Identification of Quantitative Trait Loci Associated with Grain Zinc Biofortification in Rice

Sung Woo Lee [1*], Hye Jin Choi [2]

[1] Department of Applied Plant Science, Chungnam National University, Daejeon, South Korea
[2] Department of Agricultural Biotechnology, Kyungpook National University, Daegu, South Korea

*Corresponding author: **Sung Woo Lee**

Received: 21 May 2025
Revised: 05 Jun 2025
Accepted: 26 Jun 2025
Published: 16 Jul 2025

E-ISSN: 2989-5340
DOI: https://doi.org/10.54660/jafi.2025.5.2.43-47

ABSTRACT

Zinc (Zn) deficiency is a major global public health problem, particularly in developing countries where rice (*Oryza sativa* L.) serves as the primary staple food. Modern elite rice cultivars are typically poor sources of essential micronutrients, especially after the milling process. Genetic biofortification via molecular breeding presents a sustainable approach to enhance grain Zn concentration without compromising agronomic yields. In this study, a Recombinant Inbred Line (RIL) population (F_{7:8}), derived from a cross between an elite indica variety (IR64, low grain Zn) and a wild-derived landrace donor (Madukar, high grain Zn), was evaluated across two consecutive environments to identify stable Quantitative Trait Loci (QTLs) for grain zinc concentration (GZnC) and related agronomic traits, including grain yield per plant (GY) and days to 50% flowering (DTF). A high-density genetic linkage map consisting of 1,245 Single Nucleotide Polymorphism (SNP) markers spanning 1,542.3 cM across the 12 rice chromosomes was used. Composite Interval Mapping (CIM) identified a total of nine QTLs for GZnC across both environments. Notably, two major, stable QTLs—*qGZnC1.1* and *qGZnC7.1*—were identified on chromosomes 1 and 7, explaining 18.4% and 14.2% of the phenotypic variance, respectively. Co-localization analysis revealed that *qGZnC7.1* overlaps with QTLs for grain iron concentration, suggesting a shared transport mechanism. In silico analysis within the stable QTL intervals revealed promising candidate genes, including members of the ZIP (*ZRT/IRT-like Protein*) transporter family and Nicotianamine Synthase (*OsNAS2*). These stable QTLs and flanking SNP markers provide robust tools for marker-assisted selection (MAS) to accelerate the development of zinc-dense, high-yielding rice varieties.

Keywords: Rice (*Oryza sativa* L.), Zinc biofortification, Grain zinc concentration, Quantitative Trait Loci (QTL), Marker-assisted selection (MAS), Single nucleotide polymorphism (SNP), Genetic linkage mapping, Molecular breeding

1. Introduction

Zinc (Zn) is an indispensable micronutrient required for numerous structural, catalytic, and regulatory functions in biological systems. It acts as a cofactor for more than 300 enzymes and plays a foundational role in DNA synthesis, transcription regulation, protein folding, cell division, and immune system function (Broadley *et al*., 2007) [1]. Despite its biological significance, approximately one-third of the global human population suffers from zinc deficiency, a silent epidemic leading to stunted growth, impaired cognitive development, susceptibility to infectious diseases, and increased child mortality (Black *et al*., 2008) [2]. This nutritional crisis is heavily concentrated in sub-Saharan Africa and South Asia, where resource-poor populations rely almost exclusively on polished white rice for their daily caloric intake (Bouis and Saltzman, 2017) [3].

Polished or milled rice consists primarily of the starchy endosperm, as the nutrient-rich aleurone layer and embryo are removed during milling. Typically, elite indica rice varieties contain only about 12–16 mg/kg of zinc in their milled grains, which falls far short of the target concentration (~28–30 mg/kg) required to meet the recommended daily intake (RDI) for adults (Swamy *et al*., 2016) [4]. While agronomic biofortification via foliar or soil application of zinc fertilizers offers a temporary remedy, it is labor-intensive, dependent on soil properties, and economically unsustainable for smallholder farmers (Zou *et al*., 2012) [5]. Transgenic interventions face strict regulatory hurdles and low public acceptance in several major rice-producing countries. Consequently, genetic biofortification—the breeding of crop varieties with inherently high grain micronutrient content using molecular breeding techniques—is recognized as the most cost-effective and long-term solution (Garg *et al*., 2018) [6].

The genetic architecture of grain zinc accumulation in rice is highly complex, polygenic, and significantly influenced by genotype-by-environment (G×E) interactions (Jiang *et al*., 2007) [11] (Garcia-Oliveira *et al*., 2009) [12]. Deciphering the physiological pathways of zinc accumulation is crucial for targeting genetic loci. Zinc must be taken up from the soil solution by the roots, translocated through the xylem, remobilized via the phloem during grain filling, and ultimately deposited into the grain endosperm.

As detailed in Figure 1, this complex physiological network is mediated by multiple transporter gene families, including the ZRT/IRT-like Protein (ZIP) family, Heavy Metal ATPases (HMAs), Yellow Stripe-Like (YSL) transporters, and Natural Resistance-Associated Macrophage Proteins (NRAMPs), along with the synthesis of metal chelators like nicotianamine (NA) and deoxymujianic acid (DMA) (Bashir *et al*., 2013) [8] (Ramesh *et al*., 2003) [15] (Ishimaru *et al*., 2011) [16] (Lee *et al*., 2009) [17] (Johnson *et al*., 2011) [18].

To systematically map the genomic regions controlling these variations, Quantitative Trait Locus (QTL) mapping serves as a foundational approach. Mapping populations, such as Recombinant Inbred Lines (RILs) or Double Haploids (DHs), derived from crosses between low-Zn elite cultivars and high-Zn landraces or wild relatives (*Oryza rufipogon*), facilitate the identification of chromosomal intervals associated with elevated grain zinc concentration (GZnC) (Anuradha *et al*., 2012) [9]. While several GZnC QTLs have been reported in the past decade, many exhibit low phenotypic variance explained (PVE) or fail to show stability across diverse agro-climatic environments (Zhang *et al*., 2014) [10]. Furthermore, a critical challenge in biofortification breeding is the frequent negative correlation observed between grain zinc concentration and total grain yield, which creates a breeding bottleneck (Jiang *et al*., 2007) [11].

To overcome these limitations, this study utilizes a highly structured F_{7:8} RIL population derived from a cross between the high-yielding, zinc-deficient elite indica variety IR64 and the high-zinc traditional landrace Madukar. By leveraging high-density Single Nucleotide Polymorphism (SNP) markers, we aim to:

1. Map high-confidence, stable QTLs regulating grain zinc concentration across distinct environmental seasons.
2. Identify and isolate genomic regions where GZnC co-localizes with agronomic traits to avoid negative yield drag.
3. Conduct an in silico candidate gene analysis within major stable QTL intervals to provide a molecular baseline for marker-assisted backcrossing (MABC) and gene editing programs.

2. Materials and Methods

2.1. Plant Material and Field Layout

The experimental mapping population comprised 180 Recombinant Inbred Lines (RILs) at the F_{7:8} generation, developed via the single-seed descent (SSD) method from a bi-parental cross between IR64 and Madukar. IR64 is a widely cultivated, high-yielding elite tropical indica variety characterized by low grain zinc levels (14.2 mg/kg). Madukar is an indigenous, traditional landrace originating from India that exhibits high grain zinc levels (34.5 mg/kg) but poor agronomic features, including tall architecture and susceptibility to lodging. The 180 RILs along with both parents were evaluated across two distinct environments: Environment 1 (E1: Wet Season, June–October) and Environment 2 (E2: Dry Season, January–May) at the Experimental Research Farm. The field design in both environments was a Randomized Complete Block Design (RCBD) with three replications. Each plot consisted of three rows of 15 plants per row, planted with a spacing of 20 cm between rows and 15 cm between individual plants. Standard agronomic packages and crop management practices recommended for irrigated lowland rice production were strictly adhered to, except that no zinc-containing fertilizers were applied to ensure that differences in zinc uptake reflected genetic variation.

2.2. Phenotypic Evaluation and Agronomic Traits

At maturity, data were collected from 10 competitive, interior plants from each replication per RIL for three core traits:

- **Days to 50% Flowering (DTF):** Recorded as the number of days from sowing until 50% of the panicles in a plot had emerged from the leaf sheath.
- **Grain Yield per Plant (GY, g):** Determined by harvesting, threshing, drying, and weighing the total grains produced by an individual plant, adjusted to a standard 14% moisture content.
- **Grain Zinc Concentration (GZnC, mg/kg):** Harvested panicles were manually threshed using plastic screens to avoid heavy metal contamination from mechanical threshers.

The rough rice grains (paddy) were thoroughly cleaned, dried to ~12% moisture, and dehulled using a non-contaminating laboratory de-husker equipped with polyurethane rollers. The resulting brown rice samples were polished for 60 seconds using a heavy-metal-free grain polisher to yield milled rice. Polished rice samples (2.0 g) from each replication were digested in a closed-flask microwave digestion system using an optimized ultra-pure acid mixture of HNO_3 (65%) and H_2O_2 (30%). The elemental concentration of zinc was determined via Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Standard reference material from the National Institute of Standards and Technology (NIST) was included in each analytical batch to ensure accuracy and reproducibility.

2.3. Statistical and Genetic Analysis

Phenotypic data across both environments were analyzed using SAS software (v9.4). Analysis of Variance (ANOVA) was executed using the GLM procedure based on a mixed-effects model where genotypes were treated as fixed effects, while environments, replications within environments, and genotype-by-environment (G \times E) interactions were treated as random effects. Broad-sense heritability (h2) was estimated.

2.4. Genotyping and High-Density Linkage Map Construction

Genomic DNA was isolated from young, healthy green leaves of 3-week-old seedlings of the 180 RILs and both parents using a modified Cetyltrimethylammonium Ammonium Bromide (CTAB) extraction method. Genotyping was performed using a high-density rice SNP array platform containing 4,800 uniform markers. The initial pool of SNP markers was strictly filtered using the following criteria:

1. Polymorphic between parents (IR64 and Madukar).
2. Missing data less than 5%.
3. Minor allele frequency (MAF) greater than 0.05.
4. Conforming to the expected 1:1 segregation ratio in RILs via chi-square (\chi2) analysis.

A total of 1,245 high-quality SNP markers were retained for linkage map assembly. Genetic linkage maps were constructed using JoinMap 4.1 software. Linkage groups were assigned based on a logarithm of odds (LOD) threshold score greater than or equal to 4.0. Recombination frequencies were translated into genetic distances (centiMorgans, cM) using the Kosambi mapping function.

2.5. Quantitative Trait Loci (QTL) Mapping and Candidate Gene Identification

QTL analysis was conducted independently for each environment (E1 and E2) as well as for the pooled mean data across environments using Windows QTL Cartographer v2.5. Composite Interval Mapping (CIM) was applied with a walking speed of 1.0 cM. The threshold for declaring a statistically significant QTL was determined via 1,000 permutation tests at a significance level of $P < 0.05$, which yielded an empirical minimum LOD threshold ranging from 2.8 to 3.1 depending on the specific trait.

QTLs detected in both environments with overlapping confidence intervals (defined by a 1-LOD drop from the peak) were classified as stable QTLs. The proportion of phenotypic variance explained (PVE, %) by each individual locus and its corresponding additive effect were derived directly from the CIM model. The naming convention for QTLs followed standard international nomenclature guidelines (e.g., *qGZnC1.1* denotes the first QTL for grain zinc concentration discovered on chromosome 1).

To identify potential structural or regulatory candidate genes within the major stable QTL boundaries, the physical positions of the flanking SNP markers were projected onto the reference genome of *Oryza sativa* L. ssp. *indica* (ASM465v1) and ssp. *japonica* via the Rice Annotation Project Database (RAP-DB) and Gramene database. Functional annotations, gene ontology (GO) classes, and expression profiles during early grain-filling stages were scanned for all genes resident within the peak genomic intervals.

3. Results

3.1. Phenotypic Performance and Distribution

The phenotypic data revealed substantial variation for grain zinc concentration (GZnC) and agronomic traits among the 180 RILs (Table 1). The paternal line Madukar exhibited a consistently high GZnC across both seasons (mean of 34.5 mg/kg), whereas the maternal line IR64 showed a significantly lower concentration (mean of 14.2 mg/kg). Within the RIL population, GZnC exhibited a wide range, spanning from 11.1 mg/kg to 38.6 mg/kg in Environment 1 (E1) and 12.3 mg/kg to 40.1 mg/kg in Environment 2 (E2).

Table 1: Phenotypic evaluation, distribution parameters, and heritability estimates for grain zinc concentration and agronomic traits across two environments.

Trait	Parent 1 (IR64)	Parent 2 (Madukar)	RIL Range (E1)	RIL Mean ± SD (E1)	RIL Range (E2)	RIL Mean ± SD (E2)	Heritability (h2)
GZnC (mg/kg)	14.2 \pm 0.8	34.5 \pm 1.4	11.1 – 38.6	22.4 \pm 4.3	12.3 – 40.1	24.1 \pm 4.8	0.78
GY (g/plant)	28.4 \pm 2.1	12.3 \pm 1.1	9.4 – 33.2	21.5 \pm 3.9	10.1 – 35.6	23.0 \pm 4.2	0.64
DTF (days)	84.0 \pm 1.5	108.0 \pm 2.0	78.0 – 114.0	92.5 \pm 6.4	75.0 – 110.0	90.1 \pm 5.9	0.85

Continuous, near-normal distributions were observed for GZnC in both environments. This transgressive segregation demonstrates that both parents contribute complementary alleles for zinc accumulation, confirming the typical polygenic nature of the trait (Garcia-Oliveira *et al*., 2009) [12] (Ghandilyan *et al*., 2006) [13].

The analysis of variance (ANOVA) detailed in Table 2 shows that while genotypes and environments exerted highly significant effects ($P < 0.001$) on GZnC, the genotype-by-environment interaction effect was relatively minor compared to the main genotypic effect. This indicates a high potential for identifying stable genetic loci across seasons (Jiang *et al*., 2007) [11] (Zhang *et al*., 2014) [10]. Broad-sense heritability (h2) was estimated at 0.78 for GZnC, 0.64 for GY, and 0.85 for DTF, supporting the reliability of the phenotypic data for downstream linkage mapping (Jiang *et al*., 2007) [11] (Anuradha *et al*., 2012) [9].

Table 2: Analysis of Variance (ANOVA) for GZnC, GY, and DTF across tested environments. (*ns*: non-significant; *:$P < 0.05$;: $P < 0.01$; ***: $P < 0.001$).

Source of Variation	Degrees of Freedom (df)	GZnC (Mean Square)	GY (Mean Square)	DTF (Mean Square)
Genotype (G)	179	64.85{***}	42.12{***}	112.45{***}
Environment (E)	1	112.30{***}	94.60{**}	185.30{***}
G × E Interaction	179	12.40{*}	15.10{ns}	8.40{*}
Replication (Env)	4	3.12	2.05	1.14
Error	716	4.85	5.32	2.10

3.2. Trait Correlations

Pearson correlation analysis demonstrated an expected, statistically significant negative correlation between grain zinc concentration (GZnC) and grain yield per plant (GY) across both environments ($r = -0.32$ in E1, $r = -0.29$ in E2, $P < 0.01$). This confirms a partial dilution effect, where higher grain mass can dilute the total accumulated zinc concentration. However, several high-yielding RIL lines broke this linkage drag, exhibiting both high grain yield and elevated zinc content, which suggests that independent genetic mechanisms can co-exist. GZnC showed weak, non-significant correlations with days to 50% flowering (DTF) ($r = 0.08$).

3.3. Linkage Map Construction and QTL Hotspots

The assembled linkage map spanned a total genetic length of 1,542.3 cM across the 12 chromosomes of rice, with an average inter-marker distance of 1.24 cM. Chromosome 1 presented the largest linkage group (165.4 cM), while Chromosome 10 was the smallest (92.1 cM).

Using Composite Interval Mapping (CIM), a total of nine QTLs governing GZnC were discovered on chromosomes 1, 3, 5, 7, 8, and 11. These results are compiled in Table 3.

Table 3: Properties of quantitative trait loci (QTLs) detected for grain zinc concentration (GZnC), grain yield (GY), and days to 50% flowering (DTF) in the RIL population.

QTL Name	Chr.	Flanking Markers	Position (cM)	Peak LOD (E1 / E2)	PVE (%) (E1 / E2)	Additive Effect (E1 / E2)	Source Allele
qGZnC1.1	1	SNP_0124 – SNP_0158	114.2	4.2 / 4.8	16.5 / 18.4	2.45 / 2.85	Madukar
qGZnC3.1	3	SNP_0412 – SNP_0439	45.8	2.9 / 1.5	6.2 / --	1.10 / --	Madukar
qGZnC5.1	5	SNP_0781 – SNP_0798	88.1	-- / 3.0	-- / 7.1	-- / 1.34	IR64
qGZnC7.1	7	SNP_1102 – SNP_1145	64.5	3.9 / 4.1	12.8 / 14.2	1.95 / 2.15	Madukar
qGZnC8.1	8	SNP_1420 – SNP_1452	12.4	3.1 / 2.8	8.4 / 7.9	1.40 / 1.22	Madukar
qGZnC11.1	11	SNP_2104 – SNP_2130	95.2	3.4 / 2.2	9.1 / --	1.55 / --	IR64
qGY1.1	1	SNP_0188 – SNP_0210	142.6	3.8 / 3.5	11.2 / 10.5	-3.10 / -2.95	IR64
qGY3.1	3	SNP_0488 – SNP_0512	112.4	4.5 / 4.9	14.1 / 15.3	-3.85 / -4.10	IR64
qDTF6.1	6	SNP_0945 – SNP_0970	52.1	5.2 / 5.8	22.1 / 24.5	5.10 / 5.45	Madukar

Two highly stable major QTLs—*qGZnC1.1* and *qGZnC7.1*—were consistently identified in both individual environments and the combined environment analysis.

- ***qGZnC1.1*** peaked at 114.2 cM on chromosome 1, explaining up to 18.4% of the phenotypic variance in E2 with a high LOD score of 4.8. The positive additive effect indicates that the allele from Madukar increases grain zinc concentration by 2.85 mg/kg.
- ***qGZnC7.1*** mapped to chromosome 7 between markers SNP_1102 and SNP_1145, explaining 14.2% of the variance in E2. The favorable allele was also derived from Madukar.

The spatial distribution of these critical loci across the respective chromosomes is visualized below in Figure 2.

Importantly, the yield-associated QTLs *qGY1.1* (142.6 cM) and *qGY3.1* (112.4 cM) mapped to distinct chromosomal positions away from *qGZnC1.1* and *qGZnC7.1*. This physical separation suggests that the major zinc-dense alleles can be introgressed into IR64 without triggering negative yield linkages.

4. Discussion

4.1. Genetic Architecture and Locus Stability

Grain zinc biofortification in rice is governed by a delicate network of multiple small-to-large-effect genetic loci (Garcia-Oliveira *et al*., 2009) [12]. In the present study, the identification of nine QTLs dispersed across six chromosomes demonstrates this multi-genic control. However, the majority of breeding programs fail during line fixed trials due to the environmental instability of discovered QTLs caused by complex G \times E dynamics (Ghandilyan *et al*., 2006) [13]. The discovery of *qGZnC1.1* and *qGZnC7.1* as highly stable expressions across both wet and dry seasons suggests these loci are robust candidates for marker-assisted breeding (Zhang *et al*., 2014) [10] (Anuradha *et al*., 2012) [9].

The positive additive values associated with the Madukar alleles at *qGZnC1.1* and *qGZnC7.1* confirm that landraces retain valuable alleles lost during modern high-yield selection regimes (Huang *et al*., 2012) [14]. The lack of overlapping confidence intervals between major zinc loci and yield QTLs (qGY1.1, qGY3.1) provides a genetic baseline for simultaneous optimization of nutritional value and grain yield (Zhang *et al*., 2014) [10] (Anuradha *et al*., 2012) [9].

4.2. Candidate Gene Identification

To establish a functional link between statistical mapping and molecular mechanisms, the physical genomic boundaries of the two major stable QTLs were annotated (Table 4).

Table 4: High-priority candidate genes localized within the genomic intervals of major stable zinc QTLs.

QTL Region	Physical Interval (Mb)	Candidate Gene ID	Functional Annotation / Molecular Mechanism
qGZnC1.1	24.15 – 25.40	*Os01g0584400*	OsZIP1: Zinc transporter, controls cellular zinc loading and root-to-shoot axial transport.
		Os01g0622300	OsHMA9: Heavy metal ATPase, facilitates metal ion pumping across plasma membranes.
qGZnC7.1	18.62 – 19.85	*Os07g0502100*	OsNAS2: Nicotianamine synthase 2, catalyzes chelation for phloem-mediated zinc translocation.
		Os07g0535900	OsZIP3: Zinc-regulated transporter, responsible for vascular unloading into developing grain.

The *qGZnC1.1* interval spans approximately 1.25 Mb on chromosome 1. In silico analysis highlighted OsZIP1, a well-documented zinc-regulated transporter gene (Ramesh *et al*., 2003) [15]. ZIP family proteins are responsible for driving zinc ions across hydrophobic cell membranes into cytoplasm. Previous studies indicate that tissue-specific overexpression of OsZIP1 enhances root Zn uptake capability (Ishimaru *et al*., 2011) [16]. Another candidate within this block is OsHMA9, an uncharacterized heavy-metal transporting ATPase that likely functions in heavy-metal detoxification and cellular loading pathways.

The *qGZnC7.1* interval on chromosome 7 contains OsNAS2 (Nicotianamine Synthase 2). Nicotianamine is an essential organic chelator that binds to transition metal cations, preventing precipitation in alkaline or vascular conditions (Lee *et al*., 2009) [17]. The Zn-NA complex represents the structural configuration required for efficient phloem loading and subsequent translocation through the maternal vascular tissue into the aleurone and endosperm compartments (Johnson *et al*., 2011) [18]. Another gene in this interval, OsZIP3, functions in vascular unloading. Collectively, these candidate genes provide highly prioritized targets for functional validation via CRISPR/Cas9 gene editing or allele-specific expression monitoring.

5. Conclusions

This study identified two stable, major quantitative trait loci—*qGZnC1.1* and *qGZnC7.1*—that regulate grain zinc accumulation in polished rice without causing negative yield drag. The delineation of these loci to precise physical intervals containing functional candidate genes (*OsZIP1*, *OsNAS2*) links quantitative genetic variation to specific molecular transport

mechanisms. The SNP markers flanking these stable QTL intervals can be directly integrated into marker-assisted selection (MAS) pipelines, enabling forward breeding strategies to develop zinc-enriched, high-yielding elite rice cultivars that can help mitigate micronutrient malnutrition.

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