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Association mapping uncovers maize *ZmbZIP107* regulating root system architecture and lead absorption under lead stress

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Lead (Pb) is a highly toxic contaminant to living organisms and the environment. Excessive Pb in soils affects crop yield and quality, thus threatening human health *via* the food chain. Herein, we investigated Pb tolerance among a maize association panel using root bushiness (BSH) under Pb treatment as an indicator. Through a genome-wide association study of relative BSH, we identified four single nucleotide polymorphisms (SNPs) and 30 candidate genes associated with Pb tolerance in maize seedlings. Transcriptome analysis showed that four of the 30 genes were differentially responsive to Pb treatment between two maize lines with contrasting Pb tolerance. Among these, the *ZmbZIP107* transcription factor was confirmed as the key gene controlling maize tolerance to Pb by using gene-based association studies. Two 5′ UTR_variants in *ZmbZIP107* affected its expression level and Pb tolerance among different maize lines. ZmbZIP107 protein was specifically targeted to the nucleus and *ZmbZIP107* mRNA showed the highest expression in maize seedling roots among different tissues. Heterologous expression of *ZmbZIP107* enhanced rice tolerance to Pb stress and decreased Pb absorption in the roots. Our study provided the basis for revelation of the molecular mechanism underlying Pb tolerance and contributed to cultivation of Pb-tolerant varieties in maize.

KEYWORDS

maize, lead tolerance, root system architecture, GWAS, gene-based association study, ZmbZIP107

Introduction

Heavy metal pollution from industrial and agricultural activities has become one of the most important environmental concerns worldwide. Heavy metals can cause irreversible damages to plants and thus lead to serious harm to human health through the food chain (Pourrut et al., 2013; Singh et al., 2015). Among various heavy metals, lead (Pb) is widespread in the environment and easily accumulated by plants through leaf adsorption or root enrichment (Gupta et al., 2013; Pourrut et al., 2013; Schreck et al., 2013). Pb exerts negative effects on plant growth and development of plants, such as inhibiting seed germination (Lamhamdi et al., 2011), root elongation (Huang et al., 2019), chlorophyll synthesis (Prasad and Prasad, 1987), cell division (Samardakiewicz and Woźny, 2005), and mineral element absorption (Seoud and Bakr, 2020), and causing membrane cell damage and thus cell death (Sharma and Dubey, 2005).

Furthermore, excessive Pb in soils seriously affect the yield and quality of plants (Alam et al., 2003; Dinakar et al., 2008). On the other hand, plants have evolved a variety of defense mechanisms to against the toxic action of Pb, including sequestration of Pb in vacuoles (Maestri et al., 2010), chelation of Pb in cytosols (Thapa et al., 2012), bind of Pb to organic acids (Thapa et al., 2012; Gupta et al., 2013), activation of antioxidant defense systems (Verma and Dubey, 2003; Gupta et al., 2009), and modification of soil rhizosphere environment (Jia et al., 2013).

Root system is a crucial vegetative organ for plant fixation, reproduction, and nutrient uptake (Beemster and Baskin, 1998). In addition, root system plays a significant role in plant growth and development, rhizosphere environment regulation and adaptation to abiotic stress through root number, root length, root surface area, root volume, and root bushiness and biomass (Grzesiak et al., 1999; Huang et al., 2019). Pb is absorbed and transported by plants mainly through their root systems, and approximately 90% of the total Pb is maintained in the roots for most plants (Kumar et al., 1995). In addition, root system responds to Pb stress by modulating root volume and diameter, such as inhibiting lateral root formation and thus reducing the volume of total roots. Under Pb stress, the viability of rice root cells was affected and cell death was promoted (Huang and Huang, 2008). The previous studies showed that Pb toxicity negatively affected *Lemna minor* root growth by inhibiting root tip cell division (Eun et al., 2000; Samardakiewicz and Woźny, 2005). Under Pb treatment, the root length and root dry weight were significantly reduced in wheat (Kaur et al., 2013), maize (Kozhevnikova et al., 2009), pea (Malecka et al., 2009), and *Sedum alfredii* (Gupta et al., 2010). Moreover, Pb treatment resulted in significantly inhibited root length and surface area in *Elsholtzia argyi* and *Elsholtzia splendens* (Peng et al., 2005). The recently developed 3D image reconstruction technology facilitates the studies on original traits of plant roots, such as root bushiness (BSH), root volume, and root diameter, relative to classical root traits (e.g. root length, root surface area, and total number of roots) (Mollier and Botany, 1999). Among these original traits, bushiness is described as ratio of the maximum of roots to the median of roots, which is considered an important indicator to evaluate the lush degree of plant roots and has been widely studied in soybeans, maize (Rangarajan and Lynch, 2021), Arabidopsis (Liang et al., 2018), and rice (Ambreetha et al., 2017).

Some genes involved in heavy metal tolerance have been reported in plants. Nature resistance-associated Macrophage Protein (NRAMP) family was previously demonstrated to play important roles in absorption and transport of heavy metals in plants. Among the NRAMP members, *OsNRAMP5* prevents the transport of cadmium to xylem and significantly reduced the cadmium (Cd) content in rice grains and shoots (Chang et al., 2020). However, heterologous expression of *OsNRAMP1* promoted Cd and arsenic accumulation in Arabidopsis (Tiwari et al., 2014). Yellow Stripe-Like (YSL) proteins are a kind of transporters encoding metal nicotinamides, which regulate metal uptake, transport, and distribution in a variety of plants. Among the YSL family, *BdYSL3*, *OsYSL16*, and *OsYSL2* modulate transport of intracellular copper, iron, and manganese (Mn) (Ishimaru et al., 2010; Zhang et al., 2018; Foroozani, 2021). Metal tolerance proteins (MTPs) are involved in metal homeostasis and tolerance in plants. Overexpression of *OsMTP8.1* enhanced Mn accumulation and tolerance in yeast and rice (Chen et al., 2013). Moreover, *OsMTP9* also participates in absorption and transport of Mn in rice (Ueno et al., 2015). In addition, several Pb stress-related genes have been reported in different plant species. Expression of *SbLRR2* in Arabidopsis conferred promoted root growth and reduced Pb accumulation in the transgenic Arabidopsis plants (Zhu et al., 2013). Expression of *AtATM3* in Indian mustard enhanced its tolerance to Pb and Cd stresses (Bhuiyan et al., 2011). *PSE1* that encodes an unknown NC domain-containing protein regulates Pb tolerance in Arabidopsis by activating expression of ABC transporter *PDR12/ABCG40* (Fan et al., 2016). In our previous study, the *ZmHIPP* gene, which encodes a heavy metal-associated isoprenylated plant protein was demonstrated to positively mediate Pb accumulation and tolerance in maize seedlings (Ma et al., 2022). Plant basic leucine zipper (bZIP) transcription factors are a relatively conserved and large gene families. A number of bZIP members have been identified in the plants, animals, and yeasts (Newman and Keating, 2003; Nam et al., 2021). Previous studies indicated that the bZIP transcription factors play significant roles as regulators in signal transduction and response to diverse biotic/abiotic stresses, such as drought, cold, heat, ABA, and salt stresses (Weltmeier et al., 2006; Hossain et al., 2010; Liu et al., 2014; Li et al., 2020b). These studies on the bZIP function mainly focused

on rice, maize, soybean, tomato, wheat, Arabidopsis, and banana (Kobayashi et al., 2008; Liao et al., 2008; Nijhawan et al., 2008; Hsieh et al., 2010; He et al., 2013; Yang et al., 2013). To date, only few studies reported the involvement of the bZIP genes in heavy metal stresses in plants.

Genome-wide association study (GWAS) is widely used to identify genetic variations and candidate genes of abiotic stress response-related traits in maize. A total of 21 single nucleotide polymorphisms (SNPs) for Pb accumulation were identified in a panel of 269 maize accessions using GWAS (Zhao et al., 2018). In a maize panel consisting of 230 inbred lines, 37, 12, 13, 27, and 23 SNPs were significantly associated with mercury accumulation in the grains, axes, stems, bracts, and leaves, respectively, by using GWAS, with the phenotype variation explained (PVE) by each SNP ranging from 6.91% to 15.87% (Zhao et al., 2017). In addition, 328 SNPs associated with root traits under high/low nitrate conditions were detected in an association panel of 461 maize inbred lines, with the PVE of a single SNP varying between 5.21% and 8.81% (Xsa et al., 2020). Our previous study performed GWAS for seed chilling-germination in maize and uncovered 15 significant associations controlling seed germination under cold stress (Zhang et al., 2021). By combining gene-based association studies, four genes were further confirmed as the potential causal genes (Zhang et al., 2021). In the present study, GWAS was performed in an association panel of 312 maize inbred lines to detect SNPs and candidate genes associated with relative root BSH under Pb stress. Combined with transcriptome data derived from our previous studies, the potential causual genes were identified from these candidate genes. Candidate gene association studies further confirmed that the genetic variants in the 5' UTR of *ZmbZIP107* affected Pb tolerance of root BSH among maize lines. Finally, we investigated the role of *ZmbZIP107 in vivo* by heterologous expression of *ZmbZIP107* in rice, which validated the effects of *ZmbZIP107* on Pb tolerance and accumulation in rice seedlings. Collectively, these results suggest that *ZmbZIP107* is a positive regulator of Pb tolerance in maize seedlings, which inhibits Pb absorption in the roots.

Materials and methods

Plant materials, growth conditions, and phenotypic investigations

A maize association panel of 312 diverse inbred lines, which included the Tropical, Stiff Stalk (SS), and non-Stiff Stalk (NSS) germplasms (Zhang et al., 2016a), was used to identify the genetic associations with Pb tolerance in maize seedlings. Seeds of these 312 lines were cultivated in quartz sands with a photoperiod of 16/8 h (day/night) and a temperature of 25/22 °C

(Abdel-Ghani et al., 2016). At the two-leaf stage, the consistent-growth seedlings of each line were divided into two groups, with one group transplanted into a Hoagland solution supplemented with 1 mM $Pb(NO_3)_2$ (as Pb treatment, T) and the other into a Hoagland solution without $Pb(NO_3)_2$ (as control check condition, CK). pH of these two solutions were maintained at approximately 4.5 with titration of KOH or HCl when required (Cunningham, 1996; Małkowski et al., 2005; Bashmakov et al., 2017). On the 7th day of culture, root bushiness (BSH) was measured for each line using an EPSON Expression 10000XL Root Scanner and assessed using the ARIA software (Pace et al., 2014). The 312 maize lines were arranged in a randomized complete block design with three replications. The BSH of each line under Pb treatment and normal conditions was represented by BSH-T and BSH-CK, respectively. The lead-tolerance coefficient (LTC) of BSH was calculated as the ratio of the BSH under Pb treatment to that under CK, which was used to evaluate maize tolerance to Pb treatment in GWAS (Ma et al., 2022).

Statistical analysis of phenotype data

For each line, the average of BSH across three replicates was used as the phenotype of BSH. The SPSS (v. 20, IBM, N, USA) software was used for descriptive statistical analysis of phenotypic data, including mean, standard deviation (SD), skewness, kurtosis, frequency distribution, and coefficient of variation (CV).

Genome-wide association studies and candidate gene identification

The genotypes of 312 association panel were constructed using a maize SNP chip (56K) in our previous study (Zhang et al., 2016a). The following criteria were used for SNP quality filtering in this study: minor allele frequency (MAF) < 0.05, missing rate > 0.2, and the SNPs with heterozygosity > 20% (Ma et al., 2021a). A total of 43,799 high-quality SNPs were remained for GWAS. Fixed and random model circulating probability unification (FarmCPU) model was used to detect the associations between the phenotypes and SNPs (Zhang et al., 2016a; Li et al., 2020a; Ma et al., 2021a). The simpleM program in R was used to calculate the number of effective markers (n=24876), and a stringent p threshold of 0.05/n (p = 0.05/24876 = $2.01×10^{-6}$) was used to detect SNPs significantly associated with BSH-LTC. Based on the B73 (RefGen V2) genome, all the genes located in the LD (Linkage disequilibrium) = 300 kb regions of these significant SNPs were excavated and identified as putative candidate genes for BSH-LTC (Zhang et al., 2020a; Ma et al., 2021b).

Gene-based association analysis

To verify whether variants within the candidate genes affected BSH-LTC among different maize lines, 77 inbred lines randomly selected from the association panel were subjected to gene-based association analysis for each candidate gene. In detail, the genomic DNA of the 77 lines were used as the templates to amplify the gene body and its 2000 bp upstream for each gene by PCR. The B73 genome sequence (RefGen V4) was used as the reference sequence to align the amplified sequences, and the DNAMAN software (v. 5.2.2, Lynnon Bio-soft, Canada) was employed to detect insertions/deletions (InDels) and SNPs (Zhang et al., 2020b). The associations between these genetic variants and BSH-LTC were calculated based on the general linear model (GLM) in TASSEL v. 5.0 (Zhang et al., 2021). The significance threshold was set as p = 0.05 according to the previous study (Ma et al., 2022). Haploview software (http://www.broad.mit.edu/mpg/haploview) was used to calculate the LD between the markers (Hou et al., 2021). For each gene, haplotypes were identified according to these trait-associated SNPs and a t-test was used to analyze significance of BSH-LTC between contrasting haplotypes.

Determination of Pb concentration

On the 14th day of Pb treatment, rice seedlings were soaked in 20 mM Na_2-EDTA and then rinsed with distilled water for three times. The roots were sampled and dried at 105°C for 2 h and 70°C to a constant weight. These samples were then digested with 10 mL nitric acid (HNO_3) using the MARSX (CEM) microwave digester. Finally, Pb content was determined by the Inductively Coupled Plasma Mass Spectrometry (NexION 1000 ICP-MS). Pb concentration (mg/kg DW) was represented by Pb content per unit dry weight (DW).

Real-time quantitative reverse-transcription PCR

Nine tissues were collected from maize line B73 and subjected to expression analysis of *ZmbZIP107*, including roots (three-leaf stage and silking stage), leaf (three-leaf stage and silking stage), stems (silking stage), female flowers, male flowers, grains on the 6th day after pollination (6 DAP) and 12 DAP. Moreover, maize seedlings were cultured in Hoagland solutions supplemented with 1mM $Pb(NO_3)_2$ and without $Pb(NO_3)_2$ for seven days, respectively. The shoots and roots were individually sampled for total RNA extraction by using TRIzol Reagent (Invitrogen, California, USA). The first-strand cDNA was synthesized from 2 μg total RNA using a reverse transcription kit (Novoprotein, Nanjing, China) following the manufacturer's instruction. The gene expression level of *ZmbZIP107* was quantified by performing RT-qPCR with SYBR qPCR SuperMix Plus (Novoprotein, Nanjing, China). The *OsActin* gene was used an internal reference, and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of *ZmbZIP107* (Chen et al., 2021). Three biological replicates and three technical replicates were included for each sample.

Subcellular localization of ZmbZIP107

To verify the subcellular localization of the ZmbZIP107 protein, we cloned the coding sequence (CDS) of *ZmbZIP107* without the stop codon into the *pCAMBIA2300-eGFP* vector, generating the *p35S:ZmbZIP107-eGFP* fusion expression vector. Then then fusion vector was transformed into *Agrobacterium tumefaciens* strain GV3101 and the transformed Agrobacterium suspension with $OD_{600 =} 0.8$ were injected into the *Nicotiana benthamiana* leaves for transient expression. The *p35S:eGFP* vector was used as the negative control. eGFP fluorescence signal in the transformed tobacco leaves was detected at 48 h after culture with a confocal microscope (Zeiss LSM 800, Baden-Württemberg, Germany).

Rice transformation and phenotypic identification

The full-length CDS of *ZmbZIP107* was cloned from the favorable-haplotype line (B73) and integrated into the *pCUB* vector under the control of Ubi promoter. The recombinant plasmid was transferred into the rice cultivar ZHONG HUA 11 through Agrobacterium-mediated transformation to produce *ZmbZIP107*-overexpressed lines. Positive transgenic plants were detected by using *ZmbZIP107*-based PCR. Seeds from the transgenic and wild-type plants were incubated on the germinating paper at 37°C in the darkness. Germinated seeds were transferred to a germinating box filled with distilled water and cultured for seven days. Then, the seedlings with uniform growth were transferred to nutrient solutions (pH = 4.5) with 50 mg/L $Pb(NO_3)_2$ and without $Pb(NO_3)_2$, respectively (Chen et al., 2007). These rice plants were then grown in a culture room with a photoperiod of 14/10 h (day/night) and a temperature of 28/25 °C for 14 days (Nam et al., 2021). Shoot length (SHL) were measured using a ruler. The following root traits were measured using EPSON expression 10000XL root scanner: BSH, total root length (TRL), total surface area (TSA), primary root length (PRL), secondary root length (SEL), and maximum number of roots (MNR).

Results

Statistical descriptions of BSH under CK and T conditions

To evaluate the natural variations of BSH, we investigated BSH under normal and Pb treatment conditions in a maize association panel composed of 312 inbred lines. The phenotypic frequency of BSH displayed a normal distribution under both conditions among this panel (Figure 1). The mean values of BSH were 2.70 and 3.77 under CK and T conditions with the range from 1.67 to 6.00 and 1.67 to 7.51, respectively, showing a significant difference ($p < 0.001$) between the two conditions (Table 1). The CVs of BSH were 25.56% (CK) and 30.24% (T) (Table 1), implying that Pb treatment improved the phenotypic variation of BSH among different lines. Furthermore, we calculated the lead-tolerance coefficient (LTC) of BSH to evaluate Pb tolerance for each line. The averaged BSH-LTC was 1.45 among the association panel, with the range from 0.33–3.26 (Table 1), suggesting that Pb treatment had a generally positive effect on BSH of this panel.

Genetic loci and candidate genes controlling BSH-LTC

To explore the genetic architecture of BSH-LTC in maize, we performed a GWAS for BSH-LTC using the FarmCPU model with 43,799 high-quality SNPs. The quantile-quantile plot (Q-Q plot) showed a sharp deviation from the expected p value specially in the tail region (Figure 2A), suggesting that FarmCPU did not result in many false positives in our GWAS. Finally, four SNPs were significantly associated with BSH-LTC at a stringent p threshold of 2.01×10^{-6} (0.05/24876) (Figure 2B). These significant SNPs were located on chromosomes 2, 8 and 10, with p value ranging from 3.24×10^{-7}

(PZE-110032152) to 2.87×10^{-8} (PZE-108077298). The PVE of each SNP varied between 1.89% (PZE-108062009) and 4.80% (PZE-108077298) (Figure 2B, Table 2), implicating that BSH-LTC in maize were mainly controlled be several minor loci. According to our previous studies, the linkage disequilibrium (LD) decay distance of this panel was approximately 300 kb. Accordingly, a total of 30 gene models were identified in the 300 kb flanking regions of the four BSH-LTC-associated SNPs (Table S1).

Integration of transcriptome data to identify causal genes for Pb tolerance in maize

To further select out the key genes of BSH-LTC from the 30 candidate genes identified by GWAS, we analyzed expression patterns of each gene in response to Pb stress by using the transcriptome data. Our previous study performed transcriptome sequencing for the roots of a Pb-sensitive line (SCL177) and a Pb-tolerant line (SCL280) during the response to Pb stress (Ma et al., 2022). Finally, relative to CK conditions, six candidate genes were differentially expressed under Pb treatment in at least one line, with |log2 Ratio (48 h/0 h)| > 1 and FDR < 0.05 (Table S2). Among the six genes, *Zm00001d011012* and *Zm00001d007827* were differentially expressed under Pb treatment in both lines (Figure 2C). While *Zm00001d024160*, *Zm00001d011134*, *Zm00001d010404*, and *Zm00001d040356* were specifically differentially expressed in SCL177 under Pb stress (Figure 2C), which were considered the genes differentially responding to Pb treatment between the two contrasting lines (Figure 2C). These four genes were thereby considered the potential causal genes responsible for maize tolerance to Pb stress. According to the B73 RefGen_V4 reference genome (http://www.gramene.org), *Zm00001d024160* was annotated as a TGA4 transcription factor (ZmbZIP107) whereas
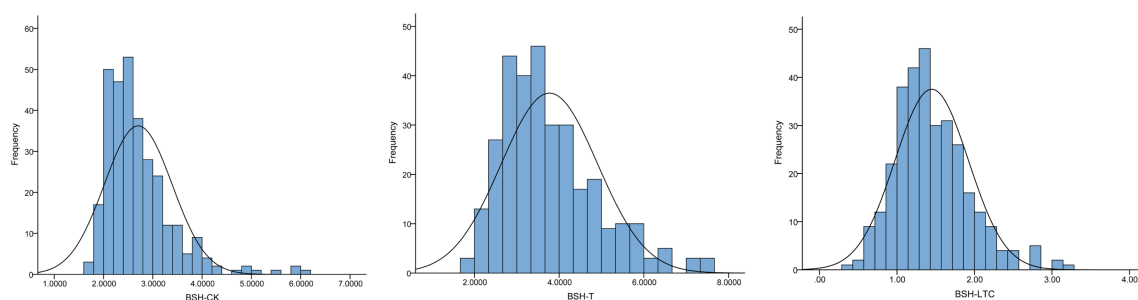
Frequency distribution of BSH under different conditions. BSH, root bushiness; CK, normal condition; T, Pb treatment; LTC, lead-tolerance coefficient.

TABLE 1 Phenotypic variations of BSH in the maize association panel.

Trait	Range	Mean	SD	Skewness	Kurtosis	CV (%)	Sig.
BSH-CK	1.67-6.00	2.70	0.69	1.89	5.32	25.56	***
BSH-T	1.67-7.51	3.77	1.14	0.92	0.71	30.24	
BSH-LTC	0.33-3.26	1.45	0.47	0.86	1.27	32.41	-

BSH, Bushiness; LTC, lead-tolerance coefficient; CK, normal condition; T, Pb treatment; SD, standard deviation; CV, coefficient of variation; *** significant at p < 0.001 level between BSH-CK and BSH-T.

Zm00001d011134 encodes aputative AP2/EREBP transcription factor superfamily protein (*WRI1*). *Zm00001d010404* and*Zm00001d040356* encode a putative cyclin superfamily protein and geranylgeranyl hydrogenase 2, respectively (Table S2).

Variations in ZmbZIP107 affecting BSH-LTC among different maize lines

To verify whether genetic variants in the above four genes influenced Pb tolerance among diverse lines, we conducted gene-based association studies for each gene. Using PCR, we amplified and sequenced the gene body and 2000 bp upstream of the four genes. Though variant calling, a total of 22 (21 SNPs and 1 InDel), 8 (7 SNPs and 1 InDel), 25 (25 SNPs), and 5 (5 SNPs) polymorphisms were identified in *Zm00001d010404*, *Zm00001d040356*, *Zm00001d024160*, and *Zm00001d011134*, respectively (Table S3). Association studies indicated that two SNPs (S8_113171481,C/G and S8_113172367, C/T) located in *Zm00001d010404* were significantly (p < 0.05) associated with BSH-LTC, which were situated in the seventh intron (Figure S1A). One SNP (S3_39647996, G/A) located in *Zm00001d040356* was significantly (p< 0.05) associated with BSH-LTC, which resided in the first exon and led to a synonymous mutation (Figure S1B). Moreover, one SNP

FIGURE 2
Combined genome-wide association study (GWAS) and transcriptome data revealing potential causal genes of maize tolerance to Pb stress. **(A, B)** Quantile-quantile (Q-Q) plot **(A)** and Manhattan plot **(B)** of the GWAS for BSH-LTC by the FarmCPU model. BSH, root bushiness; LTC, lead-tolerance coefficient. The dashed red line shows the significance threshold ($2.01×10^{-6}$) **(C)** Wayne diagram displaying the Pb treatment-responsive genes among 30 candidate genes identified by GWAS. SCL177, a Pb-sensitive maize line; SCL280, a Pb-tolerant maize line.
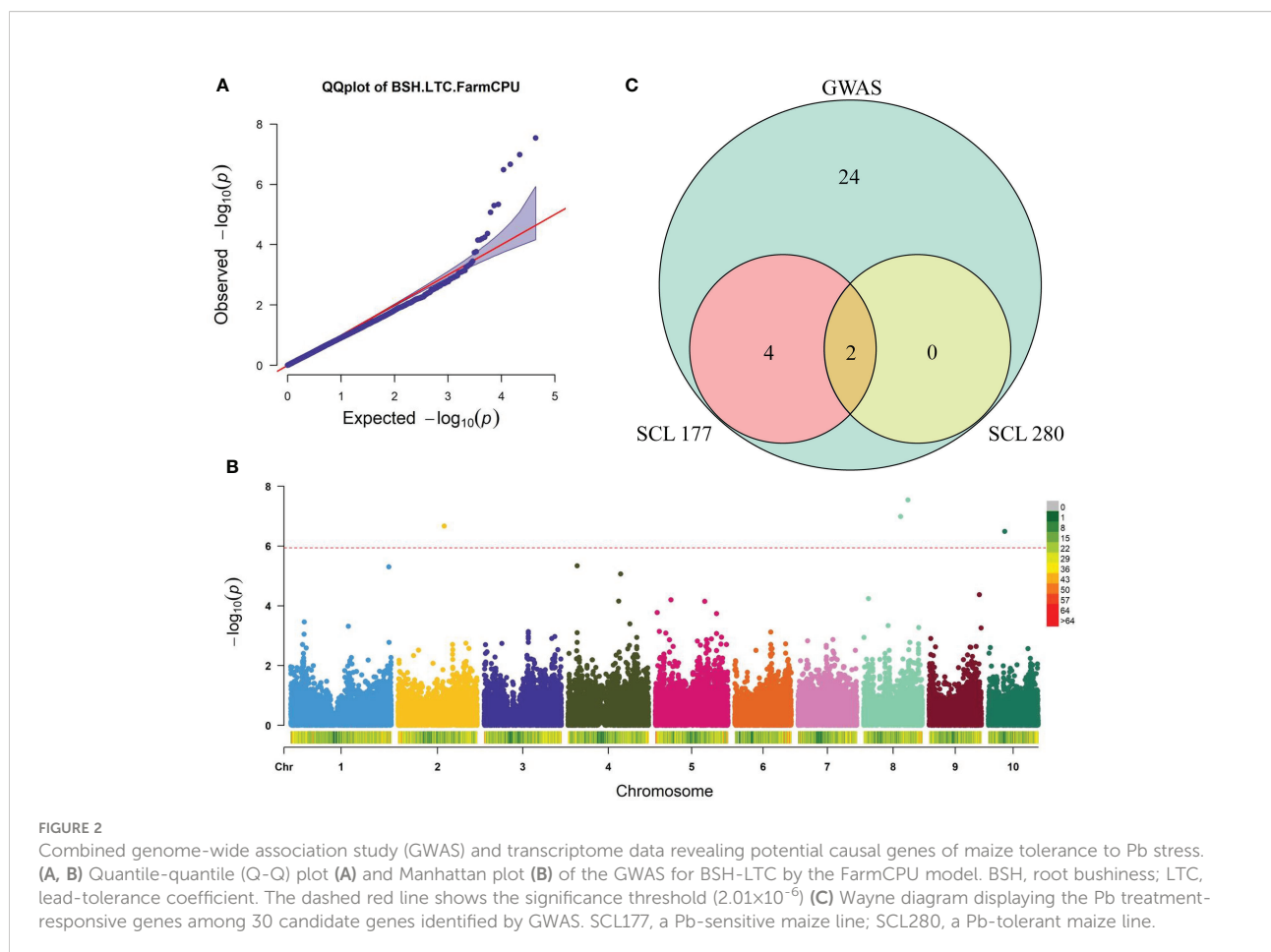
TABLE 2 Significant SNPs associated with BSH-LTC identified by GWAS using FarmCPU model.

Trait	SNP	Chr	Alleles	Position (bp)	P-value	PVE (%)
BSH-LTC	PZE-108077298	8	C/A	132854286	2.87E-08	4.80
	PZE-108062009	8	A/G	110849647	1.03E-07	1.89
	PZE-102108114	2	A/C	137985405	2.15E-07	4.03
	PZE-110032152	10	C/G	49992422	3.24E-07	2.19

BSH, root bushiness; LTC, lead-tolerance coefficient; Chr, chromosome; SNP, single nucleotide polymorphism.

(S8_140016556, C/A) located in the 5` UTR of *Zm00001d011134* was significantly (p < 0.05) associated with BSH-LTC, nevertheless, no significant difference in BSH-LTC value was observed between the lines with allele C and those with allele A (Figures S1C, D). Remarkably, two SNPs (S10_51323856, C/A and S10_51324804, C/A) located in the 5` UTR of *Zm00001d024160* (*ZmbZIP107*) were significantly (p< 0.05) associated with BSH-LTC (Figure 3A). The two significant SNPs divided the 77 lines into two major haplotypes (Hap1: AA; Hap2: CC), a *t*-test showed that the BSH-LTC (1.178) of Hap1 was significantly (p< 0.05) lower than that (1.417) of Hap 2 (Figure 3B). In contrast to synonymous variants and intron_variants, those in the 5' UTR tend to cause changes in gene transcriptional levels and thus lead to phenotypic variations (Bashirullah et al., 2001; Zhang et al., 2016b). We then detected the expression of *ZmbZIP107* in the two contrasting haplotypes under Pb treatment by using RT-qPCR. The results showed that the expression level of *ZmbZIP107* in Hap1 (AA) was significantly (p< 0.01) lower than that in Hap2 (CC) under Pb treatment (Figure 3C). Collectively, these results suggested that the two 5` UTR_variants in *ZmbZIP107* regulated its expression level and thus influenced BSH-LTC among different maize lines. Therefore, *ZmbZIP107* was confirmed as the key gene for maize tolerance to Pb treatment in the present study.
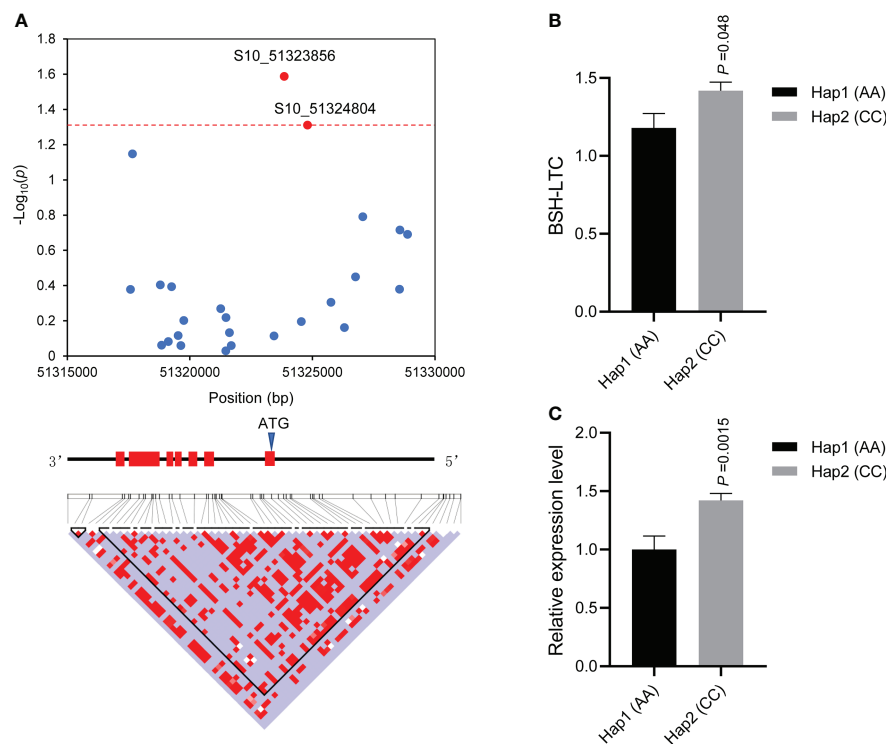

FIGURE 3
Association analysis of the candidate gene *ZmbZIP107*. **(A)** The SNPs (S10_51323856 and S10_51324804) significantly (p < 0.05) associated with BSH-LTC. The dashed red line shows the significance threshold (p = 0.05). The gene simulation structure is shown in the middle and the red filled boxes represent the exons of *ZmbZIP107*. The bottom image indicates the pairwise LDs (linkage disequilibriums) between the SNP markers. BSH, root bushiness; LTC, lead-tolerance coefficient. **(B)** Comparison of BSH-LTC between the two haplotypes, Hap1 (AA, n=12) and Hap2 (CC, n=46). **(C)** Comparison of *ZmbZIP107* expression level between the two haplotypes under Pb treatment.

Spatial expression pattern of *ZmbZIP107*

To detect subcellular localization of the ZmbZIP107 protein, we fused *ZmbZIP107* with *eGFP* and transformed it into tobacco leaves. The eGFP fluorescence signal was specifically observed in the nucleus of the leaves transformed with the *p35S:ZmbZIP107-eGFP* fusion expression vector (Figure 4A). In contrast, the fluorescence signal was observed in both the nucleus and cytoplasm of the leaves transformed with the *p35S:eGFP* vector (Figure 4A). These results showed that *ZmbZIP107* protein was specifically localized in the nucleus. Furthermore, *ZmbZIP107* expression level was detected in different maize tissues using RT-qPCR. The results displayed that *ZmbZIP107* showed the highest expression in the roots (three-leaf stage), followed by the leaves (three-leaf stage) and roots (silking stage). However, the female flower had the lowest expression level of *ZmbZIP107*. These findings supported the role of *ZmbZIP107* in mediating root system architecture (Figure 4B).
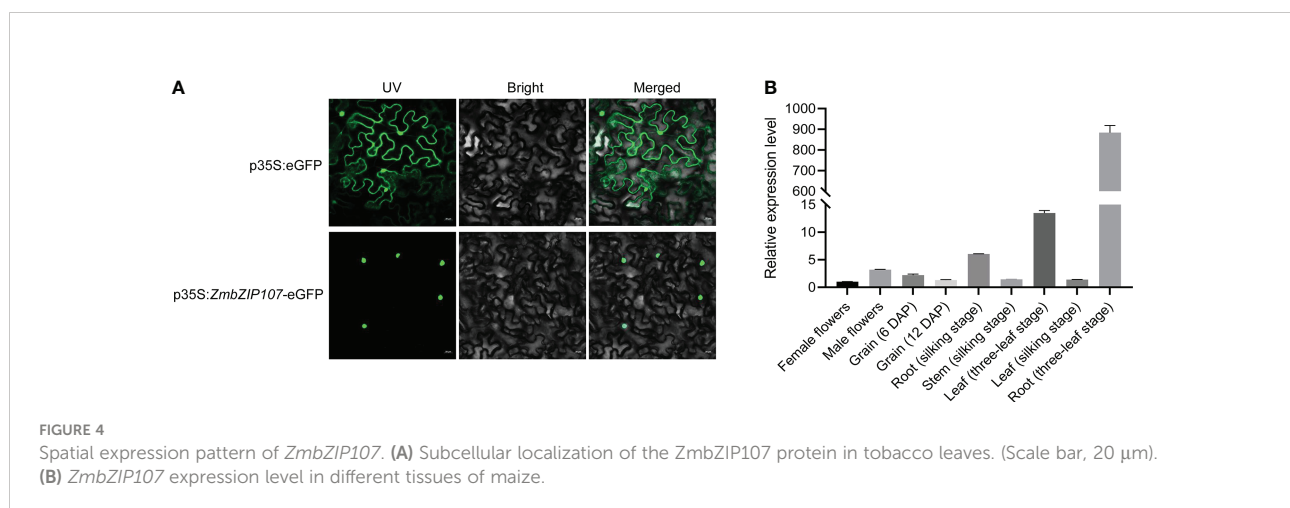
Heterologous expression of *ZmbZIP107* enhancing Pb tolerance in rice seedlings
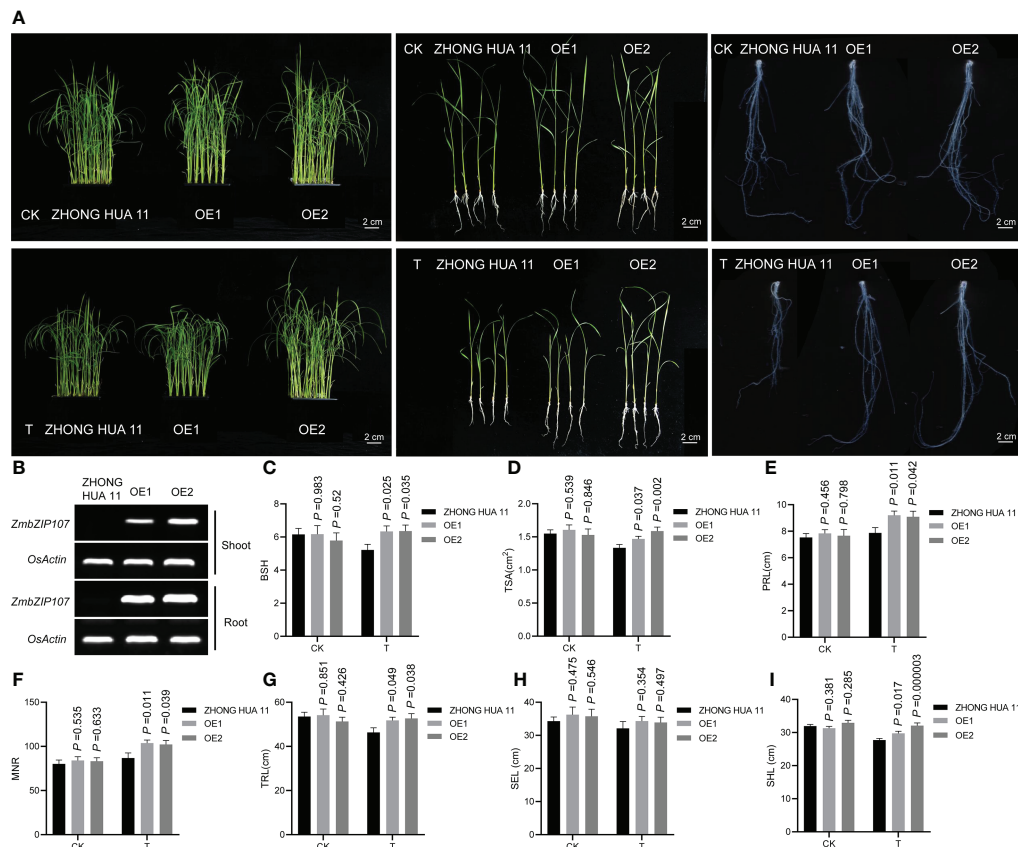
To investigate the regulatory role of *ZmbZIP107* in Pb tolerance, we obtained two independent rice transgenic lines (OE1 and OE2) overexpressing *ZmbZIP107* with the favorable haplotype (Hap2) (Figure 5A). Semi-quantitative RT-PCR showed that *ZmbZIP107* was efficiently expressed in both roots and shoots of OE1 and OE2, with higher expression levels in OE2 than those in OE1 (Figure 5B). We then compared the BSH between the OE lines and wild type under Pb and CK conditions. Under CK conditions, no significant difference in BSH was observed between each of the OE lines and wild type (Figure 5C). Under Pb treatment, the BSH was significantly ($p < 0.05$) increased by 21.5% (OE1) and 21.9% (OE2) relative to the wild type (Figure 5C). Meanwhile, the BSH-

LTC values of these OE lines were 1.026 (OE1) and 1.098 (OE2), which were higher than that (0.846) of the wild type. To further test the overexpressed *ZmbZIP107* influenced the root system architecture under Pb stress, we investigated five root architecture-related traits (TRL, TSA, PRL, SEL, and MNR) and shoot length (SHL) in the OE lines and ZHONG HUA 11 under normal conditions and Pb treatment. Ultimately, *ZmbZIP107* overexpression had no significant effect on TRL, TSA, PRL, SEL, MNR, and SHL in the early vegetative stage of rice under normal conditions (Figures 5D-I). Notably, compared with those in ZHONG HUA 11, the phenotypes of four root architecture traits (TRL, TSA, PRL, and MNR) and SHL were significantly ($p < 0.05$) increased in the two OE lines under Pb stress, with increasing average percentage varying between 7.3% (SHL) and 19.5% (MNR) (Figures 5D-I). Moreover, a higher SHL was observed in OE2 than that in OE1, which was probably owing to a higher *ZmbZIP107* overexpression level in OE2 than that in OE1. However, the overexpressed *ZmbZIP107* did not affect SEL of rice under Pb treatment (Figure 5H). Combined these results suggested that heterologous expression of *ZmbZIP107* enhanced Pb tolerance of rice roots and shoots.

Heterologous expression of *ZmbZIP107* decreasing Pb uptake in rice roots

To investigate the effect of *ZmbZIP107* on Pb accumulation, we measured Pb content in the roots of the *ZmbZIP107*-OE lines and ZHONG HUA 11 under the condition of 50 mg/L Pb treatment. The results showed that Pb concentration in these OE lines was significantly decreased by 42.3% (OE1) and 41.4% (OE2) relative to the wild-type ZHONG HUA 11 under Pb treatment (Figure 6). These suggested that increased *ZmbZIP107* expression in rice transgenic lines effectively inhibited Pb absorption by the roots, alleviating Pb toxicity and improving root and shoot growth under Pb stress.

FIGURE 4
Spatial expression pattern of *ZmbZIP107*. **(A)** Subcellular localization of the ZmbZIP107 protein in tobacco leaves. (Scale bar, 20 µm). **(B)** *ZmbZIP107* expression level in different tissues of maize.

FIGURE 5

Heterologous expression of *ZmbZIP107* improving lead tolerance in rice. **(A)** Phenotypic comparison between ZHONG HUA 11 and the overexpression lines (OE1 and OE2) under CK and T conditions. CK, normal condition; T, Pb treatment, (Scale bar, 2 cm). **(B)** Expression of *ZmbZIP107* in the roots and shoots of the OE lines under Pb treatment, detected using semi-quantitative RT-PCR. **(C–I)** Root system architecture (RSA) of ZHONG HUA 11 and the OE lines under CK and T conditions. CK, normal condition; T, Pb treatment. BSH, root bushiness; TSA, total surface area; PRL, primary root length; SEL, secondary root length; MNR, maximum number of roots; TRL, total root length; SHL, Shoot length.

Discussions

Using BSH-LTC as an indicator of maize seedlings tolerance to Pb stress

Plant roots play an important role in plant growth including anchoring plants in the soil, absorbing water and nutrients, and providing the site for the rhizosphere to interact with beneficial organisms. Meanwhile, root system is the foremost organ of direct contact with heavy metal ions in the environment (Lu et al., 2013). Pb toxicity to plant roots mainly involves rapid inhibition of root growth (Godbold and Kettner, 1991; Gzyl et al., 1997), and the symptom of Pb toxicity in plant roots is the result of restriction in cell division (Przymusiński and Woźny, 1985; Wierzbicka and Botany, 1989; Wozny and Plantarum, 1991). Reduction in root length and biomass caused by Pb stress has been reported in various plants, including tomato (Van der

Merwe et al., 2009), rice (Xiao-Ming et al., 2009), maize (Kozhevnikova et al., 2009), and wheat (Kaur et al., 2013). Root length of rice seedlings was reduced by 40% under the treatment of 0.5–1.0 mM Pb^{2+} (Verma and Dubey, 2003). Peng et al. showed that the root length and surface area were significantly inhibited under Pb stress in Elsholtzia argyi and Elsholtzia splendens (Peng et al., 2005). Pb stress also inhibited lateral root formation by affecting root diameter and volume (Hou et al., 2021). Currently, root dry weight has thereby been widely considered a reliable indicator for evaluating plant tolerance to abiotic stresses during early growth stages (Pour-Aboughadareh et al., 2020; Ma et al., 2022).

Root bushiness that is equal to the ratio of the maximum of roots to the median of roots has been extensively used to evaluate the lush degree of plant roots in different species (Ambreetha et al., 2017; Liang et al., 2018; Rangarajan and Lynch, 2021). Herein, for the first time, we used BSH-LTC that
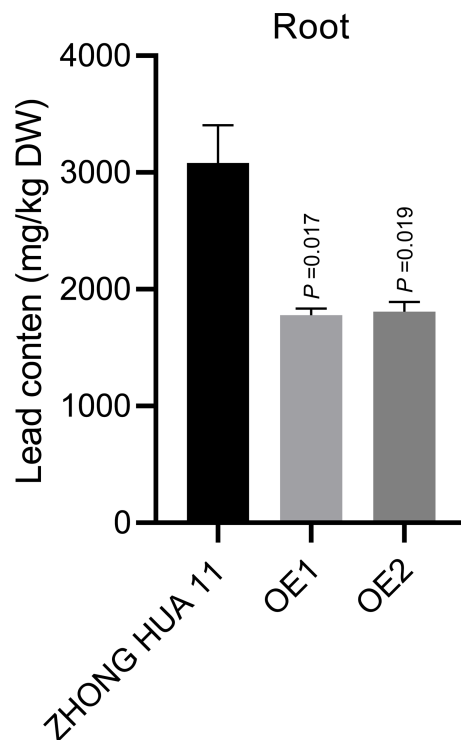
Root

FIGURE 6
Pb content in the roots of *ZmbZIP107*-overexpressed lines (OE1 and OE2) and ZHONG HUA 11. DW represents dry weight of roots.

reflects the relative BSH under Pb treatment as an indicator for evaluating maize seedlings tolerance to Pb stress. The CV of BSH-LTC was 32.41% among the maize panel and even exceeded BSH under CK conditions (25.56%) and Pb treatment (30.24%) (Table 1), suggesting that BSH-LTC can comprehensively reflect the variations of Pb tolerance-related traits among different lines. Moreover, the broad-sense heritability of BSH-LTC in maize was > 30%, which was similar to those of other root traits in maize (Pace et al., 2014). It suggested that BSH-LTC was significantly affected by genetic factors and suitable for phenotyping the maize panel in GWAS. Finally, overexpression of the *ZmbZIP107* gene that was identified by using BSH-LTC caused increased BSH-LTC in rice, verifying the reliability of employing BSH-LTC to evaluate Pb tolerance.

Combining GWAS and gene-based association analysis to identify causal gene of maize tolerance to Pb stress

GWAS is an effective method to dissect the genetic basis of quantitative traits. It has been widely applied to identify a variety of complex traits in plant species, including Pb accumulation

(Zhao et al., 2018), protein abundance (Jiang et al., 2021), root configuration-related traits (Wang et al., 2019), yield-related traits (Sharma et al., 2018), salt tolerance (Ma et al., 2021b), and so on. Gene-based association analysis is the most widespread method for associating the functional variation loci of candidate genes with the phenotypes they produce (Li et al., 2019). In soybean, a combination of GWAS and candidate gene association study identified a phosphorus efficiency-related gene *GmACP1* and a functional marker Indel170 (Zhang et al., 2014). Similarly, using this strategy, an InDel located in the *ZmDREB2.7* promoter was found to confer maize resistance to drought stress (Liu et al., 2013). Moreover, Chao et al. used 149 Arabidopsis lines to perform candidate gene association analysis for a *Heavy Metal Associated* gene (*AtHMA3*), uncovering five SNPs acting on Cd accumulation in maize leaves (Chao et al., 2012).

In the present study, four significant SNPs were identified using GWAS (Table 2), and a total of 30 gene models were located in the LD regions of these loci (Table S1). Among the 30 genes, four were differentially responsive to Pb treatment between two maize lines with contrasting Pb tolerance, which were thus considered the potential causal genes for Pb tolerance (Table S2). To further identify the key gene, we performed gene-based association studies for each of the four genes. Finally, only

two variants in the 5` UTR of *ZmbZIP107* were significantly associated with BSH-LTC (Figure 3). In summary, we selected out a causal gene (*ZmbZIP107*) from the GWAS results by combining transcriptome analysis and gene-based association mapping, which suggested the high efficiency of using the integration strategy in excavating functional genes. On the other hand, the identified favorable haplotype in *ZmbZIP107* can be used for developing functional markers in molecular marker-assisted selection.

Application of *ZmbZIP107* in breeding Pb-tolerant varieties and soil phytoremediation

Increased heavy metal concentration in soils has caused serious health threats for plants, animals, and human beings. It is vitally significant to remediate soil environment and cultivate plant varieties tolerant to heavy metals. In our study, we cloned a transcription factor *ZmbZIP107* that was responsible for Pb tolerance and accumulation in the roots of maize seedlings. The heterologous expression of *ZmbZIP107* enhanced Pb tolerance and inhibited Pb absorption in rice. In the future, using transgenic technologies to increase *ZmbZIP107* expression in other crops is desirable to improve Pb tolerance of germplasm resources and cultivate varieties with low Pb accumulation. As a widely grown crop with large biomass, maize has a great potential of soil bioremediation (Ma et al., 2022). CRISPR/Cas9 and RNAi can be used for generating *ZmbZIP107*-knockout and knockdown maize lines, respectively, breeding maize varieties with high Pb accumulation for soil phytoremediation.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://ngdc.cncb.ac.cn/, CRA004789.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1015151/full#supplementary-material

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