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Genome-wide Association Mapping of Quantitative Trait Loci (QTLs) for Contents of Eight Elements in Brown Rice (*Oryza sativa* L.)

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Supporting Information

ABSTRACT: An association mapping of quantitative trait loci (QTLs) regulating the concentrations of eight elements in brown rice (*Oryza sativa* L.) was performed using USDA mini-core subset cultivated in two different environments. In addition, correlation between the grain elemental concentrations was also studied. A total of 60 marker loci associated with 8 grain elemental concentrations were identified, and these loci were clustered into 37 genomic regions. Twenty new QTLs were found to be associated with important elements such as Zn, Fe, and P, along with others. Fe concentration was associated with the greatest number of markers in two environments. In addition, several important elemental/metal transporter genes were identified in a few mapped regions. Positive correlation was observed within all grain elemental concentrations. In summary, the results provide insight into the genetic basis of rice grain element accumulation and may help in the identification of genes associated with the accumulation of Zn, Fe, and other essential elements in rice.

KEYWORDS: *association mapping, USDA Mini-Core Rice Collection, grain element concentration, molecular markers*

INTRODUCTION

Mineral malnutrition is one of the most serious global challenges to humankind and is avoidable (Copenhagen Consensus 2004; http://www.copenhagenconsensus.com). It is estimated that >60% of the world's population are iron (Fe) deficient and that >30% are zinc (Zn) deficient.[1] This situation is attributed to crop production in areas with low mineral phytoavailability and/or consumption of (staple) crops with inherently low mineral concentrations.[1−3] The improvement of mineral content by plant breeding, also referred to as biofortification,[4] is advocated as an immediate strategy to address mineral malnutrition.[5,6] As one of the most important staple crops, rice provides >40% of the daily calories for the world's population[7] and is considered as a concentrated source of minerals and vitamins, for those dependent on a rice subsistence diet.[8] Despite the importance of essential minerals for human and plant health, and the well-developed use of rice as a plant model for genetic analysis, the genetic mechanisms controlling the accumulation of the various mineral elements in rice remain largely unknown.[8]

So far, linkage mapping based on biparental populations is widely used to identify rice metabolic traits. For example, QTLs of grain elemental concentrations are usually identified using experimental populations such as recombinant inbred lines (RILs) in rice. Although these mapping populations are powerful sources for detecting QTLs, because each allele is present in 50% of the recombinant lines, they have limited resolving power due to the limited number of recombination events that occur during their development.[9−12] Consequently, the identified QTLs often span relatively large genomic regions, making it very difficult to pinpoint the causal genes. Because traditional mapping population is generated from a cross between two parents, it potentially captures only two alternative alleles of any locus. This leads to very limited sampling of natural allelic diversity in a population and the low probability of detecting important minor alleles.[13]

Another effective approach for QTL analysis is genome-wide association (GWA) mapping based on experimental recombinant populations. This approach maps the QTLs, either among extant breeding lines with known pedigree relationships or in a diverse germplasm collection. GWA mapping has been successfully used for the identification of various QTLs in *Arabidopsis thaliana*[10,14,15] and maize.[16,17] It has also been used in rice for the unraveling of complex physiological traits.[18−20] Advances in genome-wide association studies (GWAS) with high-throughput genotyping techniques have made it possible to land close to the genes of interest for agronomically important traits in rice.[18] The development of the USDA Rice Mini-Core collection has provided a genetically diversified panel for mining genes of interest.[10,21] The present study was aimed to explore the power of GWAS in the identification of QTLs of grain mineral concentration traits in rice by using the USDA Mini-Core accessions.

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Table 1. Statistical Analysis of Grain Elemental Concentration Traits Generated at E1 and E2, Hangzhou, China, in 2012 among the USDA Rice Mini-Core Collection

trait	environment type	mean ± SE	minimum	maximum	heritability (%)	genotype[a] (G)	environment[a] (E)	genotype × environment[a] (G×E)
micronutrients (mg/kg)								
Zn	E1	32 ± 0.36	19	55	85.9			
	E2	26 ± 0.29	16	49	92.6	44.70**[b]	342.51**	39.71**
Fe	E1	17 ± 0.35	7	46	74.3	27.39**	362.52**	16.91**
	E2	11 ± 0.27	4	47	96.1			
Mn	E1	31 ± 0.44	13	59	84.6	24.25**	4.28**	14.41**
	E2	31 ± 0.46	14	56	86.8			
Cu	E1	6 ± 0.07	3	9	88.1			
	E2	3 ± 0.07	2	9	95.7	26.62**	2114.30**	17.97**
macronutrients (mg/kg)								
P	E1	3474 ± 27	2082	4862	72.3	28.39**	2.09**	21.95**
	E2	3296 ± 36	1775	5613	69.6			
Ca	E1	126 ± 1.5	18	274	63.6	22.77**	2007.60**	20.70**
	E2	129 ± 2.4	19	280	91.4			
K	E1	2919 ± 21	1744	3699	72.9	24.68**	5.55**	19.22**
	E2	2789 ± 29	1256	4361	61.2			
Mg	E1	1406 ± 12	848	2978	56.3	17.94**	0.96**	13.68**
	E2	1323 ± 17	674	2536	69.4			

[a]Depicts relevant effect (F value). [b]**, values are highly significant at Pr > F = <0.001.

MATERIALS AND METHODS

Plant Material. The USDA Rice Mini-Core Collection was introduced from the Genetic Stock *Orzya* collection (managed by the Dale Bumpers National Rice Research Center of the USDA ARS, Stuttgart, AR, USA), which consists of 211 *O. sativa*, 5 *O. glaberrima*, 2 *O. rufipogon*, and 1 *O. nivara* accession. In this study, 219 accessions were cultivated in two field locations: the Experimental Farm of Zhejiang University, Zijingang Campus in Hangzhou (environment 1, E1) and the Experimental Farm of Zhejiang Zhijiang Seed Co. in Yuhang (environment 2, E2) during the growing season of 2012. The samples were grown in triplicate in randomized complete block design in both locations. The two locations are not too distant from one another, but the soils in the two locations were quite different; the field in E1 was recently reclaimed and had low fertility made up of fluvo-aquic soils, whereas the field in E2 was a traditional rice-growing area with purplish clayey soils. Routine agronomic practice was adopted during the growth of rice plants. The wild relatives, *O. glaberrima*, *O. rufipogon*, and *O. nivara*, were excluded from association mapping analysis as they contain many rare alleles that may increase the risk of type I errors or spurious associations.[22] Of 219 accessions grown, only 175 flowered early enough to produce seeds. Those with late flowering could not set seeds and hence were excluded from our analysis (Table S1 in the Supporting Information).

Preparation, Extraction, and Measurement of Grain Mineral Elements. The harvested rice grains were dried, cleared, and dehusked in an electrical dehusker (model B-76, Huangyan, Zhejiang, China) and subsequently used for the detection of four microelements (Zn, Fe, Cu, and Mn) and four macroelements (P, Ca, K, and Mg), respectively.

The dehusked rice samples were ground to pass a 100-mesh screen with a cyclone grinder (model 3010-019, Fort Collins, CO, USA). About 0.15 g of powdered material was digested with 6 mL of nitric acid (HNO_3) and 0.2 mLof hydrogen peroxide (H_2O_2) using a microwave digestion system (Microwave 3000, Anton PAAR, Graz, Austria). The microwave digester was programmed to ramp in high temperature and pressure set at a final power of 1200 W for 20 min. The digested samples were adjusted to a volume of 25 mL with double-deionized water. Finally, multielement analysis on grain digests was carried out by inductively coupled plasma optical emission spectrometer or ICP-OES (Optima 8000DV, PerkinElmer, USA). To control the variation within samples, each sample was measured three times, and the mean of triplicate observations was used to represent the content of mineral elements in each sample. For error control among samples, all samples were measured in a set of 20 samples containing two blanks and two standard reference samples with known concentrations of mineral elements. Standard reference samples of rice grains were used to compensate for possible matrix interference variations during microwave digestion, whereas external standards were used to construct the standard curve for calibration of ICP.

Data Analysis. Correlation analysis was performed to characterize the relationships between the examined elements. The Pearson correlation coefficients between traits were calculated on the basis of the mean values over three replications using two significance levels (p < 0.001), via the Correlate Procedure in SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

The analysis of variance for eight essential elements was conducted by the MIXED Procedure in SAS version 9.2, where genotype was defined as a fixed factor and replication and interaction between replication and genotype were random factors. Broad-sense heritability was calculated as $H^2 = \sigma g^2/ (\sigma g^2 + \sigma e^2/n)$, where σg^2 is the genotypic variance, σe^2 is the environmental variance, and n is the number of replications.

Genotyping. A total of 155 molecular markers covering the entire rice genome, approximately one marker per 10 cM on average, were used for the genotyping of the 175 accessions in the USDA Mini-Core Collection. Among the markers, 149 SSRs were obtained from the Gramene database (http://www.gramene.org/) and 5 other SSRs, AP5652-1, AP5652-2, AL606682-1, con673, and LJSSR1, with the remaining marker an indel at the *Rc* locus, named Rid 12.[21] The genetic and physical positions of SSR markers were determined using the map of Gramene Annotated Nipponbare Sequence 2009 (http://www.gramene.org/) and Rice TOGO Browser (http://agri-trait.dna.affrc.go.jp/). The obtained information on these markers was thus used to align the SSR marker maps and is reported here on the basis of the markers' cM genetic locations.

Association Mapping. The association between molecular markers and grain elemental content was carried out by the General Linear Model (GLM) and Mixed Linear Model (MLM), implemented in Tassel version 3.0.167 software (http://www2.maizegenetics.net/). Aimed at GLM, the Q method was performed where population membership estimates served as covariates. In MLM analysis markers tested and subpopulation data (Q matrix) were considered as fixed-effect factors, whereas the kinship matrix was considered as a random-effect factor,[23] taking the gross level population structure (Q) and kinship (K) into account.[24,25] The P value (marker) determining

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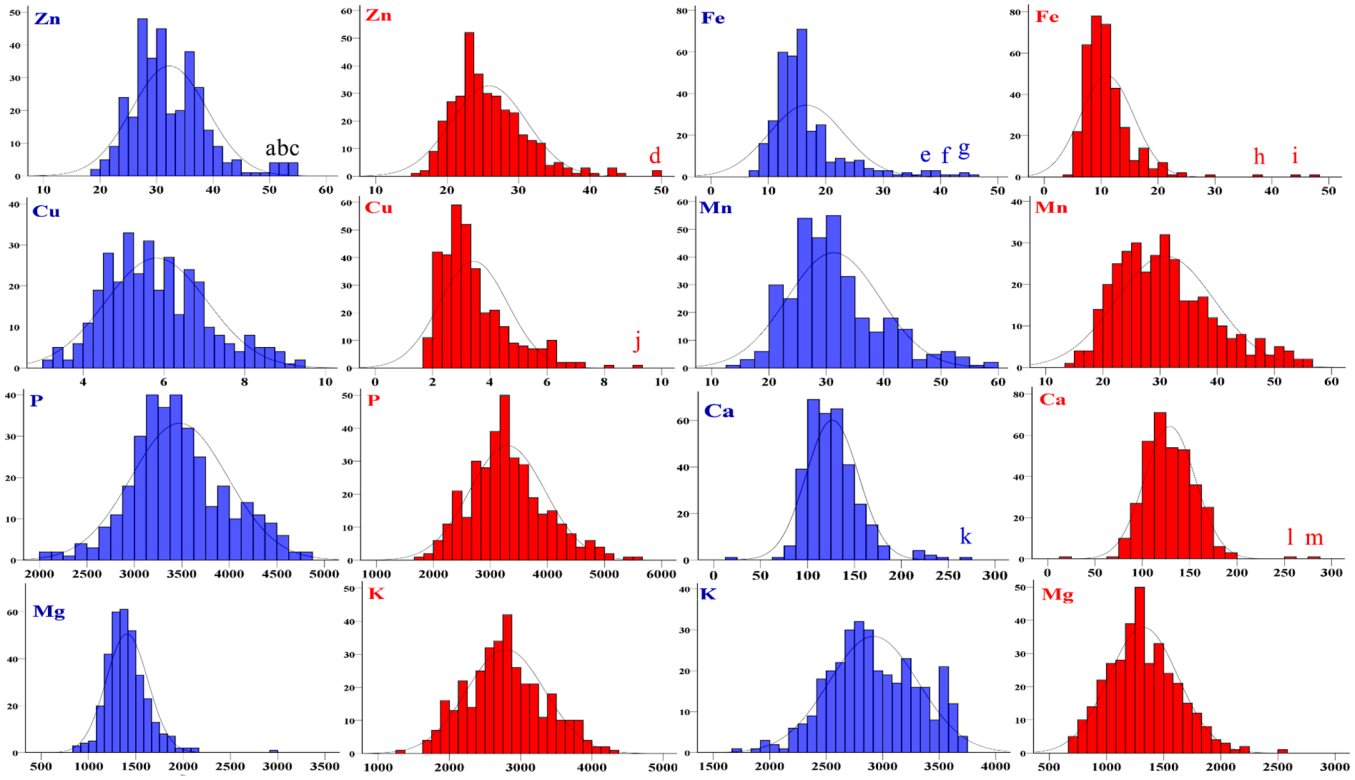

Figure 1. Histograms showing frequency distribution of micro- and macro-elements among USDA mini-core germplasm cultivated in E1 (shown in blue color) and E2 (shown in red), Hangzhou, in 2012. The values are shown in mg/kg for micro- and macro-elements. The *y*-axis denotes the value of frequency, whereas the *x*-axis shows resultant groups of accessions. *a*–*z* are the out groups, where *a* = 311180, *b* = 310724, *c* = 311206, *d* = 311689, *e* = 310598, *f* = 310131, *g* = 310998, *h* = 311635, *i* = 311643, *j* = 311269, *k* = 311236, *l* = 311689, *m* = 311140, *n* = 310102, and *o* = 311677 are the accessions of these outgroups as given in Supporting Information Table S1.

Table 2. Pearson Correlation for Micro- and Macroelements among the USDA Rice Mini-Core Collection, Evaluated in Replicated Tests at E1 (Upper Right) and E2 (Lower Left) in Hangzhou, China, in 2012

	Zn	Fe	Mn	Cu	P	Ca	K	Mg
Zn		**0.503**** [a]	**0.40****	**0.398****	**0.427****	**0.364****	**0.463****	**0.424****
Fe	0.459**		**0.36****	**0.255****	**0.353****	**0.326****	**0.456****	**0.409****
Mn	0.456**	0.275**		**0.436****	**0.528****	**0.26****	**0.428****	**0.394****
Cu	0.525**	0.363**	0.46**		**0.50****	**0.489****	**0.439****	**0.449****
P	0.637**	0.609**	0.452**	0.460**		**0.353****	**0.728****	**0.80****
Ca	0.452**	0.459**	0.238**	0.536**	0.567**		**0.430****	**0.324****
K	0.59**	0.532**	0.427**	0.563**	0.882**	0.64**		**0.659****
Mg	0.572**	0.60**	0.379**	0.423**	0.963**	0.564**	0.825**	

[a]**, correlation is significant at the 0.01 level (two-tailed).

whether the SSR marker is associated with the trait and the R^2 (marker) indicating the fraction of the total variation explained by the marker were recorded. Markers with the adjusted *P* value >0.001 were regarded as significant. Genetic maps were constructed using MapDraw V2.1.[26]

RESULTS

Genotypic and Environmental Effects on Grain Elemental Concentration. The mean values, ranges, heritability and related data of seed mineral concentrations are presented in Table 1. Analysis of variance (ANOVA) demonstrated significant ($p < 0.05$) genotypic and environmental variations for all the elements. Nearly all fell into similar range of concentrations in both environments.

The effects of environment, genotype, and interaction of genotype × environment on concentrations of micro- and macroelements were all highly significant ($p < 0.00001$). The maximum genotypic and genotype × environment effect was observed for Zn and Mg. The greatest effect of environment was detected for Cu (2114.30) and the lowest for Mg (0.96) (Table 1).

The average concentrations of both micro- and macro-elements in grains of E1 were significantly higher than in grains of E2, except Ca and Mn, which were similar in both environments. P was found to be the most abundant grain element, with concentrations of 3474 and 3296 mg/kg in E1 and E2, respectively, followed by K, Mg, and Ca (Table 1). Among the micronutrients, Mn was the most abundant, with concentrations of 31 mg/kg in both E1 and E2, followed by Zn, Fe, and Cu (Table 1).

Frequency distributions of accessions' phenotype from both locations showed clear differences due to the strong environmental effect on the concentrations of micro- and macroele-

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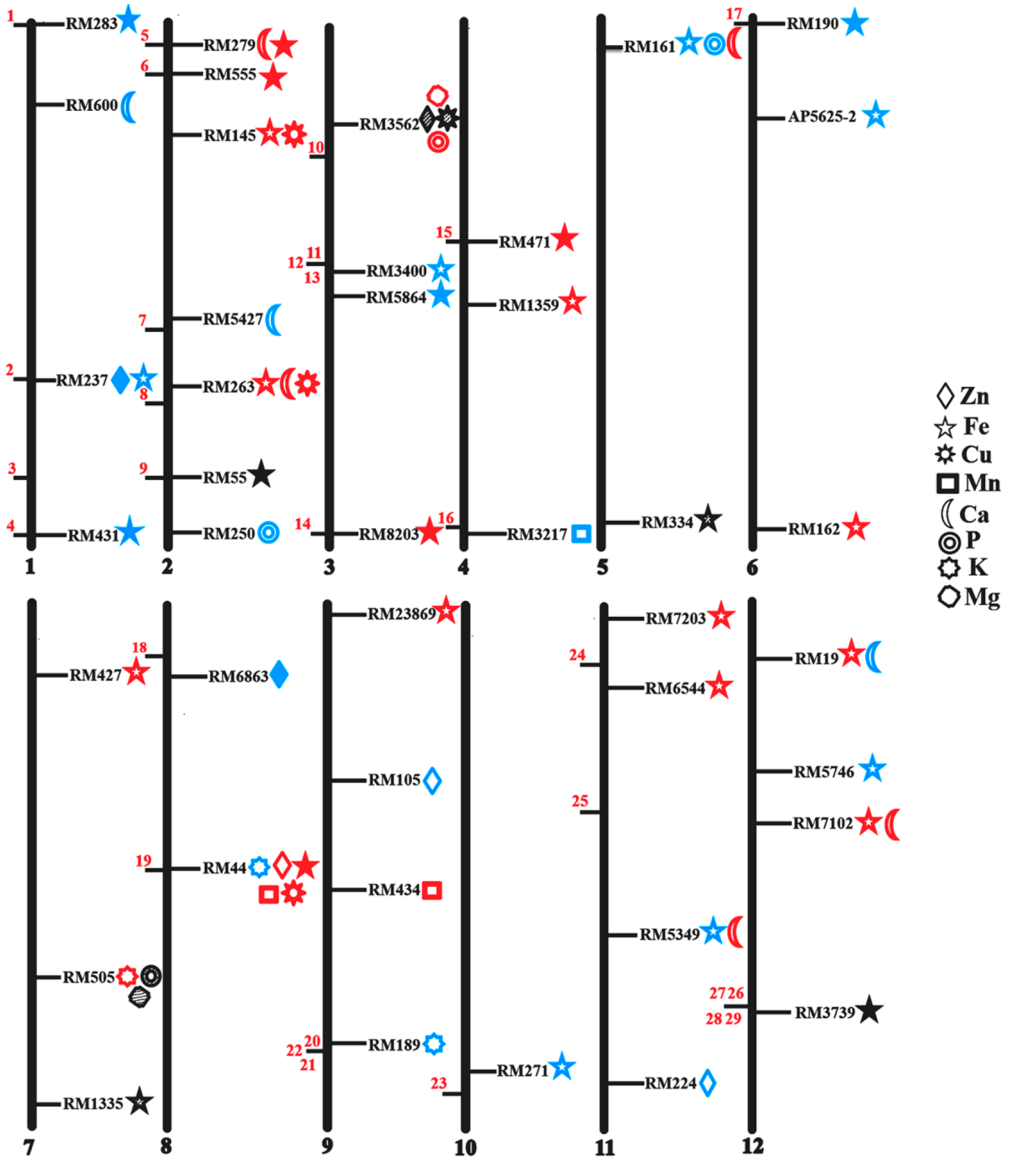

Figure 2. Sketch genetic maps of SSR markers associated with grain (brown rice) Zn, Fe, Cu, Mn, Ca, P, K, and Mg concentrations in different chromosomes. Chromosome numbers are indicated below; horizontal bars represent loci with associated marker name labeled to the right. The location information on these markers was downloaded from Gramene (http://gramene.org/) and Rice Togo Browser (http://agri-trait.dna.affrc.go.jp/). The genetic maps were drawn by Map Draw v 2.1xls. Every element is represented by a specific symbol; solid fill represents association similar to a reference at the same location; striated for consistency of marker across two locations; blue color indicates marker association with data from E1 (Zijingang), red for E2 (Yuhang), and black for both locations. The numbers on the left of each chromosome show the co-located reference from the literature: 1, 3, 14 = Norton et al.;[38] 2 = Stangoulis et al.;[32] 4, 8, 15, 16, 28 = Ishikawa et al.;[30] 5, 6, 10, 11, 12, 18, 18, 20, 21, 22, 23, 25, 26, 27 = Gracia-Oliveira et al.;[37] 7, 13, 24 = Lu et al.;[45] 9, 17, 19, 29 = Zhang et al.[8]].

ments (Figure 1). This variation existed among individual elements in the same environment as well as the same element in different environments. Among the micro and macroelements, highest variation was observed in Cu and K, respectively, in E1. The lowest variation was shown by Fe in both environments, whereas Mg showed greater variation in E2.

Correlations of Grain Elemental Concentrations and Heritability. Correlation analyses revealed a strong positive correlation between the concentrations of macro- and microelements. Overall greater correlations were found among macroelements than among microelements, where stronger correlations were present among the macroelements than among microelements. Element P strongly correlated with other macro- and microelements. The strongest correlation was exhibited among P and Mg, followed by P−K and P−Ca, across E1 and E2. Similarly, Mg and K were strongly correlated from E1 and E2, respectively. Among microelements, P strongly correlated with Fe and Zn (Table 2).

Microelements showed strong to medium correlations that were more or less consistent in the two locations. It was observed that microelements were more strongly correlated with macroelements, as compared to the microelements. The correlation among Zn and Fe was strongly positive in E1 and E2, respectively, followed by Mn and Cu. Among macroelements, Zn showed higher correlations following the descending order K < Mg < Ca.

The proportion of phenotypic variance explained by genotype is also known as broad sense heritability (H^2). The

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heritability of each of the eight elements in the two environments was >60%, indicating that these traits were suitable for association mapping (Table 1). Generally high heritability was observed for Fe followed by Cu, Zn, and Ca, which had H^2 > 90% from E2. Microelements exhibited high levels of heritability as well, whereas medium to high levels of heritability were observed for macroelements. Among the macroelements, high heritability was observed for K, followed by P, Ca, and Mg from E1. Although the heritability of Mg was <60% from E1, it was considered for association mapping as it exhibited 69.39% heritability from E2. Similar variation was observed in values of H^2 among microelements across two environments (Table 1).

QTLs/Markers Associated with Grain Elemental Concentrations. A total of 60 QTLs, that is, 30 each in E1 and E2, were identified to have significant association with grain elemental concentrations. Among the eight elements, Fe concentration was associated with the highest number of markers (16/E1 in 13/E2), followed by Zn (5/E1, 2/E2) and Ca (3/E1, 4/E2). Mg was associated only with one marker in each environment. In E1, 16 of the 30 markers were associated with grain Fe, 5 with Zn, 3 with Ca, 2 each with K and P, and 1 each with Cu, Mg, and Mn. Likewise, 13 of the 30 markers in E2 were associated with grain Fe, 5 with Cu, 4 with Ca, 2 each with Zn, Mn, and P, and 1 each with K and Mg (Figure 2).

The 60 element marker associations were often found linked or grouped into multielement clusters, where this clustering was especially common in E2. Three such synchronous associations were detected in E1, for example, RM 237 co-associated with Fe and Zn and RM3562 with Zn and Cu, whereas RM161 co-associated with Fe and P. Similarly, 5 co-associations were identified in E2, for example, RM3562 with Zn, Cu, P, and Mg; RM44 with Zn, Cu, and Mn; RM263 with Fe, Cu, and Ca; RM279 with Fe and Ca, Cu; RM7102 with Fe and Ca; and RM145 with Fe and Cu, respectively (Figure 1 and Figure S2 in the Supporting Information). Markers RM3562, RM3739, RM55, RM1335, RM334, and RM505 were consistently true among both locations. For example, RM3562 associated with Zn and Cu, RM505 with Mg, and the rest with Fe. In addition, the same marker also associated with different traits across two locations. RM44 proved to be a unique marker associating with K (E1), Zn, Fe, Cu, and Mn (E2) (Tables S2 and S3 in the Supporting Information).

DISCUSSION

In this study, we identified 60 QTLs/marker loci associated with rice grain elemental concentrations by using GWA mapping approaches. We also identified co-located markers for different elemental concentrations. Moreover, adequate genetic variations in concentrations of zinc, iron, and other essential elements were also observed among USDA Mini-Core accessions. The USDA Mini-Core Rice Collection has a unique genetic diversity and has been characterized for identification of genes and QTLs for a number of traits. These traits include grain yield, harvest index, sheath blight resistance, hull silica concentration, and protein concentration.[10,27,28] About 20 new marker-grain elemental concentration associations are observed in the present study. These new associations are concerned with important grain elements such as Zn, Fe, and P along with Mn, Cu, Ca, and K. Zn, Fe, and P are considered important as their deficiency is causing hazards for the human race.

A wide range of variation was observed in contents of micro and macroelements of rice grains. These results are in accordance with the values reported by other researchers in different rice populations.[8,29−32] We found that a few accessions performed consistently across the two environments, indicating that these rice varieties can be grown and developed for biofortification programs from a grain elemental concentration perspective, especially zinc and iron. The genotypes with high grain Zn content can be a genetic source for breeding high-Zn varieties (data not shown).

Correlation analysis of different characteristics is considered very useful in exploring interrelationships.[33] In our case, numerous significant correlations were found between the different grain nutrient concentrations. Particularly, close associations existed among all four macronutrients and micronutrients or with each other. These correlations may be due to the impact of a single gene on multiple grain elemental concentrations or co-association of physically close located genes. Our observations are in agreement with previous studies.[8,31−33] The pattern of the frequency distribution in the USDA Mini-Core Collection revealed a continuous variation in all elements analyzed, suggesting that elemental uptake and subsequent accumulation in rice may be controlled by multiple genes.

It is reported that rice accessions derived from different geographic regions react to environmental signals differently.[34] Our data showed that grain concentration traits for all elements were significantly affected by environment and environment × genotype interaction, suggesting genotypic sensitivities to differences in environmental conditions in the two locations. The USDA Mini-Core represents a wide geographical and ecological diversity of rice. Therefore, the prevailing genotype × environment interaction for the accumulation of elements may be the reason for having different association results across the two locations.[35] Further elucidation of genotype × environment interaction for grain elements and related traits is required.

To comprehend the underlying genetics for the accumulation of grain elemental concentration, many researchers used different populations of rice as experimental material such as double haploids, introgression lines, recombinant inbred lines (RILs), and backcross introgression lines (BILs). RILs and BILs maintain more or less different environmental conditions.[8,19,30,32,34,36,37] The lines derived from a biparent cross shared a relatively simple genetic background. The present study is the first report of its kind about GWA mapping of grain elemental concentration in rice. Table S4 in the Supporting Information shows a comparison of a few previous works. Some of the regions associating with markers identified in this study are confirmatory, as they have been previously identified as regions controlling elemental concentration in other rice mapping populations (Table S5 in the Supporting Information).[34,38] For example, in our study zinc was associated with RM237, RM224, RM105, RM6863, RM3562, and RM44 across the two locations. Previously, a number of QTLs/SSR markers linked with zinc were reported around the same regions.[32,37]

Micro- and macroelements were positively correlated, which is reflected in the co-location of associated makers in the current study. The co-locations of markers associated with grain elemental concentration suggest the importance of these regions for mineral accumulation in rice grain and may be due to physiological coupling of the accumulation of certain minerals or tight linkage of different genes.[37] Co-locations of QTLs/markers linked with grain concentration of Zn and Fe have been described by earlier workers.[8,32,37−39] RM505 co-

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Table 3. Co-location of Molecular Markers Associated with Grain Element Concentration and Genes for Metal Transport

						physical distance (Mbp)		genetic distance (cM)	
no.	marker	element	chr	gene and locus	gene function	marker	gene	marker	gene
1	RM237	Zn	1	vacuolar ATP synthase subunit E, vacuolar proton pump E subunit Os01g0659200	proton-transport, ATP hydrolysis coupled proton transport	28.57	28.57	113	113
2	RM431	Fe	1	*OSZIP1* Os01g0972200	metal ion transport, iron, zinc ion transport	40.08−40.21	44.8	149.1−150.7	180
3	RM145, RM555, and RM279	Fe	2	1. *OsHMA4* Os02g0196600 2. aspartate aminotransferase	metal transporter, metal efflux, transferase activity	7.74/4.04/2.67	5.26/7.74	36.8/18.4/12.8	25.8/36.8
4	RM279 and RM555	Ca, Fe	2	*OsACA2* Os02g0176700	Ca transport, cation transport	2.67/4.04	3.83	12.8/18.4	17.6
5	RM3562	Cu, Mg, P, Zn	3	1. *OsNAS1* Os03g0307300 2. *OsNAS2* Os03g0307200 3. *OsDMAS1* Os03g0237100 4. similar to 14 kDa zinc-binding protein (protein kinase C inhibitor) Os03g0322500 5. cytidine and deoxycytidylate deaminase zinc-binding region family protein Os03g0321900	Zn, iron, and other metal transport, zinc binding	11.58	11.29−11.58/ 11.29−11.58/ 6.71−6.78/ 11.29−11.58/ 11.29−11.58	50.8	49.3−50.8/ 49.3−50.8/ 31−31.3/ 49.3−50.8/ 49.3−50.8/
6	MRG4864/ RM5864 and RM3400	Fe	3	*OsZIP2* Os03g0411800	metal ion transporter, zinc ion transport, transmembrane transport	23.10− 23.14/ 17.6	17.59	88.7/83.3	83.3
7	RM8203	Fe	3	*OsCNGC14* Os03g0758300	Ca transport	32.36	32.36	140.1	140.1
8	RM3217	Mn, Fe	4	*OsZIP3* Os04g0613000	metal ion transport	30.72	31.2	100.7	102.7
9	RM161	Fe	5	1. *OsZIP5* Os05g0472700 2. *OsZIP9* Os05g0472400	metal ion transport, zinc ion transport/cation transport, zinc ion transport, transmembrane transport	20.88	22.97	80.7	95.8
10	RM162	Fe	6	1. *OsHMA2* Os06g0700700 2. ABC transporter domain containing protein Os06g0607700	metal ion transport, metal ion binding, ATP binding	24.92	30.28/24.9	87.5	115.9/88.5
11	RM1335	Fe	7	1. *OsNAS3* Os07g0689600 2. peroxidase Os07g0677300 3. potassium transporter 7 *OsHAK7* Os07g0669700 4. heavy metal transporter Os07g0671400	metal ion transport, metal ion binding, K ion transporter,	29.34−29.39	30.25/29.67/ 29.34−29.39/ 29.34−29.39	115.5− 115.8	118.6/116.6/ 115.5−115.8/ 115.5−115.8
12	RM6863 and RM44	Zn	8	*OsZIP4* Os08g0207500 lies between rm6863 and rm44	zinc ion transport, metal ion transport	2.06/11.67	6−7.4	16.4/54	44.6−45.6

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Table 3. continued

no.	marker	element	chr	gene and locus	gene function	physical distance (Mbp)		genetic distance (cM)	
						marker	gene	marker	gene
13	RM105	Zn	9	outer mitochondrial membrane protein porin -boltage-dependent anion- selective channel protein Os09g0361400	anion transport, regulation of anion transport, zinc ion transmembrane transporter activity	12.41	12.41	36	36
14	RM189	K	9	*OsCNGC9*, Os09g0558300	conducting Ca^{2+} through the plant cell plasma membrane	23.12	23	91.8	91.5
15	RM271	Fe	10	Os10g0456800 CHY zinc finger family protein	zinc ion binding, metal ion binding	17.04	17.04	41.9	41.9
16	RM19	Ca, Fe	12	*OsACA9* Os12g0136900	Ca transport	2.25	1.61−1.87	12.2	10.3−11.5

associated with K, P, and Mg at chromosome 7, making a cluster of all macroelements except Ca, whereas all microelements shared the same loci at chromosome 8 with the exception of K. Zhang et al.[29] reported two loci associated with elements, P, Mg, and K, one each on chromosomes 5 and 7. This co-location of markers is also in congruence with correlation existing among respective elements. The co-locations of multiple traits for element concentration at different loci may be due to underlying genes involved in maintaining elemental/metal homeostasis. We can infer that the co-locations observed in the current study may have resulted from close linkage of genes.

The results of various earlier and recent studies point toward the existence and connection of a complex coordinated system involving many genes associated with the regulation, uptake, sequestration, distribution, mobilization, and localization of minerals in rice.[8,34] We found 16 genetic regions where genes responsible for metal ion binding and transport lie at the same locus or close to the molecular markers associated with element grain concentration (Table 3 and Figure S2 in the Supporting Information). This can be a very important finding for unraveling the mechanism of acquisition and transport of metal/element in rice seeds. Of these, RM3562, which co-associated with Cu, Mg, P, and Zn, has seven genes around this region, including *OsNAS1*, *OsNAS2*, *OsDMAS1*, 14 kDa zinc-binding protein, and cytidine and deoxycytidylate deaminase zinc-binding region family protein. Members of the NAS family have been shown to be involved in enhancing Fe and Zn in rice endosperm.[40,41] Likewise, the role of *OsDMAS1* in iron acquisition was demonstrated by Bashir et al.[42] Five ZIP transporters were also located in the regions associated with Fe and Zn. This information can be useful for future analysis of these genetic regions, linked genes, and traits. We analyzed the *OsZIP4* (Os08g0207500) gene lying in the region of RM6863 and RM44 and found many polymorphic regions (data under process). Future elucidation may result in some novel findings relating to these transporters. The current study presents a novel method of exploiting data and information provided by bioinformatics for exploring genes/factors associated with certain phenotypes.

Another important feature of the current study is the consistency of 6/60 markers across the two environmental locations. This reflects a large environmental effect on the traits under study. Zhao et al.[20] and Norton et al.[35] have previously reported a similar environmental influence on flowering time and grain elemental contents. It has also been demonstrated that the environment greatly affects the accumulation of elements in rice grains.[8,13,29,35,38,43,44]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01191.

Tables S1−S5 and Figures S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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