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New insights unveiled: Identifying novel genomic regions governing zinc content in rice for enhanced biofortification

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ABSTRACT

Micronutrient deficiencies, notably zinc (Zn) and iron (Fe), affect approximately two billion people worldwide, significantly burdening developing countries. Diets predominantly based on rice, which sustains over 90 % of the Asian population, are frequently deficient in these essential micronutrients. This investigation evaluated the zinc concentration in milled rice (MR) across 90 Advanced Breeding Lines (ABLs), revealing a substantial variation in zinc content ranging from 13.7 to 33.80 ppm. A GWAS using the FarmCPU model effectively controlled for population structure and kinship, revealing genetic loci associated with zinc concentration in rice grains. The analysis uncovered 11 significant marker-trait associations (MTAs) related to zinc content, with several MTAs co-localizing with known zinc-associated traits, suggesting the potential for simultaneous enhancement of these traits. These robust MTAs present valuable targets for biofortification strategies aimed at increasing the zinc content in rice grains, thereby contributing to the mitigation of zinc deficiency among rice consumers. The 11 identified MTAs for Zn traits can fast-track marker-assisted breeding for developing zinc-enriched rice varieties. These findings are pivotal for ongoing initiatives to address micronutrient malnutrition and improve the nutritional quality of rice. Additionally, these MTAs facilitate gene pyramiding and genomic selection to boost breeding precision and efficiency.

1. Introduction

Rice (*Oryza sativa* L.) stands as the world's most critical food crop, sustaining more than half of the global population (Kumar et al., 2023; Mohidem et al., 2022). Rice not only fulfills daily caloric needs for millions but also supplies key nutrients, including protein, thiamine, riboflavin, niacin, and essential micronutrients like iron (Fe) and zinc (Zn) (Jauhar and Wani, 2021; Mohidem et al., 2022). With the rising living standards of rice consumers, there is an increased need for high-quality, micronutrient-rich rice grains. This need is exacerbated by difficulties such as climate change, limited agricultural land, and a burgeoning world population, which is anticipated to reach 9 billion by 2050 (Alkahtani, 2022). However, rice generally contains low levels of Fe and Zn, especially after processing (Bollinedi et al., 2020). To address this, consuming fortified foods rich in essential micronutrients like Fe and Zn is vital for meeting metabolic needs, maintaining optimal health, and combating hidden hunger (Ibeanu et al., 2020). Boosting the Fe and Zn levels in rice, the world's most widely consumed staple, is a vital

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approach to meeting these nutritional objectives (Pradhan et al., 2020).

The human body contains significant amounts of essential trace minerals, particularly Fe and Zn. These minerals are crucial for various biological functions, including immune responses, gene expression regulation, cell growth, development, and reproduction (Bollinedi et al., 2020; Yu and Zhou, 2009; Brown et al., 2001, 2004). Fe and Zn hold paramount significance in human nutrition due to their widespread presence in dietary sources and their analogous characteristics that influence their absorption or antagonism (Lim et al., 2013). An adult human with an average weight of 65 kg typically contains approximately 3.4 g of iron and 1.5–2.5 g of zinc within their body composition (King et al., 2006). Inadequate levels of zinc in the body can precipitate severe deficiency disorders, including but not limited to delayed wound healing, skeletal abnormalities, hypogonadism, anorexia, compromised immune function, diminished stature, and cognitive deficits (Prasad, 1991; Salgueiro et al., 2000; Song et al., 2022). Micronutrient malnutrition, often referred to as "hidden hunger," is a widespread global health issue mainly caused by a lack of essential minerals like iron and zinc. This predicament is particularly pronounced in developing nations, impeding their progress towards attaining the Sustainable Development Goals (SDGs). A significant contributor to diminished workforce productivity in these regions, attributable to mineral deficiencies, hampers overall gross national product (GNP) outcomes. Zinc inadequacy afflicts approximately two billion individuals worldwide and stands as the seventh most significant health risk factor in developing nations (Zhang et al., 2018). Additionally, Zn deficiency is associated with more than 16 million disability-adjusted life years (DALYs) globally (Black et al., 2008; Bollinedi et al., 2020; Caulfield et al., 2004).

Cereals are a key dietary staples for a substantial portion of the worldwide population, especially in nations with limited development. However, their micronutrient content is frequently insufficient, particularly when subjected to severe processing. As a consequence, diets relying mostly on cereals, with inadequate diversification from other nutritious food sources, leads to an increased risk of deficiencies in micro nutrients, a condition termed hidden hunger (Kumar et al., 2025). Therefore, in regions where rice is the primary staple and households lack the financial means to diversify their meals with mineral-rich fruits, vegetables, and meat, zinc deficiency is common (Harvest Plus, 2021). Modern high-yielding rice cultivars contain Zn concentrations in the brown rice (BR) within the range of 15–35 ppm, much of which is lost during polishing, resulting in the consumption of grains with only 12–28 ppm Zn (Bollinedi et al., 2020; Pradhan et al., 2020). Genetic fortification of rice grains appears to be a promising option to address the problem of micronutrient deficiencies. Zinc biofortification is a global breeding initiative aimed at addressing micronutrient malnutrition. This is especially crucial for infants in developing countries, who are at risk of zinc deficiency due to their primary food source, rice, having inadequate zinc content (Rakotondramanana et al., 2022). Developing biofortified rice varieties with higher zinc concentrations in the grains could provide an effective solution. Intake of fortified foods that are high essential to meet metabolic needs, promote overall health, and address nutritional deficiencies effectively. Enhancing the iron and zinc content in commonly consumed foods is an effective way to achieve these remarks (Bukomarhe et al., 2023). To improve biofortification using targeted breeding strategies, it is critical to comprehend the genes and pathways that control mineral equilibrium and dispersion, as well as the molecular elements that influence intake and transport. In the past few years, GWAS is identified as a very effective means for determining the genetics behind the plant features.

Using ABLs (Advanced Backcross Lines) of diverse origin in GWAS enhances the power and resolution of MTA detection by capturing a wide range of allelic variation and recombination events, which are critical for identifying robust and novel loci associated with complex traits like grain zinc content. The genetic diversity introduced from multiple backgrounds of elite lines broadens the phenotypic spectrum and improves the accuracy of association mapping (Zhu et al., 2018; Huang et al., 2010). Such diversity also increases the likelihood of discovering stable, environment-responsive MTAs that are transferable across breeding programs. According to Zhao et al. (2011), utilizing diverse rice panels significantly enhances the identification of loci linked to agronomic and nutritional traits through genome-wide association studies (GWAS).

GWAS utilizes genetic variation identified in Advanced Breeding Lines (ABLs) to pinpoint the molecular processes that regulate traits such as grain accumulation and mineral absorption. By the use of past recombination instances, GWAS efficiently assigns genetic factors to more confined genomic loci. Furthermore, GWAS leverages single-nucleotide polymorphism (SNP) markers to comprehensively survey the entire genome, facilitating the identification of functional variants underlying the trait of interest. In our GWAS conducted during the kharif (wet season) of 2021 in Raipur, India, we analyzed a diverse panel of 90 ABLs of Indica rice background, cultivated at Indira Gandhi Krishi Vishwavidyalaya, Raipur. The primary objectives of this study were to identify genomic regions and marker-trait associations (MTAs) correlated with zinc concentration in rice grains and to pinpoint elite high-potential parental lines as sources for the bio-fortification program of rice.

2. Materials and methods

2.1. Plant material and field experimentation

A panel of ninety Advanced Breeding Lines (ABLs) created at IGKV in Raipur, India, was used in the study. Advanced Breeding Lines (ABLs) are developed through strategic elite × elite crosses, where parents are selected for high yield, disease resistance, adaptability, and zinc (Zn) content. The objective is to combine these desirable traits in the progeny. Pedigree selection is used across generations to track genetic lineage and select superior individuals based on phenotypic performance. By the F6 generation, the lines approach genetic homozygosity, ensuring trait stability. These lines are then evaluated in multi-environment trials (METs) to assess yield performance, stability, and adaptability under diverse conditions. The ultimate aim is to identify ABLs with consistently high performance and enhanced nutritional quality, particularly increased Zn content. The entire genotype set was assessed at the Research and Instructional Farm, Indira Gandhi Agricultural University, Raipur, during the wet season of 2021. The studies were carried out in inceptisols of clay loam soil, with an organic carbon content of 0.32–0.34 % and a pH range of 6.8–7.4. To ensure precise data collection, a randomised block statistical design was used for field evaluation. To ensure consistent germination, seeds were pre-germinated on an elevated seedbed. Seedlings that were 21–25 days old were subsequently shifted into a rice field that had been prepared methodically, with a planting spacing of 15 cm amongst plants and 20 cm among rows. A robust crop stand was guaranteed by putting the suggested package of procedures into practice. At maturity, central row plants were selected, and their grains were harvested, threshed, and dried to 13 % moisture. The dried grains were then stored at room temperature in zip-lock bags for further analysis.

2.1.1. Sample Preparation and micronutrient estimation

One gram of polished rice from each genotype was digested with 10 mL of a di-acid mixture (nitric acid and perchloric acid in a 9:4 ratio) using a Kjeldahl digestion unit at 280 °C until the solution became colorless. After cooling, the digested samples were filtered through Whatman No. 40 filter paper, and the filtrate was diluted to a final volume of 50 mL with deionized water. Zinc (Zn) and iron (Fe) concentrations were estimated following the HarvestPlus protocol using a Thermo Scientific iCE 3000 Series Atomic Absorption Spectrophotometer (AAS) located at the Nutritional Laboratory, Department of Plant Molecular Biology and Biotechnology, IGAU, Raipur.

2.2. SNP genotyping

SNP-based marker genotyping involves the collection of leaf samples from various genotypes. The sample collection process was meticulously designed to ensure accurate and reliable results. Here's a detailed description of the revised sample collection procedure: For each rice genotype, 4–6 leaf discs of uniform size (4–5 mm diameter) were carefully taken. To ensure consistency, an equal amount of tissue was collected from all genotypes. Special attention was given to avoid the midrib of the leaf during punching to obtain representative samples. A labelled plate was used to collect the punched leaf discs. This plate was divided into two sets to ensure redundancy and prevent re-sampling in case of any analysis failures. Set 1: Primary Plate - This plate contained the initial samples collected during leaf collection. Set 2: Back-up Plate - As a precautionary measure, a duplicate set of samples was stored in the backup plate with the primary plate samples. Throughout the leaf collection process, utmost care was taken to preserve sample integrity. The 96-well sampling sets were kept in cooled conditions at all times to prevent degradation and maintain sample quality. This revised sample collection process ensures the reliability of SNP genotyping results and offers a fail-safe mechanism by having a backup plate. The collected leaf discs from tender seedlings of the studied materials were then sent to Intertek (https://www.intertek.com/agriculture/agritech/), Hyderabad, for genotyping using the High-Throughput SNP (HT-SNP) platform, supported by IRRI-TRB and HTPG. The controlled and careful handling of samples during the entire process minimizes the chances of experimental errors, contributing to the accuracy of the SNP genotyping analysis.

2.3. Analysis of linkage disequilibrium and population structure

Two methods were applied in order to determine the quantity of subgroups in the association mapping panel: PCA (principal component analysis) and a model-based approach with the program STRUCTURE 2.3.4 (Pritchard et al., 2000). A Bayesian framework and ADMIXTURE (Alexander et al., 2009) were used in the model-based study to account for correlated allele frequencies across subpopulations. For this study, a selection of 194 genome-wide SNP markers with less than 20 % missing data and a minor allele frequency (MAF) of at least 0.05 (Fig. 2) were used. Each assumed subgroup (K) was repeated ten times, with a range of 2–10. Each run included 100,000 burn-in iterations followed by 100, 000 MCMC steps. The optimal number of subpopulations (K) was identified using STRUCTURE HARVESTER (Earl and vonHoldt, 2012) by plotting ΔK against LnP(K), following the Evanno et al. (2005) method. Furthermore, the "rmvp" package (genomic association and prediction integrated tool) was used in the R environment to carry out PCA analysis (Lipka et al., 2012). Based on the screen plot that "rmvp" produced, the number of significant main components that sufficiently explained the population structure was ascertained. In order to assess linkage disequilibrium (LD) among the SNP markers, TASSEL v5.2.20 was utilized to compute r^2 values (Bradbury et al., 2007). For LD decay analysis, only r^2 values inside each chromosome with $p < 0.05$ were taken into account. Upon segmenting marker pairs into 5 kb bins, we charted the mean r^2 value of each bin with the distance. LD decay was defined as the distance at which the r^2 value declined to half of its maximum mean level (Huang et al., 2010).

2.4. Genome-wide association analysis

For the GWAS analysis, we utilized data from 194 SNP markers, applying filters to ensure a maximum of 20 % missing data per genotype and SNP, along with a minimum minor allele frequency (MAF) threshold of 0.05. Data analysis was performed using multiple statistical approaches, including FarmCPU (Liu, 2015; Liu et al., 2016), MLM, and GLM. Quantile-quantile (Q-Q) plots were constructed by plotting observed p-values against expected values to assess the adequacy of model adjustments for population structure and familial relatedness. Following a modified Bonferroni correction for multiple testing ($p < 3.21E-05$; $-\log10(p) > 5.79$), based on the reciprocal of the total number of markers used in the analysis, we identified significant marker-trait associations (MTAs). To quantify the impact of individual SNPs on the phenotype, we computed the percentage of phenotypic variance explained (PVE) through single-marker analysis. This allowed us to gauge the impact of each SNP on the trait under investigation.

2.5. Evaluation of the novelty of the MTAs identified

Comparing the physical locations of the discovered marker-trait associations (MTAs) in our investigation to previously published quantitative trait loci (QTLs) linked to zinc (Zn) concentration allowed us to evaluate the uniqueness of the MTAs. To identify the precise locations of previously documented Zn-related QTLs, we conducted a comprehensive literature study and examined the QTL database from Gramene as well as the QTL annotation rice online database (QTARO). This study used the Rice Annotation Project Database (RAP-DB) genome browser to learn more about the putative candidate genes that might be connected to the MTA regions. This allowed us to investigate and annotate the genes located in proximity to the MTAs and potentially unravel the underlying genetic mechanisms governing Zn content in rice grains.

3. Results

3.1. Genetic markers distribution and linkage disequilibrium analysis

Following filtration, 194 polymorphic SNPs remained for GWAS and LD decay analysis. The count of markers varied, with chromosome 10 having 7 SNPs and chromosome 6 having the highest count at 36 SNPs, averaging 16.16 SNPs per chromosome. The SNP marker distribution and density across the 12 rice chromosomes has been depicted in Fig. 1.

3.2. Population structure

Model-based simulations were employed to analyze the population structure, revealing diverse subpopulations within the panel. Plotting the analysis's number of groups against Δ K revealed a significant peak at K = 7 (Fig. 3a). Fig. 3c illustrates population structure, where each vertical bar represents an individual partitioned into segments reflecting inferred membership proportions across populations. Out of the 90 ABL population lines studied, only 18 % were classified as pure types, meaning they had minimal admixture (<10 %). About 7 % of the lines showed admixture ranging from 10 to 20 %, while the majority of lines (more than 73 %) exhibited a higher degree of admixture (>20 %). Additionally, a PCA was conducted to identify the presence of structure in the population. Furthermore, an admixture analysis was performed, showing that around 18 % of the accessions (17 out of 90) exhibited low admixture (0–10 %), 7 % (7 out of 90) showed moderate admixture (10–20 %), and the remaining 73 % showed a high degree of admixture (>20 %). To understand the linkage disequilibrium (LD) patterns in the association panel, LD analysis was carried out. The findings indicated that the average r^2 peaked at 0.5 over shorter distances, approximately 50 kb, and decreased to a minimum of 0.04 around 450 kb (Fig. 2). Overall, these analyses provide insights into the population structure and admixture patterns within the ABL population, as well as the variation explained by different PCs and the LD patterns within the panel.

A vertical bar is used to denote each accession. Each vertical bar's-colored subsections show the accession's membership coefficient (Q) for each distinct cluster. Compared to other values of K, the ΔK value was significantly larger for the model parameter K = 7.

3.3. Grain zinc content in the association panel

The association panel (Table 1) shows heterogeneity in the grain Zn

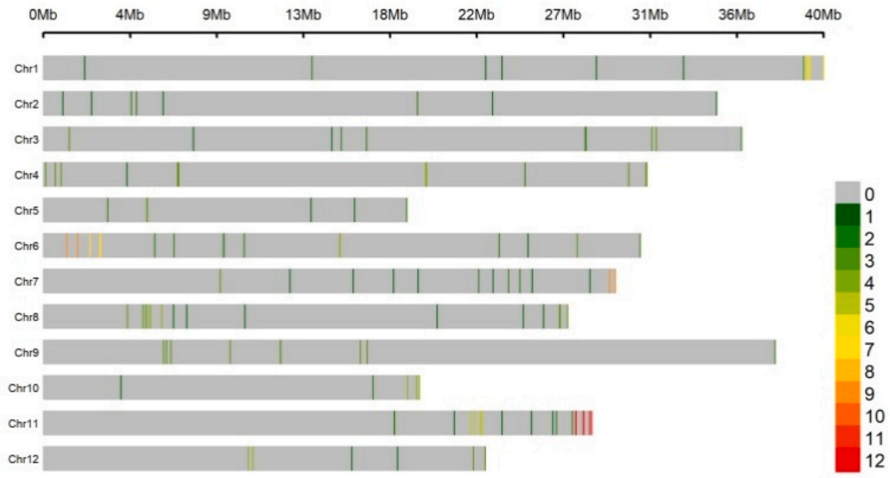

Fig. 1. The SNP marker distribution and density across the 12 rice chromosomes are depicted. Chromosome length is shown on the horizontal axis, with SNP density visualized using various color gradients. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

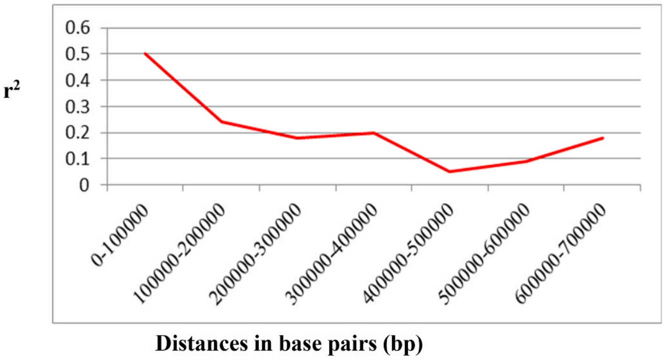

Fig. 2. Linkage disequilibrium (LD) decay plot plotted against physical distance in bp and r.^{2}.

concentration where Zn content showed a normal distribution (Fig. 4). The mean value of grain zinc was 21.58 + 0.48 ppm, with a standard deviation of 4.59. It ranged from 13.7 to 33.8 ppm. Polishing resulted in a significant loss of zinc.

3.4. Detection of specific and stable MTAs by GWAS

In the *Kharif* (wet season) season of 2021, GWAS was carried out to find correlations between genetic markers and Zn attributes. This study uses a mixed linear model (MLM), while Q-Q plots showed an excellent fit, with the observed and expected p-values roughly matching (Fig. 5). Eleven major MTAs were found to be linked with Zn characteristics in rice grains based on the MLM data. These MTAs were dispersed throughout multiple chromosomes; some showed significant and moderate effects on the phenotypic variance, while others showed just small effects. On seven of the twelve chromosomes, significant MTAs were

Table 1
Descriptive statistics values for Zn content.

Mean	21.59
Standard Error	0.48
Median	20.65
Mode	19.55
Standard Deviation	4.59
Minimum	13.70
Maximum	33.80

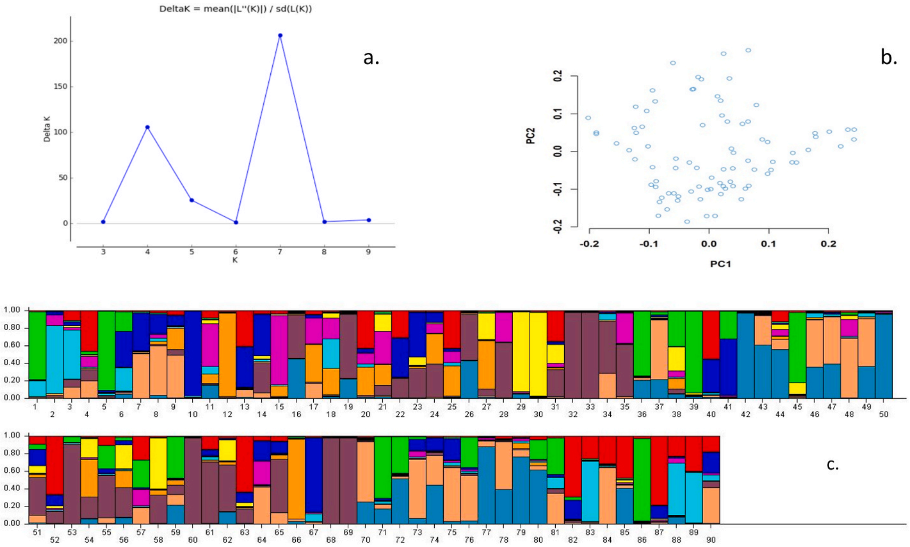

Fig. 3. (a) Delta k vs. k, (b) PC1 vs. PC2, and (c) posterior probabilities assigning each rice variety to one of six subpopulations using STRUCTURE software.

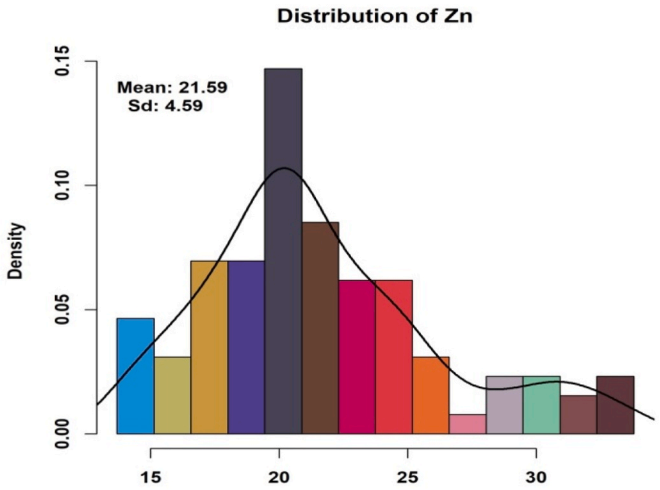

Fig. 4. Frequency distribution of grain Zn in the GWAS panel.

found (Table 2). Two of these MTAs, with phenotypic variance explained (PVE) of more than 20 %, were deemed major effects of MTAs. These two chromosomes were home to these large MTAs. On chromosomes 4 and 7, three MTAs were identified as having a moderate influence, which was defined as accounting for 10 %–20 % of the phenotypic variation. The remaining six MTAs, which were spread over chromosomes 1, 6, 10, 11, and 12, were regarded as modest effects because their PVE was less than 10 %. With three significant MTAs found overall, chromosome 12 had the most, followed by chromosomes 4 and 6, each of which had two significant MTAs. There was a single substantial MTA on chromosomes 1, 7, and 11. Notably, chromosomes 2, 3, 5, 8, and 9 did not have any MTAs linked to Zn characteristics in rice grains. Manhattan plots were generated to visually represent significant SNP markers surpassing the modified Bonferroni threshold (Fig. 5). These graphs give a concise summary of the statistical significance of the detected MTAs and show the genomic areas where they are situated.

3.5. Analysis of candidate genes for bio-fortification and novel QTLs for blast resistance

The Rice Annotation Project (RAP) database genome browser1 was used to look for potential genes from earlier research. Genes associated with desirable qualities were mapped to their physical locations, and genes that were geographically close to identified QTLs were deemed potential candidates. Using the Gramene database2, more research was done to find previously published QTLs that co-localized with the current QTLs for agronomic traits.

In this study, we identified a total of 11 significant MTAs associated with Zn traits in rice grains (Table 2). Among these MTAs, only one SNP region on chromosome 1 had been previously reported for Zn traits. The rest of the SNPs were novel or associated with different traits. Interestingly, we found several intriguing co-localizations with candidate genes and previously identified QTL areas. On chromosome 1, we reported one MTA for Zn at a distance of 33.14 M. Stangoulis et al. (2007) had also reported QTL for milled rice and brown rice at 35 M distance on the same chromosome, suggesting a tight linkage in this region. Additionally, this region was also reported to potentially harbor a Blast resistance gene. Through rice genome annotation research, we discovered that chromosome 4 contains two moderate MTAs for grain Zn. This region may be linked to resistance to Brown Plant Hopper (BPH), and it also co-localized with the ZIP3 region that was identified by Bollinedi et al. (2020). Similarly, two MTAs for Zn were identified on chromosome 6, at

Table 2
Marker-trait associations for grain Zn content.

Marker	Chr. No.	Position	p	MarkerR^2
SNP107	6	10388389	9.81E-07	0.36599
SNP7	12	22273450	3.07E-04	0.23404
SNP9	4	6930004	0.00405	0.16048
SNP8	4	6940994	0.00405	0.16048
SNP153	7	29323078	0.01153	0.1056
SNP6	12	22274141	0.01388	0.07082
SNP90	12	22885896	0.01388	0.07082
SNP64	11	22303236	0.01662	0.06698
SNP63	10	4003666	0.02116	0.09059
SNP108	6	10389352	0.03554	0.05121
SNP91	1	33144909	0.04922	0.04468

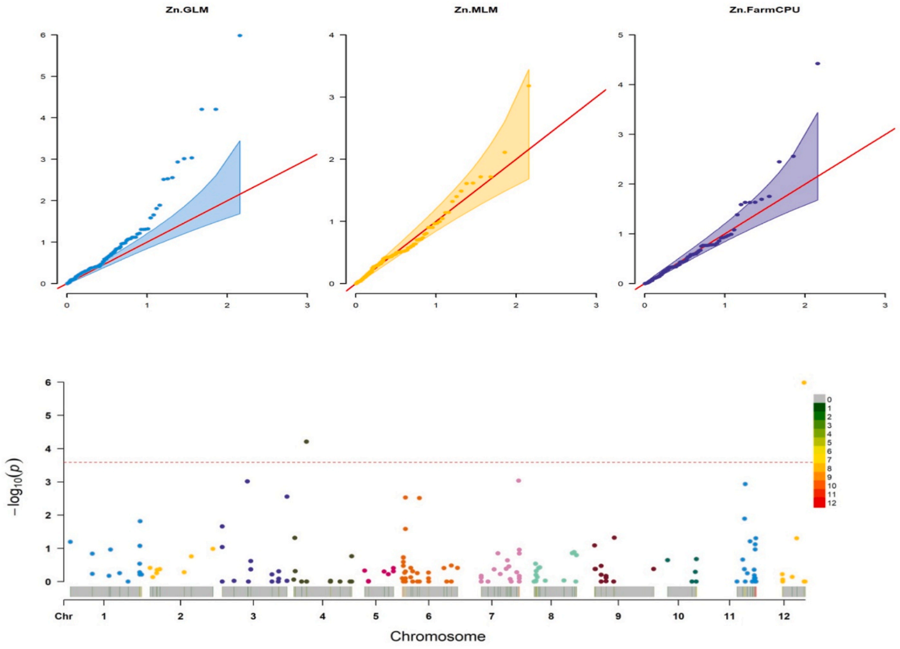

Fig. 5. Manhattan plot and Q-Q plot generated using the MLM model across the 12 rice chromosomes specifically for Zinc.

a 10.38 M distance. This region was found to be linked and co-localized with the findings of Bollinedi et al. (2020), and it is also potentially associated with Blast resistance. Notably, NAM B1, OsNAAT1, and OsNAAT4 genes responsible for metal homeostasis and ion transporters were also located at distances of 13.80 M and 13.83 M, respectively, on chromosome 6. A significant MTA for Zn was observed on chromosome 7, in the region of the NAS 3 gene, which is potentially responsible for zinc transportation and assimilation.

This finding of this study aligns with the results reported by Anuradha et al. (2012) and Evanno et al. (2005). For chromosome 10, only one MTA for Zn was reported at a distance of 4.0 Bollinedi et al. (2020) had also reported an MTA for Zn at 12.68 M distance on the same chromosome. On chromosome 11, a single MTA for Zn was reported at a distance of 22.30 M, and the Rice Genome Annotation Project indicated that the same region is associated with stress tolerance. Three MTAs for Zn were identified on chromosome 12, between 22.27 and 22.88 M distances. Interestingly, this region is also responsible for BPH, as reported in previous studies. Interestingly, six of the eleven MTAs that we detected in our analysis were found to be adjacent to candidate genes and previously described QTL areas. This highlights the significance of these genomic areas in relation to certain features and stress resistance and offers insightful information about the genetic foundation of Zn attributes in rice.

4. Discussion

The pervasiveness of mineral deficiency in micronutrients, particularly in populations that eat rice in developing countries with little dietary diversification, underscores how urgently effective solutions are needed to reduce hidden hunger. Improving the mineral micronutrient content of commonly grown rice varieties, especially zinc (Zn), is a viable strategy. However, mapping numerous genes using standard biparental populations becomes challenging due to the polygenic nature of grain micronutrient accumulation in rice. This challenge is compounded by the sparse distribution of these genes within the gene pool and their low individual effects. In this study, we utilized GWAS to leverage the extensive trait variation observed among 90 Advanced Breeding Lines (ABLs) developed at IGKV Raipur, India. The focus was on assessing zinc levels in milled rice (MR) during the wet season of 2021. Our analysis revealed that the ABL accessions exhibited significant genetic diversity for zinc, adding to the existing evidence of wide variability among rice accessions, particularly involving ABLs and brown rice (BR). Moreover, we demonstrated that this variation in zinc concentration is also present in MR, which is more commonly consumed. Similar to previous studies, we observed a polygenic inheritance pattern for zinc concentration, indicating a complex and multifactorial control over the trait. The bran of rice grains is where zinc is mostly found and makes up a large amount of the mineral content. In their study, Bollinedi et al. (2020) identified a strong correlation between zinc concentrations in brown rice (BR) and milled rice (MR), suggesting that BR zinc levels serve as a reliable predictor of MR zinc levels. With the goal of enhancing MR, this observation offers a non-destructive and effective way to assess the Zn content of breeding populations. Zn was retained in the endosperm at a significantly higher rate, with mild to negligible losses after polishing. Nevertheless, a more thorough analysis of each genotype showed differences in the percentage retention after polishing, indicating the possibility of finding genotypes that retain more nutrients in milled grains. Further research is needed to determine if the variation arises from genetic differences in mineral translocation from the aleurone to the endosperm or from aleurone layer thickness. All things considered, our research highlights the significance of applying GWAS to examine broad phenotypic variations in rice germplasm and to pinpoint several chromosomal areas affecting zinc concentration. This research contributes to the battle against malnutrition caused by a lack of specific minerals and enhances the nutritional value of rice for people who are more vulnerable by expanding our understanding of the genetic foundations of zinc accumulation in rice grains.

4.1. Unlocking bio-fortification potential through GWAS

In this study, we initiated by evaluating the genetic diversity and population structure of a panel of 90 ABLs in a GWAS targeting Zn concentration in milled rice during the wet-season 2021. The presence of diverse ABL populations was confirmed through PCA and STRUCTURE analyses. Our findings revealed significant variations in Zn concentration among the panel, indicating that grain micronutrients are influenced by multiple genetic factors, which is consistent with previous studies (Descalsota et al., 2018; Huang et al., 2015; Swamy et al., 2018). When it comes to finding QTLs and candidate genes, GWAS has proven to be more accurate and efficient than biparental linkage mapping. To minimize false associations due to population structure and kinship, various statistical models such as GLM and MLM have been developed. MLM, widely used in crop GWAS since its introduction (Wang et al., 2016; Ya-fang et al., 2015; Zhang et al., 2014), is constrained by its single-locus approach, limiting its ability to capture the true genetic architecture of complex traits influenced by multiple loci simultaneously (Kaler and Purcell, 2019). To address this limitation, multi-locus models like FarmCPU, pLARmEB (Zhang et al., 2018), LASSO (Xu et al., 2017), BLASSO (Tamba et al., 2017), FASTmrEMMA (Zhang et al., 2018), and pKWmEB (Ren et al., 2018) have been increasingly utilized. Recent research has highlighted the efficacy of these models across a spectrum of plant characteristics. These include studies on traits such as yield attributes in ear morphology (Zhu et al., 2018), wheat (Ward et al., 2019), plant height and flowering time (Wallace et al., 2016), starch properties in maize (Xu et al., 2018), agronomic features in foxtail millet (Jaiswal et al., 2019), panicle structure in sorghum (Zhou et al., 2019), and disease resistance in soybean (Wei et al., 2017).

The power of QTL identification in GWAS is strongly influenced by population size (Garcia et al., 2005). Even with a smaller sample size of 90 people, we were still able to identify 11 important MTAs influencing the Zn concentration in MR. Hoang et al. (2019) found QTLs linked to water shortage tolerance in a GWAS of 180 individuals, while Descalsota et al. (2018) used 144 MAGIC lines to find QTLs linked to agronomic characteristics and biofortification in rice. Compared to allogamous crops like maize, rice has larger haplotype blocks because it is autogamous. Yonemaru et al. (2012) reported a mean haplotype block size of approximately 50 kb in rice, whereas maize, as reported by Maldonado et al. (2019), shows an average block size of 1 kb. As a result, in crops such as rice, a greater fraction of the evolutionarily conserved genomic areas can be covered by a comparatively lower number of genotypes. Over the last decade, GWAS has seen widespread application in the rice industry, facilitating the identification and mapping of QTLs associated with various traits. These include studies on plant height and grain yield (Ma et al., 2016), agronomic characteristics (Zhang et al., 2014), panicle traits (Zhang et al., 2014), grain properties (Edzesi et al., 2016; Wang et al., 2016), milling quality (Qiu et al., 2015), among others. Limited studies have conducted GWAS specifically for mineral elements, with one focusing on milled rice (MR) by Descalsota et al. (2018), and others on brown rice (BR) by Norton et al. (2014) and Yang et al. (2018). In our study, 5 out of the 11 MTAs were novel, and not reported in previous studies, making them promising targets for rice bio-fortification programs. Additionally, number of MTAs were discovered in the intervals of previously mapped QTLs, suggesting their possible importance in controlling zinc concentration and co-localization with other critical features such as stress tolerance, disease resistance, and metal homeostasis.

The MTA on chromosome 4, which lay adjacent to the candidate gene OsZip3, a rice protein from the zinc-regulated transporter (ZIP) family, is crucial for mediating zinc distribution by enabling its unloading from the xylem at nodal vascular bundles. Initially, ZIP1 and ZIP3 cation transporter proteins were hypothesized to participate in zinc uptake from the soil (Ramesh et al., 2003; Bashir et al., 2012).

Subsequent research by Sasaki et al. (2015) confirmed the localization of OsZIP3 in nodes and elucidated its function in unloading zinc from the xylem of larger vascular bundles at these sites. Together with the MTA for zinc, several essential candidate genes like NAM B1, OsNAAT1, and OsNAAT4, which are in charge of ion transporters and metal homeostasis apart from this Os06t0286700 (Defense responsive gene), were found on chromosome 6. Furthermore, OsNAS1, OsNAS2 and OsNAS3 (upstream gene variant), two members of the nicotianamine synthase (NAS) family proteins, and qFeBR3.1 shared the same linkage disequilibrium (LD) block. NAS family members catalyze the synthesis of nicotianamine (NA), which functions as a chelator for metal cations likeFeIIC and FeIIIC. NA plays a critical role in the transport of metal cations over both short and long distances (Takahashi et al., 2003; Von Wiren et al., 1999). Overexpression of NAS genes have been shown to significantly elevate levels of Fe, Zn, and NA in rice grain.

Nicotianamine, acting as a chelator for metal cations such as Fe and Zn and facilitating the transportation within plants, is synthesized by the NAS protein family, pivotal in this biological process (Johnson et al., 2011; Lee et al., 2009). Our study elucidates the genetic mechanisms governing zinc concentration in rice grains, pinpointing potential candidate genes and genomic regions for targeted rice biofortification strategies aimed at enhancing zinc content and related traits. This research deepens our understanding of the complex genetic architecture underlying micronutrient accumulation in rice, offering avenues to bolster its nutritional profile in combatting micronutrient deficiencies. Study identified eleven accessions exhibiting grain zinc concentrations surpassing 28 ppm in the MR, aligning with targets set by initiatives like HarvestPlus for rice biofortification (Bouis and Saltzman, 2017). Notably, these accessions demonstrated elevated grain zinc levels under the assessed conditions. Furthermore, our investigation unveiled several highly promising advanced breeding lines (ABLs). However, conventional breeding methods may have limitations in substantially increasing zinc concentration in rice. Advance genome editing technologies such as transcription activator-like effector nucleases and CRISPR/Cas9, offer precise and efficient tools for enhancing zinc bio-fortification in rice grains through targeted genetic modifications. These approaches facilitate the manipulation of key genes involved in zinc uptake, transport, and accumulation, thereby providing a robust and scalable strategy to improve the nutritional quality of rice (Lee et al., 2009).

5. Conclusions

The intricate genetic structure controlling the zinc (Zn) content of milled rice (MR) during the 2021 kharif (wet) season has been clarified by our extensive Genome-Wide Association Study (GWAS), which involved 90 Advanced Breeding Lines (ABLs). We have verified the genetic heterogeneity among the ABL populations and noted significant variations in Zn content by thorough analyses, such as PCA and STRUCTURE. These results demonstrate the effectiveness of GWAS in identifying quantitative trait loci and candidate genes, underscoring the impact of many genetic variables and concurring with earlier studies. Five new targets for rice biofortification initiatives have been found as a result of our improved accuracy in identifying important marker-trait connections through the use of multi-locus models. Notably, our discovery of putative genes like OsZip3 and NAS protein family members opens up exciting possibilities for improving zinc biofortification in specific ways. This study complements international programs like HarvestPlus and makes a substantial contribution to our understanding of the processes of micronutrient accumulation in rice. Traditional breeding has evolved, however its limitations demand for alternate approaches. The identified MTAs for zinc traits can be effectively utilized in marker-assisted and genomic selection to accelerate the development of biofortified rice varieties. They enable early, accurate selection of high-Zn lines and facilitate the introgression of beneficial alleles into elite cultivars. In genomic selection, these MTAs improve prediction accuracy for zinc accumulation, aiding faster and more efficient breeding. A promising method for improving zinc biofortification in rice is genetic engineering, specifically CRISPR/Cas9. Clarifying the genetic basis of zinc accumulation, improving CRISPR/Cas9 for targeted enhancement, testing stability in different environments through field trials, and integrating technological and traditional breeding for durable nutritional gains should be the main goals of future study. Functional validation through candidate gene expression analysis, gene editing (CRISPR/Cas9), and testing across diverse genetic backgrounds confirms their role and ensures the stability of their phenotypic effects. By addressing these issues, zinc-biofortified rice will be easy to deploy in practice and help reduce malnutrition worldwide.

CRediT authorship contribution statement

Abhinav Sao: Writing – original draft, Software, Resources, Methodology, Formal analysis, Data curation. **Hemant Sahu:** Writing – review & editing, Methodology, Formal analysis. **Ajit Kumar Mannade:** Writing – review & editing, Methodology, Formal analysis. **Sunil Kumar Nair:** Writing – review & editing, Validation, Investigation, Data curation. **Vinay Premi:** Writing – review & editing, Investigation. **Girish Chandel:** Writing – review & editing, Validation. **Tarun Kumar Thakur:** Writing – review & editing, Visualization. **Amit Kumar:** Writing – review & editing, Investigation, Conceptualization. **Rupesh Kumar:** Writing – review & editing. **Dharmendra Singh Tomar:** Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on reasonable request from the first author.

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