHB1 (Fig 4A). Loss of function of IKU1, IKU2, MINI3, and SHB1 genes reduces seed size due to precocious cellularization of the endosperm (Luo et al., 2005; Wang et al., 2010; Zhou et al., 2009). Therefore, SiPLATZ12 is a novel activator of genes in the IKU pathway to influence endosperm growth or grain filling. Moreover, KLU encodes the cytochrome P450 CYP78A5 and positively regulates seed size by promoting cell proliferation in maternal integuments (Adamski et al., 2009). The up-regulated expression of KLU by SiPLATZ12 overexpression in foxtail millet (Fig 4A) indicating a role of SiPLATZ12 in maternal control of seed size at least through activating KLU expression. Besides, FL3 and GL6 are
required for tRNA and 5S rRNA transcription through interaction with RNA Polymerase III (Kim et al., 2018; Li et al., 2017; Wang et al., 2019; Wang et al., 2018). Consistent with the binding sequences of FL3 and GL6, SiPLATZ12 bound A/T rich sequences in the promoters of target genes (Fig 5B-F), indicating possible similar mechanisms of SiPLATZ12 with FL3 and GL6 in regulating seed size. Thus, SiPLATZ12 controls seed length and width by regulating the expression of multiple genes involved in several signaling pathways.

Panicle size also strongly affects grain yield. SiMADS34, E-class MADS-box TF, positively regulates panicle length but negatively regulates panicle width and grain yield in S.italica by upregulating Seita.6G205500, a homology of rice SPL14 (Yang et al., 2021). A recessive mutant of Loose Panicle 1 (LP1), which encodes a group I WRKY TF, shows pleiotropic phenotypes, such as a lax primary branching pattern, aberrant branch morphology, semidwarfism, long and wide panicles, and big seeds (Xiang et al., 2017). Similar to LP1 but different from SiMADS34, SiPLATZ12 positively regulates panicle size and grain number in foxtail millet (Fig 1E-H), reflecting the complexity of panicle and grain number development.

Moreover, plant height and stem diameter are usually thought to be associated with high grain yield by changing lodging resistance of most crops. Breeding of the varieties carrying the dwarfing genes (Rht) is the main direction to reduce the risk of lodging and increase grain yield (Evans, 1998; Rebetzke et al., 2011; Tang et al., 2021). The diameter of the stem correlates with resistance to lodging in rice and wheat (Ageeva et al., 2020; Kashiwagi et al., 2008). The loci with high phenotypic effects on lodging tolerance are colocalized with loci responsible for plant height, stem diameter and stem strength (Ageeva et al., 2020). Due to the reduced plant height and improved stem diameter of SiPLATZ12 overexpression millet (Fig 1J-M, Fig 3K; Dataset EV1), SiPLATZ12 may be a novel loci controlling lodging resistance.

Besides, heading time is an important developmental transition in plants leading to successful sexual reproduction and is determined by multiple genes and environmental factors, such as day-length and temperature (Freytes et al., 2021). Several genetic factors controlling the heading date of foxtail millet have been identified. Heading date 1 (HD1), homolog gene of CO, has been identified as a candidate of a quantitative trait loci (QTL) for heading date using genome-wide association studies (Jia et al., 2013), which is under strong selection during domestication (Liu et al., 2015). Roc4 was identified by QTL sequence (Yoshitsu et al., 2017). Roc4 promotes flowering time under long days in rice (Wei et al., 2016). The delayed heading of SiPLATZ12 transgenic foxtail millet and the association of heading date with HapHSEIn9 indicate a novel genetic factor in controlling heading date and the domestication of SiPLATZ12 HapHSEIn9 in foxtail millet (Fig 1JL, N, Fig 3L; Dataset EV1).

In addition, SiPLATZ12 negatively regulates millet salt tolerance. The salt tolerance of plants has been proved to be
largely associated with cytoplasm Na⁺ homeostasis which is maintained by membrane localized NHXs and their regulatory proteins, such as SOS2 and CBLs (Halfter et al., 2000; Liu and Zhu, 1998; Quintero et al., 2011; Zhou et al., 2022). We found that SiPLATZ12 repressed the expression of most of NHX, SOS and CBL genes, and directly targeted SiNHX2, SiCBL4, and SiCBL7 genes (Fig 4B and C; Fig 5A-F; Appendix Fig S9). SiNHX2 and SiCBL4 overexpression could rescue the salt sensitivity of SiPLATZ12 overexpression foxtail millet (Fig 5G-J). Therefore, SiPLATZ12 overexpression might lead to lower Na⁺/H⁺ antiporter activities of SOS1 and NHXs, which in turn leads to the increased Na⁺ accumulation in SiPLATZ12-overexpressing foxtail millet (Fig 4D). Simultaneously, NHXs participate in vacuolar K⁺ and pH homeostasis (Bassil et al., 2011a; Bassil et al., 2011b). NHX5 and NHX6-mediated pH increasing of endomembrane compartments influences the sorting of transmembrane proteins (Bassil et al., 2011a). Thus, NHX operations are essential for many processes, including stomatal regulation (Barragán et al., 2012), flower development (Bassil et al., 2011b; Yoshida et al., 2005), plant reproduction (Bassil et al., 2012; Bassil et al., 2011b), and seed development (Reguera et al., 2015). Consistently, Na⁺, K⁺ and vacuolar pH homeostasis were disturbed in foxtail millet overexpressing SiPLATZ12 (Fig 4D-F), which might be the reason behind the multiple changed yield traits under normal conditions (Fig 1). Furthermore, TGW showed negative correlation while salt tolerance showed positive correlation with the expression levels of SiNHX1, SiNHX2, SiNHX3, and SiNHX7 (Fig 4G and H). Therefore, SiPLATZ12 regulates multiple yield traits and salt tolerance partially by suppressing the expression of NHX, SOS, and CBL genes.

Tradeoffs among agronomic traits limit the crop yield, such as penalties of yield by plant immunity, grain quality by yield and negative correlations among plant architecture components (Nelson et al., 2018; Wang et al., 2021; Song et al., 2022). Many of these tradeoffs are caused by gene pleiotropy. Our results provide strong evidence for the SiPLATZ12 pleiotropy (Fig 1, 2 and 3, Appendix Fig S3). Two tradeoffs between TGW and salt tolerance, and grain number per panicle and seed size were caused by SiPLATZ12. Changes on cis-regulatory regions can regulate quantitative traits at different levels (Rodriguez-Leal et al., 2017; Hendelman et al., 2021; Liu et al., 2021). The yield and salt tolerance are determined in different conditions, while panicle size and seed size are determined in different developmental stages. Therefore, modifying cis-regulatory regions of SiPLATZ12 may overcome the drag between yield and salt tolerance as well as grain number per panicle and seed size to outperform current elite natural alleles. In fact, a 9-bp insertion in the SiPLATZ12 promoter disrupted the HSE element and resulted in higher expression of SiPLATZ12^HapHSEIn9 than SiPLATZ12^HapHSE under normal condition (Fig S4F), supporting the efficiency of overcoming the tradeoffs between different yield traits by modifying cis-regulatory regions of SiPLATZ12. Therefore, identifying and modifying the cis-elements responding to salt stress and heading date may reduce the pleiotropic effect of SiPLATZ12 and generate a
‘ideal’ phenotype variation in foxtail millet.

In summary, our study reveals the genetic mechanisms by which SiPLATZ12 promotes multiple elite yield traits at the expense of salt tolerance in foxtail millet (Fig 6). The expression levels of SiPLATZ12 in HapHSE\textsuperscript{lab} millet accessions (landrace and cultivated varieties) are increased by a 9-bp insertion in the promoter of SiPLATZ12. On one hand, SiPLATZ12 then activates the expression of genes in the IKU pathway, including IKU1, IKU2, MINI3, and SHB1, and the maternal control genes, such as KLU, to increase seed size. On the other hand, SiPLATZ12 inhibits the expression of NHX, SOS, and CBL genes, at least directly targets to SiNHX2, SiCBL4 and SiCBL7, to increase TGW, panicle size and stem diameter, but inhibit plant height, heading date and salt tolerance of foxtail millet. Our results provide novel insights into the genetic basis of the trade-off between yield traits and salt tolerance in millet. However, further elucidating the regulatory relationships of SiPLATZ12 with other regulators controlling yield traits and salt tolerance modifying the \textit{cis}-element of SiPLATZ12 will contribute well to breeding of new elite rice cultivars with “ideal” plant architecture to improve grain yield of foxtail millet.

Materials and Methods

Plant materials and growth conditions

Foxtail millet (\textit{S. italica}) cultivars ‘Yugu1’ and ‘Ci846’, and \textit{Arabidopsis} Col-0 were used for gene expression and function analyses. Foxtail millet seeds were sterilized with sodium hypochlorite, washed three times and germinated at 28\(^\circ\)C for 3 d. For inducible expression analysis, 10-day-old seedlings of ‘Yugu1’ were treated with Hoagland solution containing 200 mM NaCl, 50 mM NaHCO\textsubscript{3}, 100 \(\mu\)M ABA, and 20\% (w/v) polyethylene glycol 6000 (PEG6000) or at 4\(^\circ\)C or 42\(^\circ\)C for 0, 3, and 24 h. After treatment, whole seedlings were collected and frozen quickly in liquid nitrogen for RNA extraction. For tissue expression analysis, roots, stems and leaves from 10-day-old seedlings, panicles from 2-month-old seedlings and seeds of ‘Yugu1’ were collected and frozen quickly in liquid nitrogen. For phenotypic analysis, seeds were germinated on filter paper wetted with water or salt solution for the times indicated. Alternatively, the 3-day-old uniformly developed seedlings were cultured for 10 d under 16/8 h light/dark period and 28 \pm 1\(^\circ\)C or in the field during summer for the times indicated. Transgenic hairy roots were prepared as described previously (Zhang \textit{et al}., 2021). Culture \textit{Arabidopsis} materials were described previously (Yu \textit{et al}., 2019). Phenotypes were photographed, and root or shoot length and fresh weight were measured.

For salt tolerance haplotype analysis, 801 germplasms, including wild, landrace and cultivated accessions, were used (Supplementary dataset1). Four-day-old seedlings were transferred to pots with matrix (nutrient soil:
vermiculite=1:1) for 7 d. Healthy developed seedlings were then saturated in 300 mM NaCl solution for three consecutive times (once every 3 days) and grown for an additional 10 d. Surviving seedlings were counted, and survival rates were calculated. Three independent experiments were carried out.

Identification of SiPLATZ, SiNHX, SiSOS1, SiSOS2 and SiCBL genes in foxtail millet

PLATZ and NHX family members and SOS2 from Arabidopsis were used to BLAST the foxtail millet genome in Phytozome V12 (https://phytozome.jgi.doe.gov) to identify SiPLATZ, SiNHX and SiSOS2 genes. SiSOS1 (Yan et al., 2021) and SiCBL genes (Zhao et al., 2013) has been identified. Isoelectric point and molecular weight were predicted using the ExPASy Proteomics Server (http://expasy.org/). An evolutionary tree was generated using MEGA 6 software and a phylogenetic evolutionary tree was constructed using the neighbor-joining analysis and bootstrap method, with 1000 replicates.

RNA extraction and quantitative RT-PCR (RT-qPCR)

Total RNA was extracted using RNAiso Plus (Takara, Ohtsu, Japan). As described previously (Yu et al., 2019), reverse transcriptions were performed using PrimeScriptTM RT reagent kit (TaKaRa, Ohtsu, Japan). RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Biotech Co., Ltd) in a three-step program on a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). 18S ribosomal RNA and SiACT7 (Li et al., 2016) were used as internal controls. Three biological replicates were performed. Primers are shown in Table S1.

Generation of transgenic foxtail millet and Arabidopsis

Transgenic foxtail millet (cultivar ‘Ci846’) plants were generated by introducing the gene-coding region of SiPLATZ12 under control of the 35S promoter via callus-based gene transformation using Agrobacterium tumefaciens strain GV3101. Transformants were selected on half-strength MS medium supplemented with 50 mg/L kanamycin and planted to soil for harvesting seeds. Transgenic foxtail millet seedlings were confirmed by RT-qPCR using primers listed in Table S1. Transgenic Arabidopsis containing 35S::SiPLATZ12 and 35S::SiPLATZ12-GFP was generated simultaneously using the floral-dipping method. T3 transgenic Arabidopsis plants were selected on half-strength MS medium supplemented with 50 mg/L kanamycin and confirmed by RT-qPCR using primers listed in Table S1.

Haplotype analysis

We collected the available data for phenotypes related to yield traits for 916 accessions in our previous study (Jia et al., 2013) The genotype data for those accessions were obtained by high-depth resequencing (unpublished). The DNA sequences, comprising promoter (3 kb upstream of ATG), gene body (2360 bp between 28665353 to 28667712 in
chromosome 7, reverse), and 2 kb downstream of SiPLATZ12, were extracted. The haplotype analysis was performed using in-house python and R scripts.

**Fluorometric GUS assays**

$pHapHSE_{in9}$ and $pHapHSE$ representing the 869 bp intergenic region upstream of ATG of SiPLATZ12 from HapHSE_{in9} and HapHSE millet accessions, and was fused to the pBI121 vector to drive GUS expression. Then $pHapHSE_{in9}$::GUS and $pHapHSE$::GUS constructs were transiently transformed into tobacco (N. benthamiana) leaves. GUS enzymatic activities in tobacco leaves were measured on an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) using 4-methylumbelliferyl-β-d-glucuronide as a substrate. Standard curves were prepared using 4-methylumbelliferone. Mean GUS activities were obtained from three independent measurements, and each assay was repeated three times.

**Immunoblotting analysis**

Root samples (0.5 g) of 7-day-old transgenic foxtail millet seedlings harboring $pHapHSE_{in9}$::GUS and $pHapHSE$::GUS obtained by via Agrobacterium tumefaciens transformation were ground in liquid nitrogen and mixed with 500 µl of non-denatured protein extract [Tris–HCl (pH 7.5), 25 mM; MgCl₂, 10 mM; NaCl, 10 mM; DL-Dithiothreitol (DTT), 5 mM; phenylmethylsulfonyl fluoride (PMSF), 4 mM; ATP, 10 mM]. The crude extract was placed on ice for 30 min and then centrifuged at 17 000 g for 15 min at 4°C. The supernatant was centrifuged again, and the supernatant was collected. Anti-GUS antibody was used to examine the protein level of GUS.

**Y1H assay**

$SiNHX2$, $SiCBL4$, and $SiCBL7$ promoters were amplified and cloned into pLacZi2µ, which contains the LacZ reporter gene, to generate $pSiNHX2$:LacZ, $pSiCBL4$:LacZ and $pSiCBL7$:LacZ, respectively. The cDNA sequence (CDS) of SiPLATZ12 was cloned into the pJG4-5 vector containing the GAL4 transcriptional activation domain (GAD) to generate PLATZ12-pJG4-5. PLATZ12-pJG4-5 and $pSiNHX2$:LacZ, $pSiCBL4$:LacZ or $pSiCBL7$:LacZ were co-transformed into yeast strain EGY48 according to the Yeast Protocols Handbook (Clontech). Transformed yeast (Saccharomyces cerevisiae) cells were plated onto selective medium without Ura or Trp, and positive clones were cultured on medium without Ura or Trp containing galactose (20%), raffinose (20%), BU-salt (50 ml: 1.95 g Na₂HPO₄, 1.855 g NaH₂PO₄·2H₂O), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for development of blue colour. Yeast cells co-transformed with pJG4-5 and $pSiNHX2$:LacZ, $pSiCBL4$:LacZ or $pSiCBL7$:LacZ were used as negative controls.
Subcellular localization analysis

To determine the subcellular localization of SiPLATZ12, transgenic foxtail millet hairy roots and Arabidopsis harboring 35S::SiPLATZ12-GFP were generated. GFP fluorescence was then observed using a LSM880 high-resolution laser confocal microscope (Zeiss, Germany). DAPI (4′,6-diamidino-2-phenylindole) was used to stain the nucleus.

ChIP-qPCR assay

ChIP assays were performed as described previously (Liu et al., 2020). Seven-day-old 35S::SiPLATZ12-GFP transgenic seedlings and GFP-specific monoclonal antibody (Beyotime Biotechnology, China) were used for ChIP. ChIP DNA products were analyzed by qPCR with primers designed to amplify the indicated DNA fragments within the SiNHX2, SiCBL4 and SiCBL7 promoter, respectively.

MST assay

MST assay was performed according to the method established by Wienken et al. (2010). SiPLATZ12-His proteins were induced in Escherichia coli Rosetta cells and purified using a His-Tagged Protein Purification Kit (CW BIO, Beijing, China) as described previously (Liu et al., 2020). Binding reaction of recombinant SiPLATZ12-His protein to different probes was measured by microscale thermophoresis (MST) in a Monolith NT.115 (Nano Temper Technologies) instrument. The SiPLATZ12-His labeled with Cy5 were diluted to 40 nM by a buffer containing 50 mM Tris-HCl (pH 7.4) and 0.05% (v/v) Tween 20. A range of concentrations of different probes in the assay buffer [50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 10 mM MgCl2, 0.05% Tween 20] was incubated with labeled protein (1:1, v/v) for 10 min. The sample was loaded into the Monolith NT.115 standard capillaries and measured with 40% MST power. The KD Fit function of the Nano Temper Analysis Software was used to fit the curve and calculate the value of the dissociation constant (Kd).

EMSA

Three indicated DNA fragments within the SiNHX2, SiCBL4 and SiCBL7 promoters were respectively amplified using biotin-labeled primers (Table S1) synthesized by Sangon Biotech (Shanghai China) and purified using a PCR purification kit (Qiagen). EMSA was conducted using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Pierce, USA) as described previously (Liu et al., 2020). Unlabeled DNA fragments of the same sequences were used as competitors. Migration of biotin-labeled probes was detected using enhanced chemiluminescence substrate (Thermo Scientific) on a ChemDoc XRS system (Bio-Rad).

Na+ and K+ content measurement
To determine Na\(^+\) and K\(^+\) contents, one-week-old seedlings were treated with 200 mM NaCl for 12 h before harvesting, drying for 48 h at 80°C and then grinding to powder. Tissue powder (100 mg) was digested in concentrated nitric acid, hydrochloric acid and hydrogen peroxide for 30 min in a microwave 3000 digestion system (Anton Paar) for element extraction. Na\(^+\) and K\(^+\) concentrations were determined using a flame atomic absorption spectrometer (Analytik Jena).

**Vacuolar pH measurement with BCECF-AM**

The pH-sensitive fluorescent dye BCECF-AM was used to measure the vacuolar pH in root cells (Bassil et al., 2011b). Four-day-old seedlings grown on vertical plates were collected and incubated in liquid medium containing one-tenth-strength MS medium, 0.5% (w/v) Sucrose, 10 mM MES (pH 5.8), 10 μM BCECF-AM, and 0.02% (v/v) pluronic F-127 (Molecular Probes) for 1 h at 22°C in darkness. Seedlings were washed once for 10 min before microscopy. Dye fluorescence images were obtained using an LSM880 high-resolution laser confocal microscope (Zeiss, Germany). The fluorophore was excited at 458 and 488 nm, and single emission between 530 and 550 nm was detected for all images. Mature root cells were used for the images. After background correction, integrated pixel intensity was measured at both 458- and 488-nm excitation. Ratio values were used to calculate pH based on a calibration curve using ImageJ software (Bassil et al., 2011b). Average ratio values were determined from more than 10 individual seedlings.

**Statistical analysis**

All experiments in this study were performed at least three times. Error bars in each graph indicate mean values ± standard error (SE) of replicates. Statistically significant differences between measurements were determined using the independent sample \(t\)-test (*\(P < 0.05\); **\(P < 0.01\), ***\(P < 0.001\)) or one-way ANOVA (\(P < 0.05\); LSD and Duncan test) in IBM Statistical Product and Service Solutions Statistics software version 24 (IBM, USA).

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**Author contributions**

C.W., C.Z. and X.D. conceived the experiments; S.X. and C.W. write the article; S.X., Y.W., L.Z., Y. B., Y.W., M.L., and J. F. performed experiments; S.T., Y. S., S.Z., J.H., G.Y., and K.Y. provided materials and suggestions. All authors
approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

All relevant data, vectors, and plant materials that support the findings of this study are available from the corresponding author upon request.


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Supporting information

**Figure S1.** Characterization and expression analysis of SiPLATZs in foxtail millet.

**Figure S2.** Transcript levels of SiPLATZ12 in transgenic foxtail millet.

**Figure S3.** Overexpression of SiPLATZ12 increases TGW but reduces growth and salt tolerance of transgenic Arabidopsis.

**Figure S4.** Relationship between seed size and survival rate with the expression of PLATZ12 in three types of selected HapHSE and HapHSE Setaria accessions.

**Figure S5.** Phylogenetic analysis of NHXs (A) and CBLs (B) from foxtail millet (Setaria italica) and Arabidopsis thaliana.

**Figure S6.** SiPLATZ12 affects expression of salt tolerance related genes in Arabidopsis.

**Figure S7.** pH calibration curve and BCECF-AM dye loaded roots.

**Figure S8.** Subcellular localization of SiPLATZ12 in hairy-root of foxtail millet (A) and root tip of transgenic Arabidopsis (B).

**Figure S9.** Diagrams of DNA fragments in the promoter of SiNHX2, SiCBL4, and SiCBL7 in ChIP-qPCR.

**Table S1.** Identification of PLATZ genes in foxtail millet.

**Table S2.** Primers and DNA sequences used in this article.

**Table S3.** Identification of NHX genes in foxtail millet.

**Dataset EV1.** Accessions used in the haplotype and correlation analysis with TGW, panicle length, main stem diameter, heading date, and SUR.
Figure legends

Figure 1. SiPLATZ12 affects multiple yield traits in foxtail millet.

A  Seed shape of 35S::SiPLATZ12 transgenic plants and ‘Ci846’ (the background material) under normal conditions. Scale bars = 2 mm.

B-D  Seed length (B), width (C) and TGW (D) of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. At least 30 seeds were measured in each replicate. Three independent replicates were conducted. Values are given as the mean ± SD, ***P < 0.001 by Student's t-test.

E  Panicle shape of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants under normal conditions. Scale bars = 5 cm.

F-I  Panicle length (F) and width (G), grain number (H) and weight (I) per panicle of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. At least 20 panicles were measured in each replicate. Three independent replicates were conducted. Data are represented as the mean ± SD, ***P < 0.001 by Student’s t-test.

J, K  Stem phenotype (J) and diameter (K) of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. Scale bars = 4 mm. At least 20 stems were measured in each replicate. Three independent replicates were conducted. ***P < 0.001 by Student’s t-test.

L  35S::SiPLATZ12 transgenic foxtail millet grew slower and heading later than ‘Ci846’ under normal conditions. Scale bars = 10 cm.

M, N  Plant height (M) and leaf number (N) of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. At least 20 plants were used in each replicate. Three independent replicates were conducted. **P < 0.01 and ***P < 0.001 by Student’s t-test.

Figure 2. SiPLATZ12 negatively regulates salt tolerance in foxtail millet.

A  Phenotypes of seven-day old Ci846’ and 35S::SiPLATZ12 transgenic seedlings under normal and salt stress conditions. Scale bars = 1 cm. Seedlings were grown in half-strength Hoagland solution containing a series of indicated concentrations of NaCl.

B, C  Relative root length (B) and fresh weight (C) of seven-day old Ci846’ and 35S::SiPLATZ12 transgenic seedlings under normal and salt stress conditions. At least 20 seedlings were used in each replicate. Three independent replicates were conducted. *P < 0.05, **P < 0.01 and ***P < 0.001 by Student's t-test.

Figure 3. A 9-bp insertion in the SiPLATZ12 promoter affects different expression of SiPLATZ12, yield traits and salt tolerance in millet.

A  Gene structure and distribution of DNA polymorphisms of SiPLATZ12.
B Chromosome and corresponding positions of variant nucleotides in *SiPLATZ12*, five major haplotypes (Hap) of *SiPLATZ12*, and the corresponding average thousand-grain weight (AVE-TGW) under normal conditions and survival rate (AVE-SUR) under salt stress. REF and ALT represent reference sequence and alternative sequence, respectively.

C Distribution of five major haplotypes among cultivated (*Setaria italica*) and wild (*Setaria viridis*) accessions. Numbers indicate frequencies (freq) of five major haplotypes. Arrow indicates domestication and breeding direction.

D Two haplotypes caused by a 9-bp insertion (red letters) at -585 bp upstream of the translational start site of *SiPLATZ12*. HapHSE contains a correct heat stress response element (HSE) (blue letters), while HapHSE<sup>ln9</sup> contains a 9-bp insertion in HSE.

E Relative expression levels of *SiPLATZ12* in every eight HapHSE and HapHSE<sup>ln9</sup> millet accessions, respectively. Three independent replicates were conducted. Values are given as the mean ± SD, **P < 0.01 by Student's t-test.

F GUS activities driven by HapHSE and HapHSE<sup>ln9</sup> promoters in tobacco leaves. 35S::GUS was used as control. Data are presented as the mean ± SD of three replicates. P < 0.05 by one-way ANOVA.

G, H Transcript (G) and protein (H) levels of *GUS* gene in tobacco leaves transformed with *pHapHSE::GUS* or *pHapHSE<sup>ln9</sup>::GUS*, respectively. 35S::GUS was used as control. Data are presented as the mean ± SD of three replicates. P < 0.05 by one-way ANOVA.

I-M TGW (I), panicle length (J), stem diameter (K), heading date (L), SUR (M) of HapHSE and HapHSE<sup>ln9</sup> millet accessions. *P < 0.05; **P < 0.01; ***P < 0.001 by Student’s t-test.

Figure 4. *SiPLATZ12* regulates the expression of seed development and salt tolerance related genes.

A Absolute expression levels of genes related to seed development in panicles at 3 day (Stage I), 10 day (stage II), and 20 day (stage III) after heading of ‘Ci846’ or *SiPLATZ12* overexpression foxtail millet. *SiACTIN7* and 18S rRNA were used as internal controls. Color scale for log2 FC (fold change) values is shown at the right. Experiments were performed in biological triplicate. *P < 0.1; **P < 0.01; ***P < 0.001 by Student’s t-test.

B, C *SiNHXs*, *SiSOSs* and *SiCBLs* in 7-day old ‘Ci846’ or *SiPLATZ12* overexpression foxtail millet treated with or without 200 mM NaCl for 24 h. *SiACTIN7* and 18S rRNA were used as internal controls. Color scale for log2 FC values is shown at the right. Experiments were performed in biological triplicate. *P < 0.1; **P < 0.01; ***P < 0.001 by Student’s t-test.

D, E Na<sup>+</sup> and K<sup>+</sup> contents of ‘Ci846’ and *SiPLATZ12* overexpression millet seedlings treated with or without 300 mM
NaCl for 6 h. Data are presented as the mean ± SD of three replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Student’s t-test.

F Vacuolar pH in roots of 4-d etiolated seedlings of ‘Ci846’ and SiPLATZ12 overexpression foxtail millet (#24), and measured by BCECF-AM. Data are presented as the mean ± SD; n > 10. ***$P < 0.001$ by Student’s t-test.

G Relative expression of SiNHX1, SiNHX2, SiNHX3 and SiNHX7 in HapHSE and HapHSE$^{109}$ millet accessions under normal and salt stress conditions. Data are presented as the mean ± SD of three replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Student’s t-test.

H Correlations of TGW and SUR with relative expression levels of SiNHX1, SiNHX2, SiNHX3 and SiNHX7 under normal and salinity conditions.

Figure 5. SiPLATZ12 binds directly the promoters of SiNHX2, SiCBL4, and SiCBL7 genes.

A Yeast one-hybrid assays show the binding of SiPLATZ12 to SiNHX2, SiCBL4, and SiCBL7 promoters. WU, Synthetic Dropout/-Trp-Ura.

B-D ChIP-qPCR analysis determine the SiPLATZ12 binding regions in the SiNHX2, SiCBL4 and SiCBL7 promoters. Data are presented as the mean ± SD of three replicates. ns, not significant; ***$P < 0.001$ by Student’s t-test.

E MST assays verify the direct binding of SiPLATZ12 to the selected selected A/T sequences in SiNHX2, SiCBL4, and SiCBL7 promoters. A mutant DNA fragment (mP5) was used as control.

F EMSAs verify the binding of SiPLATZ12 to the selected A/T sequences in SiNHX2, SiCBL4 and SiCBL7 promoters. DNA sequences and the corresponding mutant sequence used in this experiment were listed in Table S2. Arrow indicates shifted band of DNA by SiPLATZ12. The plus (+) or minus (-) denote the presence or absence of SiPLATZ12 or DNA in each sample.

G Photographs of ‘Ci846’ and ‘#28’ SiPLATZ12 overexpression seedlings transformed with empty vector (EM), 35S::SiNHX2 (SiNHX2) or 35S::SiCBL4 (SiCBL4) and cultured on MS medium with or without 150 mM NaCl for 7 days. Scale bars = 1 cm. Data represents the mean ± SD of three replicates. **$P < 0.01$, ***$P < 0.001$ by Student’s t-test.

H, I Expression levels of SiNHX2 and SiCBL4 in transgenic ‘Ci846’ (H) and ‘#28’ (I) seedlings. SiACTIN7 and 18S rRNA were used as the internal control. Data represents the mean ± SD of three replicates. **$P < 0.01$, ***$P < 0.001$ by 0.001 by Student’s t-test.

J Survival rates of ‘Ci846’ and ‘#28’ SiPLATZ12 overexpression seedlings transformed with EM, SiNHX2 or SiCBL4 and cultured on MS medium with or without 150 mM NaCl for 7 day. At least 30 seedlings were used. Data
represents the mean ± SD of three replicates. **P < 0.01, ***P < 0.001 by Student's t-test.

Figure 6. A model for SiPLATZ12-regulated yield traits and salt tolerance in millet.

A 9-bp insertion in the SiPLATZ12 promoter leads to the increased expression of SiPLATZ12 in HapHSE<sup>ho9</sup> millet accessions (landrace and cultivated varieties) compared to HapHSE (wild) accessions. On one hand, SiPLATZ12 directly targets and inhibits the expression of NHX, SOS, and CBL genes, leading to increased panicle size and TGW, but reduced plant growth and salt tolerance in foxtail millet. On the other hand, SiPLATZ12 activates the expression of genes involved in the IKU pathway, including IKU1, IKU2, MINI3, and SHB1, and in the maternal control, such as KLU, to increase seed size.
Figure 1. SiPLATZ12 affects multiple yield traits in foxtail millet.

A  Seed shape of 35S::SiPLATZ12 transgenic plants and ‘Ci846’ (the background material) under normal conditions. Scale bars = 2 mm.
B-D  Seed length (B), width (C) and TGW (D) of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. At least 30 seeds were measured in each replicate. Three independent replicates were conducted. Values are given as the mean ± SD. ***P < 0.001 by Student’s t-test.
E  Panicle shape of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants under normal conditions. Scale bars = 5 cm.
F-I  Panicle length (F) and width (G), grain number (H) and weight (I) per panicle of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. At least 20 panicles were measured in each replicate. Three independent replicates were conducted. Data are represented as the mean ± SD, ***P < 0.001 by Student’s t-test.
J, K  Stem phenotype (J) and diameter (K) of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. Scale bars = 4 mm. At least 20 plants were measured in each replicate. Three independent replicates were conducted. ***P < 0.001 by Student’s t-test.
L  35S::SiPLATZ12 transgenic foxtail millet grew slower and heading later than ‘Ci846’ under normal conditions. Scale bars = 10 cm.
M, N  Plant height (M) and leaf number (N) of ‘Ci846’ and 35S::SiPLATZ12 transgenic plants. At least 20 plants were used in each replicate. Three independent replicates were conducted. ***P < 0.001 by Student’s t-test.
Figure 2. SiPLATZ12 negatively regulates salt tolerance in foxtail millet.

A Phenotypes of seven-day old Ci846 and 35S::SiPLATZ12 transgenic seedlings under normal and salt stress conditions. Scale bars = 1 cm. Seedlings were grown in half-strength Hoagland solution containing a series of indicated concentrations of NaCl.

B, C Relative root length (B) and fresh weight (C) of seven-day old Ci846 and 35S::SiPLATZ12 transgenic seedlings under normal and salt stress conditions. At least 20 seedlings were used in each replicate. Three independent replicates were conducted. *P < 0.05; **P < 0.01; ***P < 0.001 by Student's t-test.
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B Chromosome and corresponding positions of variant nucleotides in SiPLATZ12, five major haplotypes (Hap) of SiPLATZ12, and the corresponding average thousand-grain weight (AVE-TGW) under normal conditions and survival rate (AVE-SUR) under salt stress. REF and ALT represent reference sequence and alternative sequence, respectively.

C Distribution of five major haplotypes among cultivated (Setaria Italica) and wild (Setaria Viridis) accessions. Numbers indicate frequencies (freq) of five major haplotypes. Arrow indicates domestication and breeding direction.

D Relative expression levels of SiPLATZ12, yield traits and salt tolerance. REF and ALT represent reference sequences.

E Relative expression of SiPLATZ12 in every eight HapHSE and HapHSE<sup>ind</sup> millet accessions, respectively. Three independent replicates were conducted. Values are given as the mean ± SD of three replicates. P < 0.05 by one-way ANOVA.

F GUS activities driven by HapHSE and HapHSE<sup>ind</sup> promoters in tobacco leaves. 35S::GUS was used as control. Data are presented as the mean ± SD of three replicates. P < 0.05 by one-way ANOVA.

G, H Transcript (G) and protein (H) levels of GUS gene in tobacco leaves transformed with phapHSE<sup>ind</sup>::GUS or phapHSE<sup>ind</sup>::GUS, respectively. 35S::GUS was used as control. Data are presented as the mean ± SD of three replicates. P < 0.05 by one-way ANOVA.

I-M TGW (I), panicle length (J), stem diameter (K), heading date (L), SUR (M) of HapHSE and HapHSE<sup>ind</sup> millet accessions. *P < 0.05; **P < 0.01; ***P < 0.001 by Student's t-test.
Figure 4. SiPLATZ12 regulates the expression of seed development and salt tolerance related genes.

A Absolute expression levels of genes related to seed development in panicles 3 day (Stage I), 10 day (stage II), and 20 day (stage III) after heading of ‘Ci846’ or SiPLATZ12 overexpression foxtail millet plants. SiACTIN7 and 18S rRNA were used as internal controls. Color scale for log2 FC (fold change) values is shown at the right. Experiments were performed in biological triplicate. *P < 0.1; **P < 0.01; ***P < 0.001 by Student’s t-test.

B, C SiNHXs, SiSOSs and SiCBLs in 7-day old ‘Ci846’ or SiPLATZ12 overexpression foxtail millet plants treated with or without 200 mM NaCl for 24 h. SiACTIN7 and 18S rRNA were used as internal controls. Color scale for log2 FC values is shown at the right. Experiments were performed in biological triplicate. *P < 0.1; **P < 0.01; ***P < 0.001 by Student’s t-test.

D, E Na+ and K+ contents of ‘Ci846’ and SiPLATZ12 overexpression foxtail millet seedlings treated with or without 300 mM NaCl for 6 h. Data are presented as the mean ± SD of three replicates. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test.

F Vacular pH in roots of 4-d etiolated seedlings of ‘Ci846’ and SiPLATZ12 overexpression foxtail millet (#24) measured by BCECF-AM. Data are presented as the mean ± SD; n > 10. **P < 0.01 by Student’s t-test.

G Relative expression of SiNHX1, SiNHX2, SiNHX3 and SiNHX7 in HapHSE and HapHSE-millet accessions under normal and salt stress conditions. Data are presented as the mean ± SD of three replicates. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test.

H Correlations of TGW and SUR with relative expression levels of SiNHX1, SiNHX2, SiNHX3 and SiNHX7 under normal and salinity conditions.
Figure 5. SiPLATZ12 binds directly to the promoters of SiNHX2, SiCBL4, and SiCBL7 genes.

A Yeast one-hybrid assays show the binding of SiPLATZ12 to SiNHX2, SiCBL4, and SiCBL7 promoters. WU, Synthetic Dropout-Trp-Ura.

B-D ChIP-qPCR analysis determine the SiPLATZ12 binding regions in the SiNHX2, SiCBL4, and SiCBL7 promoters. Data are presented as the mean ± SD of three replicates. ns, not significant. ***p < 0.001 by Student's t-test.

E MST assays verify the direct binding of SiPLATZ12 to the selected A/T rich sequence in SiNHX2, SiCBL4, and SiCBL7 promoters.

F EMSAs verify the binding of SiPLATZ12 to the selected A/T rich sequences in SiNHX2, SiCBL4, and SiCBL7 promoters. DNA sequences and the corresponding mutant sequence used in this experiment were listed in Table S2. Arrow indicates shifted band of DNA by SiPLATZ12. The plus (+) or minus (-) denote the presence or absence of SiPLATZ12 or DNA in each sample.

G Photographs of Ci846 and #28 SiPLATZ12 overexpression seedlings transformed with empty vector (Em), 3S::SiNHX2 (SiNHX2) or 3S::SiCBL4 (SiCBL4) and cultured on MS medium with or without 150 mM NaCl for 7 days. Data represents the mean ± SD of three replicates. **p < 0.01, ***p < 0.001 by Student's t-test.

H-I Expression levels of SiNHX2 and SiCBL4 in transgenic 'Ci846' (H) and '#28' (I) seedlings. SIACTIN7 and 18S rRNA were used as the internal control. Data represents the mean ± SD of three replicates. **p < 0.01, ***p < 0.001 by Student's t-test.

J Survival rates of 'Ci846' and '#28' SiPLATZ12 overexpression seedlings transformed with Em, SiNHX2 or SiCBL4 and cultured on MS medium with or without 150 mM NaCl for 7 days. Data represents the mean ± SD of three replicates. **p < 0.01, ***p < 0.001 by Student's t-test.
Figure 6. A model for SiPLATZ12-regulated yield traits and salt tolerance in millet. A 9-bp insertion in the SiPLATZ12 promoter leads to the increased expression of SiPLATZ12 in HapHSE9b millet accessions (landrace and cultivated varieties) compared to HapHSE (wild) accessions.

On one hand, SiPLATZ12 directly targets and inhibits the expression of NHX, SOS, and CBL genes, leading to increased panicle size and TGW, but reduced plant growth and salt tolerance in foxtail millet. On the other hand, SiPLATZ12 activates the expression of genes involved in the IKU pathway, including IKU1, IKU2, MINI3, and SHB1, and in the maternal control, such as KLU, to increase seed size.