Identification of quantitative trait loci and the exploration of candidate genes for the tolerance to Zn deficiency in maize

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Abstract

Background

Zn is essential for plants and Zn deficiency leads to great reduction in quality and quantity of crops. Maize, as one of the most important main staple crops worldwide, is more susceptible to Zn deficiency than any other cereal crops. Therefore, understanding the functional mechanisms in tolerance to Zn deficiency in maize is urgent but is still lacking. In this study, quantitative trait loci (QTL) analysis in K22 and By815 RIL population with high-density bin map was conducted to investigate genetic basis of the mechanisms in maize to tolerate Zn deficiency, subsequently some candidate genes were identified and considered as being associated with Zn metabolisms in plants.

Results

21 QTLs were detected and accounted for 5.9% - 16.6% of phenotypic variations. Based on the co-localization in this study and the comparisons with previous studies in different RIL and GWAS populations, 223 candidate genes were identified inside the reduced QTL peak intervals on chromosome 1, 2, 6, 7 and 9. Furthermore, 9 genes detected within the peak bins of valuable genomic regions are suggested to be associated with ions transportation and some redox processes affected by Zn deficiency. Additionally, 5 genes, including ZmIRT1, ZmNRAMP6, ZmEIN2 and ZmHMAs, whose homologous gene have been studied and considered to be responsible for metal cations transportation and ethylene-signaling pathway requiring a transition metal were discovered in 5 loci we mapped.

Conclusions

14 target genes identified in 9 loci we mapped in this work were explored to elucidate the potential functions in Zn homeostasis and the direct or indirect effects on mechanisms in Zn deficiency tolerance in maize. It is the first time that ZmIRT1, ZmNRAMP6, ZmHMAs were identified using linkage analysis under Zn deficiency in maize, providing genetic
evidence and foundation for further gene functional characterization. Our findings have assisted us untangling the genetic basis of possible mechanisms in response to Zn deficiency in maize.

Background

Zinc (Zn) is an essential micronutrient for plant metabolism, development and productivity. Zn deficiency is the most ubiquitous micronutrient deficiency problem in world crops. Zn deficiency leads to stunted growth, chlorosis of leaves, small leaves and spikelet (ear) sterility (Marschner 1995; Broadley et al. 2007). Moreover, Zn deficiency causes large reduction in quality of crop products and increases susceptibility of plants injured by high light intensity and temperature and infection by certain fungal diseases (Graham 1983; Cakmak 2000). Plant-based foods are significant sources of Zn for humans (Welch and Graham, 2004). In 2011, 1.1 billion people were at risk of zinc (Zn) deficiency respectively due to inadequate dietary supply (Kumssa et al. 2015).

Maize (Zea mays L.) is the most important resource for food, feed, and biofuel and is one of the most cultivated crop plants all over the world. In 2014, more than 1.0 billion tons of maize grain was produced globally (FAOSTAT 2014). With a growing world population and need for biofuel, increasing maize grain yield is necessary to meet the market demand. However, the limited cultivable land does not support the approach of increasing maize production by enlarging its planting area. Besides, zinc deficiency leads to large reductions in crop yield (Sadeghzadeh 2013). Nevertheless, maize is the most susceptible cereal crop to Zn deficiency (Alloway 2009). In fact, correction of Zn deficiency via fertilization is not always successful due to agronomic and economic factors (Hacisalihoglu and Kochian 2003). Therefore, enhancing genetic grain yield potential via breeding efforts and improve the breeding efficiency and effectiveness using molecular tools have become feasible and integral strategies for many maize researchers worldwide (Prasanna et al. 2005).
Quantitative trait locus (QTL) analysis provides an effective means of dissecting the genetic basis of complex trait in plants and animals (Zeng 1994; Alonso-Blanco et al. 2009). Marker-assisted selection (MAS) based on QTLs can greatly facilitate crop improvement (Prasanna et al. 2010; Li et al. 2018). Recently, QTL analysis was used to investigate the genetic basis of the mechanism of the tolerance to Zn deficiency in plants, including rice (Gao et al. 2013), wheat (Velu et al. 2017), barley (Lonergan et al. 2009; Sadeghzadeh et al. 2010), Arabidopsis (Ghandilyan et al. 2012). Genc et al. (2009) found that most of the QTLs linked to wheat seedling growth under Zn deficiency were associated with height genes with greater seedling biomass associated with lower Zn concentrations. In rice, four QTLs associated with plant mortality were detected, and only one of those co-localized with one of the four QTLs detected for leaf bronzing, implying that both were under independent genetic control (Wissuwa et al. 2006).

As for maize, much more studies were focus on the molecular basis of biofortification for mineral elements, especially iron and zinc, in maize grain, rather than genetic mechanism of the tolerance to Zn deficiency for the whole plants. Šimić et al. (2012) used the ratios as bioavailability traits, and found that three QTLs for Fe/P, Zn/P, and Mg/P were co-localized on chromosome 3, coinciding with SSR marker bnlg1456 which was close to previously identified phytase genes (ZM phys1 and phys2). Based on meta-QTL analysis in rice and maize reported by Jin et al. (2015), three MQTL-containing candidate genes in maize were detected and two maize orthologs of rice, GRMZM2G366919 and GRMZM2G178190, were characterized as NRAMP genes likely responsible for the natural variation in maize grain zinc and iron concentration. Combing single environment analysis with multiple environment trial (MET) QTL analysis in six environments, Zhang et al. (2017) found five candidate genes for the target traits were identified in the intervals
detected by meta-QTLs in the previous study.

Up to now, the maize genome has been thoroughly sequenced and assembled. However, systematic analysis of genes responsible for Zn metabolism in maize is still limited. The first as well as the only one gene family has been cloned and described as being associated with Zn transport in maize is ZIP family (the zinc-regulated transporter, iron-regulated transporter protein). In maize, ZmZIP genes encode functional Zn or Fe transporters that may be responsible for the uptake, translocation, detoxification and storage of divalent metal ion in plant cells (Li et al. 2013). In transgenic Arabidopsis, ZmIRT1 and ZmZIP3 are characterized to function as metal transporters with different ion selectivities, and ZmIRT1 may stimulate endogenous Fe uptake mechanisms, possibly facilitating metal uptake and homeostasis (Li et al. 2015a). So far, except for ZIP family, specific functions in some other genes associated with Zn homeostasis in maize have not yet been studied, such as HMA (heavy metal transporting P-type ATPase) and CDF (the cation diffusion facilitator family).

There is little genetic evidence on the mechanistic basis of Zn deficiency tolerance in maize. Here, K22 and By815 RIL population were utilized to investigate the genetic basis in the tolerance to Zn deficiency in maize. By the identification of QTL co-localization in different traits and the comparison between the loci detected in this work with the QTLs in our previous studies mapped by different populations, some valuable genomic regions were selected with the high-density bin map. Target genes explaining potential causes were mined by the combination of some Zn metabolism-related genes which have been characterized in other plants and some genes unknown for the functions located in the genomic regions refined by peak bins, providing genetic evidence and foundation for further gene functional characterization.

Results
**Phenotypic variation in the tolerance to Zn deficiency in maize**

Regardless of treatments, all the traits associated with the tolerance to Zn deficiency showed significant difference between K22 and By815 (Table 1, Figure 1). Shoot and root dry weights in the -Zn and -Zn/CK treatments of By815 were more than three times higher than that of K22. R/S ratios in the -Zn treatment of By815 were significantly lower than that of K22. The Best Linear Unbiased Prediction (BLUP) values indicated that the means of the RIL population for all the traits were between K22 and By815 (Figure 1). Besides, there were large variations among inbred lines and the coefficients of variation for all the traits ranged from 14.0% to 55.2%. A normal distribution was observed in each trait, suggesting that the alleles responsible for enhancement in tolerance to Zn deficiency reside in K22 and By815. The variance analysis results revealed that highly significant effects on all the traits were due to genotype and environments except for Zn score (Table 2). Broad-sense heritability of each trait under different conditions was higher than 85%, indicating that much of phenotypic variations in the RIL population were genetically controlled.

**Identification of QTLs for each traits**

On the basis of a linkage map of 1670.4 cM, twenty-one QTLs were detected in the RIL population at an empirical threshold logarithm of odds (LOD) value of 2.9 estimated by 1000 permutation tests. These loci were distributed among 21 genomic regions on chromosome 1, 2, 3, 5, 6, 7, 9 and 10, explaining 5.2% - 16.6% of phenotypic variation (Figure 2). The QTL interval averaged 9.9 Mb (5.4 cM) ranging from 0.4 to 64.6 Mb (2.0 - 13.6 cM). A total of 21 QTLs were identified to be associated with the traits: four QTLs for Zn score, four QTLs for plant height, four QTLs for shoot dry weights, six QTLs for root dry weights and 3 QTLs for R/S ratio (Table 3).
**Zn score** Four QTLs (qKB-ZnSc6-1, qKB-ZnSc9-1, qKB-ZnSc9-2, qKB-ZnSc10-1) for Zn score were detected on chromosome 6, 9 and 10. Alleles from By815, the Zn-efficient parent, had increasing effects (0.15 - 0.19) on Zn score at these four mapped loci. qKB-ZnSc6-1 located on chromosome 6 was flanked by SYN11817 and PZE-106098680, explaining 9.2% of phenotypic variation. qKB-ZnSc9-1 and qKB-ZnSc9-2 were detected on chromosome 9 in the intervals of PZE-109025227 - PZE-109051633 and PZE-109059409 - PZE-109064132, respectively. These two loci explained 6.3 - 7.7% of phenotypic variations with additive effects of 0.16 - 0.17. qKB-ZnSc10-1 was located in the genomic region between bin PZE-110104601 and SYN19780, explaining 5.2% of phenotypic variation.

**Plant height** Four QTLs (qKB-PH1-1, qKB-PH2-1, qKB-PH6-1 and qKB-PH9-1) controlling plant height were determined on chromosome 1, 2, 6 and 9 with additive effects of By815 alleles except for qKB-PH1-1. qKB-PH1-1 was mapped in the genomic region of PZE-101205031 ~ PZE-101209438, explaining 10.1% of phenotypic variation. The second largest QTL qKB-PH2-1, located in the interval of PZE-102017472 ~ SYN18069, explained 12.0% of phenotypic variation with increasing effect of 4.04 cm on plant heights. qKB-PH6-1 and qKB-PH9-1 were detected in genomic regions of PZE-106089546 ~ PZE-106098680 and PZE-109053554 ~ PZE-109075980, respectively.

**Shoot dry weight** qKB-SDW2-1, qKB-SDW2-2, qKB-SDW1-1, qKB-SDW3-1 controlling shoot weight in maize were mapped on chromosome 1, 2 and 3, explaining 8.5% - 10.1% of phenotypic variation. In the -Zn treatment, qKB-SDW2-1 and qKB-SDW2-2 were identified on chromosome 2 in the regions of PZE-102009755 ~ SYN37566 and PZE-102017472 ~ PUT-163a-60342470-2456, respectively. Alleles from By815, at these two loci, increased shoot dry weights under Zn deficiency by 0.13 g. qKB-SDW1-1, qKB-SDW3-1 was detected in the intervals of PUT-163a-31558578-1965 ~ PUT-163a-71311320-3113 and PUT-163a-86473168-4518 ~ SYN36395 in the CK and -Zn/CK treatment respectively, each loci
explaining 8.5% of phenotypic variation.

**Root dry weight** Six QTLs (qKB-RDW1-1, qKB-RDW1-2, qKB-RDW3-1, qKB-RDW1-3, qKB-RDW9-1, qKB-RDW9-2) identified at different Zn nutrition status were distributed on chromosome 1, 3 and 9. qKB-RDW1-1 controlling the root dry weight under Zn deficiency was flanked by PZE-101213588 and PZE-101217291, explaining 5.9% of phenotypic variation. In the CK treatment, qKB-RDW1-2 and qKB-RDW3-1 were localized in the genomic regions of PUT-163a-31558578-1965 ~ PZE-101026148 and SYN1579 ~ PZE-103187323, explaining 9.4% and 6.5% of phenotypic variation, respectively. Alleles from By815 increased root dry weights by 0.07 g and 0.05 g at these two loci, respectively.

Three QTLs were identified in the -Zn/CK treatment, explaining 8.8% - 11.2% of phenotypic variation. qKB-RDW1-3 detected on chromosome 1 was flanked by PZE-101225664 and PZE-101233241, with an additive effect of K22 alleles. qKB-RDW9-1, the third largest loci, explaining 11.2% of phenotypic variation, was identified in the interval of PZE-109023988 ~ PZE-109025227. qKB-RDW9-2 localized in the genomic region of PZE-109027610 ~ PZE-109053554 on chromosome 9 explained 9.8% of phenotypic variation. Alleles from By815 increased root dry weights in the -Zn/CK treatment by 0.28 and 0.23 at these two loci localized on chromosome 9, respectively.

**R/S ratio** Three QTLs for R/S ratio were identified on chromosome 5 and 7, explaining 6.4% - 16.6% of phenotypic variation. qKB-R/S5-1 detected on chromosome 5 was in the region of SYN20689 ~ PZA00069.4 with an additive effect of 0.02. qKB-R/S7-1 mapped on chromosome 7 was flanked by PZE-107011423 and SYN18112, explaining 7.8% of phenotypic variation. qKB-R/S7-2, major effect QTL in the K22×By815 RIL population, was localized in the interval of PZE-107132535 - SYN32833, explaining 16.6% of phenotypic variation.
**QTL Co-localizations and candidate genes identification**

Five co-localizations of loci for different traits were identified on chromosome 1, 2, 6 and 9 with additive effects of By815 alleles, explaining 5.5% - 12.0% of phenotypic variation (Table 4). Furthermore, the first co-localization was detected at 12.5 - 15.7 Mb on chromosome 1, containing \( qKB-RDW1-2 \) and \( qKB-SDW1-1 \) which explained 9.4% and 8.5% of phenotypic variation, respectively. On chromosome 2, the second largest-effect locus \( qKB-PH2-1 \) together with \( qKB-SDW2-2 \) were co-localized in the region of 7.7 - 8.8 Mb, explaining 12.0% and 9.2% of phenotypic variation, respectively. At 150.7 - 152.3 Mb on chromosome 6, \( qKB-PH6-1 \) and \( qKB-ZnSc6-1 \) were co-localized under Zn deficiency, explaining 5.5% - 9.2% of phenotypic variation. Two co-localizations, including \( qKB-ZnSc9-1 \) and \( qKB-RDW9-2 \) as well as \( qKB-PH9-1\)and \( qKB-ZnSc9-2 \), mapped on chromosome 9 were detected at 28.0 - 89.4 Mb and 100.9 - 107.3 Mb respectively, explaining 5.2% - 9.8% of phenotypic variation.

Combined with the high-density bin map of K22×By815 RIL population, 12 physical intervals determined by 9 QTLs where there were co-localizations as well as the largest effect QTL \( qKB-R/S7-2 \) and the single locus \( qKB-SDW2-1 \) were narrowed the range to two adjacent bins for each QTL peak, varying from 30.3 kb to 31.1 Mb (Table S1 in the additional file). And 223 genes identified by 11 refined physical intervals were annotated in total (Table S1): 7 genes for \( qKB-RDW1-2 \), 15 genes for \( qKB-SDW1-1 \), 9 genes for \( qKB-SDW2-1 \), 4 genes for \( qKB-PH2-1 \) and \( qKB-SDW2-2 \), 8 genes for \( qKB-PH6-1 \), 6 genes for \( qKB-ZnSc6-1 \), 6 genes for \( qKB-R/S7-2 \), 21 genes for \( qKB-ZnSc9-1 \), 106 genes for \( qKB-RDW9-2 \), 27 genes for \( qKB-PH9-1 \) and 14 genes for \( qKB-ZnSc9-2 \).

**Discussion**

**Comparison of loci among different populations**

Previous results pertaining to the genomic locations, confidence intervals or phenotypic
variance explained by QTLs were inconsistent due to the differences in genetic backgrounds, environments, and/or mapping populations (Jin et al. 2015). Quantifying target traits in plants is time consuming, laborious, and expensive. Consequently, comparing QTL for different traits detected by independent experiments is important. Evidences for comparative QTLs detected by different populations leading to identify candidate genes which are probably responsible for target traits have been recorded by multiple studies in maize (Prioul et al. 1999; Pelleschi et al. 1999; Duble et al. 2000; Liu et al. 2012; Osman et al. 2013; Jin et al. 2015).

By comparing our previous results of linkage analysis in different RIL populations and genome-wide association study (unpublished), we found that 7 QTLs in the present study were also detected in Wu312×Ye478 and K22×Dan340 RIL population. On chromosome 1, qKB-PH1-1 was co-localized by qKD-PH1-2 which explained 14.2% of phenotypic variation in K22×Dan340 population. Besides, qKB-RDW1-1 was also detected in the genomic regions covered by qKD-PH1-1, qKD-RDW1-1 and qKD-ZnSc1-3, explaining 11.4%-15.5% of phenotypic variation in K22×Dan340 population. On chromosome 2, qKB-PH2-1 and qKB-SDW2-2 as well as qKB-SDW2-1 were all mapped inside the major effect QTL qWY-ZnSc2-1 explaining 63.5% of phenotypic variation in Wu312×Ye478 RIL population. On chromosome 9, genomic regions mapped by qKB-ZnSc9-1 and qKB-RDW9-2 also contained the SNP (chr9.S_59587835) which control shoot and root dry weights under Zn deficiency and explained 10% of phenotypic variation in our GWAS studies (unpublished).

**Exploration of genes associated with Zn deficiency tolerance**

Genes associated with Zn nutrition in plants were widely studied, however, were rarely verified in maize. It is the first time that 7 genes (GRMZM5G855347, GRMZM2G025680, GRMZM2G118821, GRMZM2G009368, GRMZM2G000219, GRMZM2G151406 and
GRMZM2G404702) which belong to ZIP (ZRT, IRT-like protein), HMA and NRAMP (The natural resistance associated macrophage protein) families were discovered based on the natural phenotypic variations in maize in the use of single-family linkage analysis at a low level of Zn nutrition.

qKB-PH1-1 which was co-localized by qKD-PH1-2 in K22×Dan340 population, contained ZmIRT1 and ZmNRAMP6 which were also known as GRMZM2G118821 and GRMZM2G028036, respectively. ZIP family were characterized to transport various divalent cations, including Fe$^{2+}$, Zn$^{2+}$, Mn$^{2+}$ and Cd$^{2+}$ (Guerinot 2000; Colangelo and Guerinot 2006). Previous results indicated that IRT-like genes encode major Fe transporters at the root surface in plants (Eide et al. 1996; Varotto et al. 2002; Vert et al. 2002). Recently, AtIRT3 could complement the Zn and Fe uptake double yeast mutants, indicating that AtIRT3 is involved in both Zn and Fe translocation, which was recorded by Lin et al. (2009). In maize, the expression of ZmIRT1 was significantly up-regulated in shoots under Zn deficiency, and ZmIRT1 remarkably reversed the growth defects in the yeast mutants while the effect of the other proteins in ZIP family were relatively inferior, indicating that ZmIRT1 may be likely to play an essential role in Zn uptake in maize, which was evidenced by Li et al. (2013). Besides, results from Li et al. (2015a) indicated that ZmIRT1 has a high selectivity for iron transportation and overexpression of ZmIRT1 enhances Fe and Zn concentration in the roots and seeds of transgenic Arabidopsis. Therefore, ZmIRT1 was predicted to be Zn and Fe homeostasis gene and its physiological function remains unclear in maize.

ZmNRAMP6, a member of NRAMP family. NRAMP genes, in general, are considered to be associated with membrane-spanning proteins (Cellier et al. 1995) and function as transporters for a variety of divalent cations in plants (Gunshin et al. 1997; Thomine et al. 2000; Curie et al. 2000; Bereczky et al. 2003; Nevo and Nelson 2006). However, specific
function of ZmNRAMP6 still remains largely unknown and needs further functional characterization. EIN2 corresponding to GRMZM2G009368 in maize has been discovered in qKB-RDW1-1 in this study. EIN2, a central signal transducer in the ethylene-signaling pathway, contains sequence similarity (21% identity) to the NRAMP family of proteins (Alonso 1999). Physiological studies indicated that ethylene perception requires a transition metal such as Cu or Zn (Rodríguez et al. 1999) and studies in Arabidopsis thaliana have provided complementary evidence for the role of Cu in ethylene perception (Hirayama et al. 1999), but there was no further verification for Zn. These studies suggest that metal metabolism may have a critical role not only in ethylene perception but also in ethylene signaling.

The heavy metal ATPases (HMAs) belong to P_{1\text{B}} subfamily of P-type ATPase superfamily responsible for metal cations transport. 3 members of HMA family were identified on chromosome 1 and 9 in this study. GRMZM5G855347 on chromosome 1 covered by qKB-SDW1-1 whose functional characterization in Arabidopsis thaliana indicated that AtHMA8 (also known as PAA2) transports Cu into the thylakoid lumen to supply plastocyanin (Abdel-Ghany et al. 2005). Besides, qKB-PH9-1 on chromosome 9 contained GRMZM2G000219, GRMZM2G151406 and GRMZM2G404702 whose homology gene correspond to AtHMA6 (also known as PAA1) and AtHMA7 (also known as RAN1) respectively. And it is reported that both of them contributed to Cu transport (Hirayama et al. 1999; Shikanai et al. 2003). However, few genes in HMA family in maize have been cloned and described. Thus, these genes are still valuable for further functional verifications.

The QTL mapping resolution is mainly limited by population size and marker density (Mackay et al. 2009). Generally, increasing the marker density can increase the resolution of the genetic map and enhance the resolution and precision of QTL mapping. The quality
and accuracy of high density bin map for QTL detection has been validated by studies on multiple traits in maize (Pan et al. 2012; Stange et al. 2013; Unterseer et al. 2014; Guimaraes et al. 2014; Li et al. 2015b; Zhou et al. 2016). Therefore, the QTL intervals narrowed down via high density SNP map within the peak bin is more likely to exhibit potential for target genes responsible for Zn deficiency tolerance than those outside of the peak bins. In the present study, 9 genes are selected out of 223 candidate genes on chromosome 1, 2, 6 and 9.

For the largest-effect locus qKB-R/S7-2, the leading gene GRMZM2G149040 was characterized as bZIP-transcription factor 58 (also known as bZIP58) in maize. Transcription factors bZIP19 and bZIP23 in Arabidopsis thaliana were identified to regulate the mechanism in tolerance to Zn deficiency by increasing the transcription of ZIPs and other genes (Laurie et al. 2004), implying that maize bZIP-like transcription factors may play essential roles in the regulation of ZmZIP expression under Zn deficient conditions.

GRMZM2G395114, a leading gene detected in a refined interval (217 kb) of qKB-SDW2-1 which was co-localized by the largest effect locus qWY-ZnSc2-1 in WY population, is known as an expressed gene AST91 (Anti-sigma factor antagonist domain of sulfate transporter 91) and ZmSULTR3;3 in maize, also a member of SULTR family which contribute to mediate the uptake and translocation of sulfate in higher plants (Huang et al. 2018).

ZmSULTR3;3 is similar to sulfate transporters which is a high-affinity H(+)/sulfate co-transporter in Arabidopsis thaliana. Furthermore, Zn was partly absorbed by plants from soil in the way of Zn$^{2+}$ coupled with SO$_4^{2-}$. Therefore, variations in ZmSULTR3;3 might be likely to regulate the amount of sulfate transporter thus participating in the control of Zn transport by roots.

GRMZM2G059314 located inside qKB-ZnSc6-1 together with GRMZM2G004128 covered by
qKB-RDW9-2 both encode the NAD(P)-binding oxidoreductase family proteins which are considered to participate in the produce and remove of oxygen free radical (OFR), revealing that the plant growth may be influenced by the expression of GRMZM2G004128 via redox process under Zn deficiency. Under the Zn-deficient conditions, the inhibitions in the removal of OFR and the oxidation of membrane not only lead to plasma membrane leak and leaf chlorosis, but also result in the oxidative degradation of IAA thus causing suppressions in shoot growth (Marschner 1995). It is reported that IAA level of the shoot tips and young leaves in the Zn-deficient plants decreased to about 50% of that in Zn-sufficient plants (Cakmak et al. 1989). In our work, GRMZM2G344993 which is identified within qKB-RDW9-2 encodes indole-3-acetate beta-D-glucosyltransferase which is also known as indol-3-ylacetylglucose synthase (Michalczuk and Bandurski 1982), IAA-glucose synthase (Leznicki and Bandurski 1988), IAGlu synthase (Kesy and Bandurski 1990). The enzyme IAGlc synthase catalyzes the reversible reaction: IAA + UDP ↔ 1-O-IA-glucose + UDP, which is the first step in the biosynthesis of IAA-ester conjugates in monocotyledonous plants (Ostrowski et al. 2015), revealing that GRMZM2G344993 was predicted to participated in the metabolism of IAA then affecting plant growth at a low status of Zn nutrition.

AC210595.3_FG004 detected inside qKB-ZnSc9-1 encodes plasma-membrane associated cation-binding protein 1, also known as PCaP1. Previous studies Arabidopsis thaliana verified that PCaP1 binds Ca^{2+}, Cu^{2+} and other cations and is stably associated with the plasma membrane (Ide et al. 2007; Nagasaki-Takeuchi et al. 2008). Similarly, PCaP1 in maize probably functions as a metal cations transport protein. GRMZM2G034015 identified in qKB-RDW9-2, which was also the only candidate gene in the leading SNP (chr9.S_59587835) mapped in the GWAS study in maize (unpublished), was found to encode transmembrane protein in plants. To our knowledge, transmembrane proteins

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mediate signal transduction between cells and the environments, and perform important functions in lots of cellular biological processes, such as the receptors for signaling molecules and hormones, transmembrane channels for some ions and transport proteins for cations. The function of *GRMZM2G034015* was identified associated with cations transport involving in metal homeostasis.

**Conclusions**

We detected 21 QTLs associated with Zn deficiency tolerance in maize in K22×By815 RIL population using a high-density linkage map. By comparing the linkage analysis and GWAS in different populations, 14 target genes considered as being associated with the mechanistic metabolism in tolerance to Zn deficiency were mined in combination of candidate genes located in refined QTLs and the gene families already characterized to transport metal cations. It is the first time that ZmIRT1, ZmNRAMP6, ZmHMAs were identified using linkage analysis under Zn deficiency in maize. Our findings have assisted us untangling the genetic basis of possible mechanisms in response to Zn deficiency in maize.

**Methods**

**RIL population**

A recombinant inbred population, consisting of 209 lines, was derived from the cross between inbred lines K22 and By815. All the seeds are provided by Professor Xiaohong Yang from National Maize Improvement Center of China in China Agricultural University. The results of our previous studies indicated that By815 was highly tolerant to Zn deficiency and K22 was sensitive to Zn deficiency. The molecular map consisting of 2263 recombinant bins covers 1670.4 cM throughout the genome and the average interval is 0.74 cM.
**Plant culture in hydroponics**

Maize seeds were sterilized for 30 minutes in a 10% solution of \( \text{H}_2\text{O}_2 \), and washed with distilled water. After having been soaked in saturated \( \text{CaSO}_4 \) for 10 h, the seeds were then germinated on moist filter paper in the dark at room temperature. Two days later, the germinated seeds were wrapped in moist filter paper roll and grown. At the stage of two visible leaves, the seedlings were selected and transferred into a 40-L black container.

The RIL population and the parents were grown in the Zn-deficient \( (3\times10^{-4} \text{ mmol L}^{-1} \text{ Zn-EDTA}) \) and Zn-sufficient \( (1\times10^{-2} \text{ mmol L}^{-1} \text{ Zn-EDTA}) \) conditions. The adjusted Hoagland nutrient solution contained \( (\text{mmol L}^{-1}) \): 0.5 \( \text{NH}_4\text{NO}_3 \), 0.5 \( \text{CaCl}_2 \), 1.5 \( \text{Ca(NO}_3)_2 \), 0.75 \( \text{K}_2\text{SO}_4 \), 0.65 \( \text{MgSO}_4 \), 0.1 \( \text{KCl} \), 0.25 \( \text{KH}_2\text{PO}_4 \), \( 1.0\times10^{-3} \text{ H}_3\text{BO}_3 \), 0.35 EDTA-Fe(II), \( 1.0\times10^{-3} \text{ CuSO}_4 \), \( 5.0\times10^{-3} \text{ MnSO}_4 \), \( 5.0\times10^{-6} (\text{NH}_4)\text{Mo}_7\text{O}_{24} \). Solution pH was set at 5.5 - 6.0. Nutrient solution was renewed every 3 days and aerated by a pump. And growth chamber condition was set as a 14-h light period from 8:00 to 22:00 with 28 °C and a 10-h dark period with 22 °C.

The average light intensity measured at canopy was 350 \( \mu\text{mol m}^{-2} \text{s}^{-1} \) and relative humidity was 60%. Each treatment contained 3 duplicates. An each duplicate contained 200 lines and their parents.

**Phenotyping methods**

Zn deficiency symptoms were observed at the 9\(^{th}\)~12\(^{th}\) day after transplanting, and would be assessed by scoring during this period. Zn scores were scaled from 0 to 5. Score 0 indicates the severest symptoms of Zn deficiency. 0-scored plants growth was heavily depressed, showing shortened internodes and petioles. Besides, small malformed leaves
turned pale yellow and tended to be dead. Score 5 indicates great advantages in plant height, greenness, number of expanded leaves, but still being Zn-deficient when comparing with control plants. The differences in phenotypes between 5-scored plants and control plants are less than the other lines. Score 1 to 4 would be determined by specific symptoms, including internode length, chlorosis, necrotic spots, as well as the difference between the other lines and control plants. The experiment was terminated at the 15th day after transplanting, and the plant heights were measured first, then the shoots and roots were stored in the envelopes separately before drying. All samples were dried at 105 °C, then shoot and root dry weights were measured and the R/S ratios were calculated.

**Statistical analysis**

Means of different inbred lines were compared using one-way ANOVA at a 0.05 level of probability by SPSS 20.0. The correlation analysis of Pearson and Spearman were used to investigate the relationships among traits by SPSS version 20.0. The linear mixed effect function lmer in the lme4 package of R version 3.1.1 was fitted to each RIL to obtain the BLUP (Best Linear Unbiased Prediction) value for each traits: $y_i = \mu + f_i + e_i + \epsilon_i$, where $y_i$ is the phenotypic value of individual $i$, $\mu$ is the grand mean for all environments, $f_i$ is the genetic effect, $e_i$ is the effect of different environments and $\epsilon_i$ is the random error. These variance components were considered to calculate the broad-sense heritability as $h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2/e)$, where $\sigma_g^2$ is the genetic variance, $\sigma_e^2$ is the residual error and $e$ is the number of environments (Holland et al. 2003). And the 95% confidence intervals of the $h^2$ were calculated following the method of Knapp et al. (1985).

**QTL mapping**
The identification of QTL was performed using composite interval mapping (CIM) in the Windows QTL Cartographer version 2.5. The scanning interval between markers was set at 0.5 cM, and the window size was set at 10 cM. Model 6 was selected for detecting QTLs and estimating their effects. The threshold logarithm of odds (LOD) values in this study were estimated by permutation tests with minimum of 1000 replicates at a significant level of $P < 0.05$. The confidence interval of the QTL position was determined using the 1-LOD interval method.

**Annotation of candidate genes**

According to the physical distance of peak bins, genes within the refined QTL peak and their functional description were identified using the maizeB73 reference genome assembly v2 available on the MaizeGDB Genome database (http://www.maizeGDB.org). The function of candidate genes were further confirmed by the annotations of orthologs in *Arabidopsis* or rice.

**Abbreviation List**

| Abbreviation | Full name          |
|--------------|--------------------|
| ZnSc         | Zinc score         |
| PH           | Plant height       |
| SDW          | Shoot dry weight   |
| RDW          | Root dry weight    |
| R/S          | R/S ratio          |

**Declarations**

**Availability of supporting data**

All supporting data can be found within the manuscript and its additional files.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The dataset used and analyzed during the current study are included in this published article and its supplementary information files. And all the data used and analyzed in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interest.

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Authors’ contributions

Jianqin Xu performed and carried out the experiments, and wrote the manuscript; Futong Yu designed the study; Xiaoyang Zhu modified the manuscript; Xiuyi Fu assisted in analyzing data. All authors have read and approved the final version of the manuscript.

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Tables
Table 1 Statistical analysis of phenotypic variations of the parents and K22×By815 RIL population

| Trait          | Treatment | Parents  | RIL Population | P     | Mean   | Range  | CV (%) | Skewness | Kurtosis | $H^2$ (%) | 90% Confidence Interval (%) |
|----------------|-----------|----------|----------------|-------|--------|--------|--------|----------|----------|----------|-----------------------------|
| ZnSc (cm)      | -Zn       | 29.5     | 67.2           | 0.000 | 43.3   | 19.1-79.5 | 55.2   | -0.88    | -0.42    | 95.2     | 92.3-97.8                        |
| PH (cm)        | -Zn       | 0.29     | 1.88           | 0.000 | 0.78   | 0.18-2.63 | 48.8   | 0.87     | 0.64     | 91.7     | 89.5-93.5                        |
| SDW (g)        | -Zn       | 0.23     | 1.16           | 0.000 | 0.51   | 0.12-1.17 | 47.2   | 0.67     | -0.12    | 94.1     | 92.4-95.4                        |
| RDW (g)        | Zn/CK     | 0.12     | 0.64           | 0.000 | 0.26   | 0.07-0.66 | 40.8   | 0.95     | 0.97     | 89.0     | 86.2-91.4                        |
| R/S            | Zn/CK     | 0.41     | 0.29           | 0.006 | 0.35   | 0.21-0.55 | 19.5   | 0.63     | 0.00     | 78.6     | 73.2-83.1                        |
| R/S            | Zn/CK     | 1.31     | 1.12           | 0.047 | 1.23   | 0.53-1.50 | 14.0   | 0.04     | 0.25     | 85.7     | 81.9-88.7                        |

Note: $P < 0.05$ indicates significant difference between K22 and By815.

Table 2 Analysis of variance for the traits associated with the tolerance to Zn deficiency

| Variance       | Genotype          | Environment | Residuals |
|----------------|-------------------|-------------|-----------|
| ZnSc (-Zn)     | 1.804**           | 0.000       | 0.000     |
| PH (-Zn)       | 108.678**         | 3.315**     | 38.143    |
| SDW (-Zn)      | 0.112**           | 0.003**     | 0.030     |
| SDW (-Zn/CK)   | 0.105**           | 0.002**     | 0.020     |
| RDW (-Zn)      | 0.009**           | 0.000**     | 0.003     |
| RDW (-Zn/CK)   | 0.082**           | 0.001**     | 0.023     |
| R/S (-Zn)      | 0.004**           | 0.000**     | 0.003     |
| R/S (-Zn/CK)   | 0.084**           | 0.003**     | 0.042     |

Note: ** represents significant difference at $P = 0.01$.

Table 3 Quantitative trait loci (QTL) for traits associated with Zn deficiency tolerance in K22×By815 RIL population

| Trait          | Treatment | Chr | Name         | Marker Interval | Genetic Interval (cM) | Physical Interval (Mb) | LOD | Additive effect | $R^2$ (%) |
|----------------|-----------|-----|--------------|-----------------|-----------------------|------------------------|-----|-----------------|-----------|
| ZnSc           | -Zn       | 6   | qKB-ZnSc6-1  | SYNL181 7 - PZE-1060986 | 73.1 - 75.8 | 150.7 - 152.3 | 5.3 | 0.19            | 9.2       |
|                |           | 9   | qKB-ZnSc9-1  | PZE-109025227 - PZE-109051633 | 43.1 - 50.2 | 25.3 - 89.4 | 3.6 | 0.16            | 6.3       |
|                |           | 9   | qKB-ZnSc9-2  | PZE-109059409 - PZE-109059809 | 53.9 - 58.0 | 100.9 - 107.3 | 4.5 | 0.17            | 7.7       |
| Sample | Treatment | PH-Zn Sc10 | SDW-Zn | CK-Zn | RDW-Zn | -Zn/CK | PH | SDW | CK | RDW | -Zn/CK |
|--------|-----------|------------|--------|-------|--------|--------|----|-----|----|-----|--------|
| 10     | qKB-      | 1090641    | 114.6  | 146.7 | 3.1    | 0.15   | 5.2 |     |    |    |        |
|        | ZnSc10-1  | 32         | 121.9  | 148.1 |        |        |     |     |    |    |        |
|        | PZE-      | 1101046    | 0      | 46    | 3.1    | 0.15   |     |     |    |    |        |
|        | 01        | 1012050    | 0      | 80    | 3.1    | 0.15   |     |     |    |    |        |
|        | SYN1978   | 38         | 0      | 46    | 3.1    | 0.15   |     |     |    |    |        |
|        | 1012094   | 1020174    | 26.6   | 7.7   | 6.6    | 4.04   | 12.1 |     |    |    |        |
|        | 72        | 1060895    | 62.4   | 75.8  | 3.3    | 2.65   | 5.5  |     |    |    |        |
|        | 9         | 1090535    | 50.9   | 92.6  | 3.1    | 2.57   | 5.2  |     |    |    |        |
|        | PZE-      | 1060986    | 62.4   | 75.8  | 3.3    | 2.65   | 5.5  |     |    |    |        |
|        | 80        | 1090759    | 50.9   | 92.6  | 3.1    | 2.57   | 5.2  |     |    |    |        |
|        | PZE-      | 1020097    | 17.3   | 4.4   | 5.0    | 0.13   | 10.1 |     |    |    |        |
|        | 55        | SYN3756    | 6      | 4.4   | 5.0    | 0.13   |     |     |    |    |        |
|        | 1020174   | 6         | 26.6   | 7.7   | 4.3    | 0.13   | 9.2  |     |    |    |        |
|        | PUT-      | 163a-3155857 | 40.1  | 12.5  | 3.8    | 0.22   | 8.5  |     |    |    |        |
|        | 8         | 1010261    | 40.1   | 12.5  | 3.8    | 0.22   |     |     |    |    |        |
|        | 163a-3155857 | 145.3 | 149.0 | 212.2 | 3.8    | -0.15 | 8.5  |     |    |    |        |
|        | 8         | 1010261    | 145.3  | 149.0 | 212.2  | 3.8    |     |     |    |    |        |
|        | 163a-3155857 | 186.2 | 191.3 | 263.8 | 3.0    | -0.03 | 5.9  |     |    |    |        |
|        | 8         | 1012135    | 186.2  | 191.3 | 263.8  | 3.0    |     |     |    |    |        |
|        | 163a-3155857 | 188.7 | 192.8 | 230.0 | 3.1    | 0.05  | 6.5  |     |    |    |        |
|        | 8         | 1012256    | 188.7  | 192.8 | 230.0  | 3.1    |     |     |    |    |        |
|        | 163a-3155857 | 196.8 | 203.8 | 276.0 | 4.1    | -0.19 | 8.8  |     |    |    |        |
|        | 8         | 1012332    | 196.8  | 203.8 | 276.0  | 4.1    |     |     |    |    |        |
|        | 163a-3155857 | 40.3  | 43.1   | 24.1  | 4.6    | 0.28  | 11.2 |     |    |    |        |
|        | 8         | 1090239    | 40.3   | 43.1   | 24.1  | 4.6    |     |     |    |    |        |
|        | 163a-3155857 | 40.3  | 43.1   | 24.1  | 4.6    | 0.28  | 11.2 |     |    |    |        |
| Chr | Trait | Treatment | Name | Physical Interval (Mb) | LOD | Additive effect |
|-----|-------|-----------|------|------------------------|-----|-----------------|
| 1   | RDW   | CK        | qKB-RDW1-2 | 12.5 - 15.7            | 4.3 | 0.07            |
|     | SDW   | CK        | qKB-SDW1-1 | 12.5 - 16.8            | 3.8 | 0.22            |
| 2   | PH    | -Zn       | qKB-PH2-1  | 7.7 - 8.8              | 6.6 | 4.04            |
|     | SDW   | -Zn       | qKB-SDW2-2 | 7.7 - 8.9              | 4.3 | 0.13            |
| 6   | PH    | -Zn       | qKB-PH6-1  | 146.9 - 152.3          | 3.3 | 2.65            |
|     | ZnSc  | -Zn       | qKB-ZnSc6-1 | 150.7 - 152.3         | 5.3 | 0.19            |
| 9   | ZnSc  | -Zn       | qKB-ZnSc9-1 | 25.3 - 89.4           | 3.64| 0.16            |
|     | RDW   | -Zn/CK    | qKB-RDW9-2 | 28.0 - 92.6           | 3.96| 0.23            |
| 9   | PH    | -Zn       | qKB-PH9-1  | 92.6 - 122.9          | 3.07| 2.57            |
|     | ZnSc  | -Zn       | qKB-ZnSc9-2 | 100.9 - 107.3        | 4.54| 0.17            |

Note: Positive values of additive effect indicate By815 alleles are in the direction of increase; negative values indicate K2 alleles are in the direction of increase. The position refers to the B73 reference sequence Version 2.
Table 5 The information of target genes within the loci detected in the K22×By815 RIL population

| QTL      | Chr | Gene ID                  | Gene position (bp)       | Description                                                                 |
|----------|-----|--------------------------|--------------------------|-----------------------------------------------------------------------------|
| qKB-SDW1-1 | 1   | GRMZM5G855347            | 16332686 - 16338873      | ZmHMA - Heavy metal translocating P-type ATPase                            |
| qKB-PH1-1  | 1   | GRMZM2G025680            | 255995136 - 255998013    | ZmNRAMP6 - Metal transporter Nramp6                                         |
| qKB-PH1-1  | 1   | GRMZM2G118821            | 258353073 - 258355277    | ZmIRT1 - A member of ZIP family in maize                                    |
| qKB-RDW1-1 | 1   | GRMZM2G009368            | 264267252 - 264269646    | ZmEIN2 - Involved in ethylene signal transduction.                          |
| qKB-SDW2-1 | 2   | GRMZM2G395114            | 4890135 - 4895146        | AST91 - Anti-sigma factor antagonist domain of sulfate transporter 91      |
| qKB-ZnSc6-1 | 6   | GRMZM2G059314            | 151454748 - 151457344    | NAD(P)-linked oxidoreductase superfamily protein                           |
| qKB-R/S7-2  | 7   | GRMZM2G149040            | 173148589 - 173154260    | bZIP58 - bZIP-transcription factor 58                                      |
| qKB-ZnSc9-1 | 9   | AC210595.3_FG004         | 42521681 - 42524448      | Plasma-membrane associated cation-binding protein 1                         |
| qKB-RDW9-2 | 9   | GRMZM2G034015            | 59587805 - 59594040      | Transmembrane protein                                                      |
| qKB-RDW9-2 | 9   | GRMZM2G004128            | 86529826 - 86531722      | FAD/NAD(P)-binding oxidoreductase family protein                            |
| qKB-RDW9-2 | 9   | GRMZM2G344993            | 87564495 - 87566420      | Indole-3-acetate beta-D-glucosyltransferase ZmPAA1 - Encodes a putative metal-transporting P-type ATPase |
| qKB-PH9-1  | 9   | GRMZM2G000219            | 120249240 - 120251017    | ZmHMA5 - Encodes a cation-transporting ATPase HMA5                         |

Note: The position refers to the B73 reference sequence Version 2.

Additional File

Table S1 Information of candidate genes

Figures

![Diagram of ZnSc(-Zn) and PH(-Zn)]
Figure 1

Phenotypic distribution of the traits associated with the tolerance to Zn deficiency in the population. Yellow indicates K22 and blue indicates By815.
QTLs detected in this study and the co-localization of QTLs identified in *Wu312×Ye478* RIL population. Six target genes belonging to ZIP, HMA, NRAMP gene families are also depicted in black columns.
LOD values for QTL bins and candidate genes inside the peak bins. The blue lines represent the LOD profiles of the bins within a QTL interval. Red bands indicate the genes predicted to have putative functions associated with Zn deficiency tolerance; and gray bands indicate the other genes in each peak bin. For qKB-RDW9-2, only 3 target genes are shown.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Supplementary material.xlsx