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Rice

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A Genome-wide Association Study of the Grain Ionome in Rice *Oryza Sativa* Ssp. *Japonica* under Two Diverse Water Management Systems

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Abstract
Rice is an important human staple food providing calories and useful elements, even though vulnerable to heavy metal contamination. Breeding tools for improving the concentration of nutrient and reduce levels of toxic compounds can improve the nutritional value and safety of rice grains. This work presents a comprehensive analysis of the genetic bases controlling variation in the rice ionome employing genome-wide association studies (GWAS) with a diversity panel of 294 temperate and tropical *japonica* accessions, each genotyped with 36,830 SNP loci. GWAS was performed for brown rice content of 13 elements: As, Ca, Cd, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Zn for rice plants grown under two diverse water management regimes, permanent flooding and limited watering. GWAS identified 232 significant marker-traits associations (MTAs); 87 of which had high R^2 and low p-values and were selected for further analysis. Among them, 32 MTAs were consistently identified under both environments. These can represent valuable candidates for marker-assisted selection to improve the composition of essential mineral nutrients and reduce the concentration of toxic elements in the rice grain. Furthermore, co-localization regions for 60 MTAs were highlighted for two or more traits. Potential candidate genes were identified for 14 MTAs with $-\log_{10}(p)$ value < 5 and $R^2 > 6$; among them, gene functions that were related to transport/uptake, accumulation, detoxification, metal binding and root architecture, coherent with the traits of interest, were highlighted. The study provides relevant insights into the genetic basis of ionomic variations in rice and may serve as an important foundation for improvement in breeding, as well as for further studies on the genetic bases and molecular mechanisms controlling the rice grain ionome.

Keywords Rice, Ionomic traits, Water management, GWAS

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Background

Rice (*Oryza sativa* L.) is a major staple food for more than half of the world's population (Parengam et al. 2010) and is considered a concentrated source of minerals and vitamins. However, in the form that most consumers eat (white, polished rice), it contains low levels of micronutrients (Bouis and Welch 2010), thus contributing to malnutrition and hidden hunger, especially for those populations that depend on a rice subsistence diet (White and Broadley 2009; Antony et al. 2022). Indeed, it has been estimated that up to two billion people worldwide suffer from iron (Fe) and zinc (Zn) deficiencies (Zimmermann and Hurrell 2007; Smith and Myers 2018). In recent years, the nutrition and public health community has used various interventions for these deficiencies, including supplementation, fortification, and biofortification (Bouis and Welch 2010; Descalsota-Empleo et al. 2019; Tiozon et al. 2021). On the other hand, plants also take up from the soil nonessential and toxic elements, which may cause phytotoxicity or enter the food chain, posing a risk to human health (Clemens et al. 2002; Williams and Salt 2009). For this reason, several countries have reduced the maximum permitted content of some harmful elements in rice. For example, the European Community has brought the maximum content in rice for arsenic (As), cadmium (Cd), and nickel (Ni) to 0.15, 0.15, and 1.5 mg kg^{-1}, respectively, (EU Regulation 2023/915). Hence, revealing the genetic diversity for accumulation of essential or toxic micronutrients in brown rice is important to promote brown grain-based health benefits and prevent adverse effects from undesirable toxic elements.

The composition in terms of mineral and trace elements is defined as the plant ionome (Lahner et al. 2003; Salt et al. 2008), and a pivotal task in plant ionomic research is to unravel the genetic basis underlying the variations of the element concentration across the different accessions and cultivars of a specific crop. The plant ionome is a complex trait since it is affected by genetic, environmental (including water management, soil properties, and conditions that affect mineral supply and availability), and developmental factors, as well as their interaction (Pinson et al. 2015; Huang and Salt 2016). The strong influence of environment on this trait may mask the genetic variation or make quantitative trait loci (QTLs) unreproducible. Many genetic loci responsible for uptake, translocation, and storage of mineral elements in plants have been identified, and many QTLs for mineral concentrations have been reported in rice (Lu et al. 2008; Garcia-Oliveira et al. 2009; Norton et al. 2010, 2012; Cu et al. 2021; Liu et al. 2021; Pasion et al. 2023; Tanaka et al. 2025) supporting the importance of exploring the genetic variability present in the rice germplasm available worldwide (Pinson et al. 2015; Tiozon et al. 2024). To date, only a small number of these QTLs have been finely mapped, leading to the identification of the causal genes involved in the traits of interest. Among them, the transporter genes *Os-HKT1;5* (or *SKC1*) for sodium (Na), *Os-HMA3* for Cd, *NRT1.1B* for nitrogen (N), *Os-HMA4* for copper (Cu) were identified (Ren et al. 2005; Ueno et al. 2010; Miyadate et al. 2011; Hu et al. 2015; Huang et al. 2016). In addition, the gene *OsMOT1* controlling molybdenum (Mo) concentration in both straw and grain was identified (Wang et al. 2020a, b). However, the gene networks controlling mineral accumulation and homeostasis are complex and largely remain to be elucidated.

Genome-wide association study (GWAS) is a powerful tool for identifying QTLs in complex traits in rice (Islam et al. 2020; Wang et al. 2020a). Several studies that deployed GWAS for micronutrient variation in rice showed polygenic inheritance (Bollinedi et al. 2020; Descalsota-Empleo et al. 2019; Norton et al. 2010, 2012, 2014; Tan et al. 2020; Yang et al. 2018). These results agree with an extensive heritable variation observed for rice grain ionome in 1,763 accessions of diverse geographic and genetic origins (Pinson et al. 2015). In particular, GWAS was applied for the concentrations of four minerals (As, Cu, Mo, and Zn) in rice grain (Norton et al. 2014), as well as eight elements (Zn, Fe, manganese [Mn], Cu, phosphorus [P], calcium [Ca], potassium [K], and magnesium [Mg]) in brown rice (Nawaz et al. 2015). More recently, an extensive GWAS analysis for 17 elements was conducted on a diversity panel of 529 accessions, mainly represented by ssp. *indica*, grown under diverse environments and fertilization, allowing the identification of shared or environment-specific marker-trait associations (MTAs) and candidate genes located in 42 associated loci (Yang et al. 2018). A further study evaluated a germplasm panel of *indica* rice for 12 elements in two locations for two years and identified 128 loci associated with the concentration of grain elements, 14 of which resulted stable across growing environments, then representing suitable candidates for marker-assisted selection (Cu et al. 2021). Another panel of 318 re-sequenced *indica* accessions was subjected to GWAS for 12 micronutrients, allowing a detailed identification of candidate genes whose putative contribution and interaction were validated through transcriptome analysis and co-expression network analysis (Pasion et al. 2023). Even though changes in water management are expected to affect the grain ionome of rice by their effects on minerals solubility and soil bioavailability, only few investigations are currently providing information about this topic. In one of these studies, the accumulation of 16 grain elements in response to two diverse watering systems (flooded and irrigated-but-unflooded) was investigated using a biparental mapping population represented by Teqing × Lemont introgression lines, therefore representing *indica* genetic material. The study highlighted differences in QTLs between the two water management systems, and a higher number of QTLs and element-to-element correlations were detected under flooded conditions than under the alternative watering approach (Zhang et al. 2014). Finally, a total of 191 *indica*, *japonica*, and Aus accessions from the USDA Rice mini-core collection were used for GWAS for 16 ionomic traits under flooded and unflooded conditions, and a very scarce overlapping was observed for the MTAs detected in the different watering conditions (Liu et al. 2020).

Here, we provide a contribution to narrow the knowledge gap about ionome composition under various water regimes, as we describe a comprehensive GWA analysis of the rice ionome using 294 diverse rice accessions mostly belonging to *O. sativa* ssp. *japonica*, each genotyped for 36,830 SNP loci. The rice accessions were grown for two years in a typical rice growing district in Italy under two different water management conditions: permanent flooding (PF) and limited water (LW). Using high-throughput inductively coupled plasma mass spectrometry (ICP-MS), brown rice grains were analyzed for 13 mineral elements (As, Ca, Cd, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Zn). GWAS was carried out separately for the two watering conditions to identify significant and stably expressed MTAs. Investigations allowed the identification of specific loci involved in the accumulation of multiple elements in LW and PF. Subsequently, putative candidate genes located in these regions were identified. We also detected potential favourable alleles, which could be used in rice biofortification programs or in developing genotypes able to limit the accumulation of undesirable elements in the grain.

Materials and Methods

GWAS Panel and Experimental Design

A previously described panel of 294 *Oryza sativa* varieties was used for this experiment (Volante et al. 2017a, 2020; Supplementary Table S1). The panel was composed of 73 tropical *japonica*, 211 temperate *japonica*, 7 *indica* accessions, 2 aromatic and 1 Aus. Detailed information regarding the accessions is reported in Supplementary Table S1 in Volante et al. (2017a). The accessions were grown in paddy fields in Vercelli (Piedmont, Italy), coordinates 45°19’204” N, 8°22’25,35” E (WGS84). According to the USDA classification, the soil in this area is classified as a sub-acid loam-type soil. Its chemical-physical characteristics and the content in mineral micronutrients and trace elements were reported in Orasen et al. (2019) and reported in Supplementary Table S2.

Accessions were grown during two growing seasons under two different soil water management systems: forced Limited Water (LW) and Permanent Flooding (PF). The experimental design involved a completely randomized block design with three replicates per watering condition. Each plot consisted of three rows of about 60 plants for about 180 plants per plot (170 cm-long rows at a distance of 10 cm). The fertilization was performed spreading and burying 23-0-10 NPK (0.1 t ha^{-1}) at pre-sowing in pre-seed; no further N input was applied during the panicle differentiation phase (Volante et al. 2017a). The sowing was performed in dry conditions for both water treatments. In the case of PF, the field was flooded (10 cm water) when most of the cultivars reached the three-leaves stage (typically after 30 days), and it was kept in this condition throughout the whole growing season until 30 days before harvesting. In the case of LW, water was applied when ψ_{H2O} values were lower than – 30 kPa at -20 cm depth up to soil water potential (ψ_{H2O}) rise to zero (Volante et al. 2017a).

Analysis of Brown Grain Ionome

The ionome of the brown grains was defined by measuring the concentrations of 13 elements: As, Ca, Cd, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Zn. To do this, samples of 300 mg (2 samples for each biological replicate) of flour, obtained from completely milled brown grains dried to 13% humidity, were treated in Teflon tubes with 10 mL of 65% HNO_3 and then mineralized by a microwave digestion system (Anton Paar MULTIWAVE-ECO) applying a 10-min one-step temperature ramp (20–210 °C) and maintaining them at 210 °C for more than 10 min. After 20 min cooling, aliquots of the mineralized samples were diluted using MILLI-Q water (1: 40) in polypropylene test tubes. The concentration of the 13 elements in the diluted samples was determined using an Inductively Coupled Plasma Mass Spectrometer (BRUKER Aurora-M90 ICP-MS). An aliquot of 2 mg L^{-1} of an internal standard solution (^{72}Ge, 8^{9}Y, ^{159}Tb) was added to the test samples and the calibration curve samples to give a final concentration of 20 µg L^{-1}. Typical polyatomic analysis interferences were removed using CRI (Collision-Reaction-Interface) with an H_2 flow of 70 mL min^{-1} flown through a skimmer cone. Accuracy was evaluated using a certified reference material (rice flour- NIST SRM 1568a; National Institute of Standards and Technology - NIST, Gaithesburg, MD).

Statistical Analysis of Phenotypic Data

Frequency distributions of phenotypic data were tested for normality using the Shapiro–Wilk function; additionally, kurtosis and skewness were calculated using the R package dlookr. Analysis of variance (ANOVA) was performed using the “lm” function in the R environment to assess the significance of genotypes (G), year (E), and genotype × year interaction (G×E) within each environment (PF and LW). Broad sense heritability (H^2) was calculated from ANOVA as:

$$H^2 = s^2G / [s^2G + (s^2GE/E) + (s^2e/rE)]$$

where σ^2G is the genetic variance, σ^2GE is the genotype × year interaction variance, σ^2e is the residual variance, E is the number of years, and r is the number of replicates. A one-factor ANOVA was also performed to verify the effect of the water management on grain element concentration, as reported in Table 1.

Finally, to find out possible correlations among the concentration of elements, for each watering treatment Pearson coefficients of average concentration of the three biological replicates in the two years were calculated for each element, using the standard "cor" function in R corrplot package; the significance of correlations was assessed with the *t*-test implemented in the "cor.mtest" function.

Genotyping of the GWAS Panel and LD Analysis

The 294 accessions of the rice panel subjected to ionomic analyses were subjected to genotyping-by-sequencing (GBS) following a pipeline described by Biscarini et al. (2016), except for the number of tags required for the alignment to the Nipponbare reference sequence (1 instead of 5). A set of 246,084 SNPs was identified and mapped on the Os-Nipponbare-Reference-IRGSP-1.0 genome pseudomolecule sequence assembly (Kawahara et al. 2013). Markers with a call rate value lower than 90% and minimum allele frequency (MAF) lower than 5% were discarded. After filtering for call rate and MAF, a total number of 36,830 SNPs was subsequently used for GWAS. The population structure was preliminarily defined as in Volante et al. (2017a, b, 2020), with the same parameters. Briefly, results were obtained from Principal Component Analysis conducted using Tassel v5.2.41 (Bradbury et al. 2007), Jukes-Cantor phylogenetic clustering with MEGA7 (Kumar et al. 2016) and a Bayesian model-based analysis performed with Structure, v2.3.4 (Pritchard et al. 2000) on a subset of 9996 markers (i.e., a random selection of 833 SNPs per chromosome).

Association Mapping

For GWAS, the average of the three biological replicates for each element concentration of the two years was calculated for each watering treatment. A total number of 36,830 SNPs obtained after filtering was used for the analysis. GWAS was performed by running Mixed Linear Models (MLM), which account for population stratification and genotype relatedness by including the first three Principal Components (PCs) and the kinship matrix (K) calculated from the same set of SNP markers. Two separate association analyses were performed for the two different watering treatments (PF and LW) with Tassel v5.2.41. The program was run with the following parameters: no compression, genetic, and residual variance re-estimated for each marker. A p-value of the association to the phenotypic traits was calculated for each marker; the significance threshold to declare a marker as associated was set to 0.05 after correction for multiple testing using the false discovery rate (FDR) method, according to Benjamini and Hochberg (1995). Manhattan plots and Q–Q plots were drawn using the R package "qqman" (Turner 2018) for each trait. MTA loci were defined, merging adjacent significant SNPs A peak marker was defined as the SNP with the lowest p-value and highest explained variance (R^2) for each MTA region. As adopted in previous works where the same population was used, the regions defined by the peak marker/region positions, including 100 kbp upstream and downstream (corresponding to an average LD decay of 0.5 estimated on the LOESS curve, as a trade-off between accuracy and power of the analysis; Volante et al. 2017a, 2020) were screened to search for candidate genes underlying each trait. When a single marker was associated, this was assumed to be a peak. This search was conducted for MTAs showing a $-\log_{10}(p)$ value >5 and $R^2>6$. All gene loci within these intervals were extracted from the annotation of the *Oryza sativa* reference sequence (Os-Nipponbare-Reference-IRGSP-1.0 updated 07-2024; http://rapdb.dna.affrc.go.jp/download/irgsp1.html).

Results

Phenotypic Analysis

To evaluate the effect of water management system on ionomic composition of 13 elements in brown rice grains, a panel of 294 rice varieties was tested under PF or LW conditions in two consecutive seasons. The two-year average data of the element concentrations (mg kg^{-1}) are reported in Supplementary Table S1, where percentile values are also indicated, so that those accessions showing extreme values for each element in both watering conditions can be easily identified.

The frequency distribution of the average phenotypic data recorded in the two years for each of the 13 elements was evaluated for both watering conditions (Fig. 1). Although the Shapiro-Wilk test does not indicate a normal distribution, the range of values for kurtosis and skewness parameters supports the normality of the distributions for most of the elements. The distribution and statistical analyses (Fig. 1; Table 1) indicate that water management affected grain concentration of some elements, i.e., As, Cd, Cu, Ni, and, to some extent, Mn. Other elements were affected to a lower extent, i.e., Ca, Mo, Na. Only for Na the differences in average concentrations in LW and PF were statistically not significant. The largest variations were observed for Cu, Ni, and Cd, with a decrease of 38, 81 and 50% under PF, respectively, whereas Mn and As were more enriched under PF, with an increase of 30 and 225%, respectively, compared to LW (Table 1; Fig. 2).

Table 1 Descriptive statistics of brown grain element concentrations in the rice panel

Element	Environment	Min	Max	Avg	PF/LW	*p*-value	Corr	SE	H^2	CV%	Skewness	Kurtosis
		mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	(%)							
As	LW	0.01	0.11	0.04	+ 225	$2.20\cdot10^{-16}{}_{***}$	0.44	0.00	0.33	44.61	1.16	1.61
	PF	0.04	0.24	0.13			***	0.00	0.83	22.60	0.16	0.79
Ca	LW	51.29	161.00	91.93	-5	$2.61\cdot10^{-11}{}_{***}$	0.61	0.86	0.59	16.16	1.01	2.67
	PF	57.79	142.92	87.54			***	0.75	0.83	14.67	0.53	1.08
Cu	LW	2.09	6.42	4.35	-38	$2.20\cdot10^{-16}{}_{***}$	0.72	0.04	0.69	16.03	0.24	0.27
	PF	1.62	4.31	2.71			***	0.03	0.79	17.32	0.66	0.65
Cd	LW	0.01	0.39	0.06	-50	$2.20\cdot10^{-16}{}_{***}$	0.48	0.00	0.67	56.26	5.37	49.1
	PF	0.01	0.24	0.03			***	0.00	0.86	89.95	3.90	23.1
Fe	LW	4.05	16.02	8.63	-9	$2.20\cdot10^{-16}{}_{***}$	0.57	0.09	0.59	18.66	0.83	2.44
	PF	4.20	13.81	7.86			***	0.10	0.67	21.43	0.39	0.33
K	LW	2,394.44	3,650.37	2,921.17	-2	$2.35\cdot10^{-12}{}_{***}$	0.63	11.68	0.55	6.89	0.46	0.62
	PF	2,372.61	3,589.80	2,859.98			***	13.26	0.79	7.99	0.50	0.26
Mg	LW	1,091.12	1,683.45	1,334.32	-4	$2.20\cdot10^{-16}{}_{***}$	0.64	5.92	0.66	7.64	0.69	1.06
	PF	1,020.55	1,731.39	1,282.69			***	6.42	0.75	8.62	0.77	1.39
Mn	LW	10.43	23.88	15.81	+ 30	$2.20\cdot10^{-16}{}_{***}$	0.72	0.15	0.67	16.81	0.63	0.35
	PF	13.07	33.62	20.67			***	0.20	0.86	16.46	0.58	0.29
Mo	LW	0.18	1.41	0.52	+ 7	$1.43\cdot10^{-3}{}_{***}$	0.45	0.01	0.63	31.94	1.06	2.8
	PF	0.07	1.25	0.55			***	0.01	0.51	33.84	0.57	0.82
Na	LW	2.99	11.90	6.90	-	0.91 ns	0.58	0.09	0.47	21.35	0.15	0.39
	PF	1.75	11.95	6.92			***	0.10	0.79	24.45	0.05	0.41
Ni	LW	1.04	4.60	1.78	-81	$2.20\cdot10^{-16}{}_{***}$	0.59	0.02	0.56	21.82	1.85	9.53
	PF	0.13	1.18	0.34			***	0.01	0.68	35.57	2.37	12.20
P	LW	3,129.21	4,629.32	3,758.83	-5	$2.20\cdot10^{-16}{}_{***}$	0.60	15.64	0.57	7.17	0.43	0.17
	PF	2,738.62	4,675.82	3,580.18			***	19.90	0.77	9.57	0.26	0.18
Zn	LW	14.90	36.49	22.69	-7	$2.20\cdot10^{-16}{}_{***}$	0.68	0.19	0.75	14.44	1.03	1.77
	PF	14.28	38.46	21.20			***	0.20	0.88	16.13	0.70	1.84

The concentrations are the mean of three biological and two technical replicates for two years. H^2, broad-sense heritability; SE, standard error; CV%, coefficient of variation; Corr: Correlation between PF and LW data, PF/LW: percent increase or decrease of PF value compared to LW value, p-value, p-value of the ANOVA test PF vs. LW

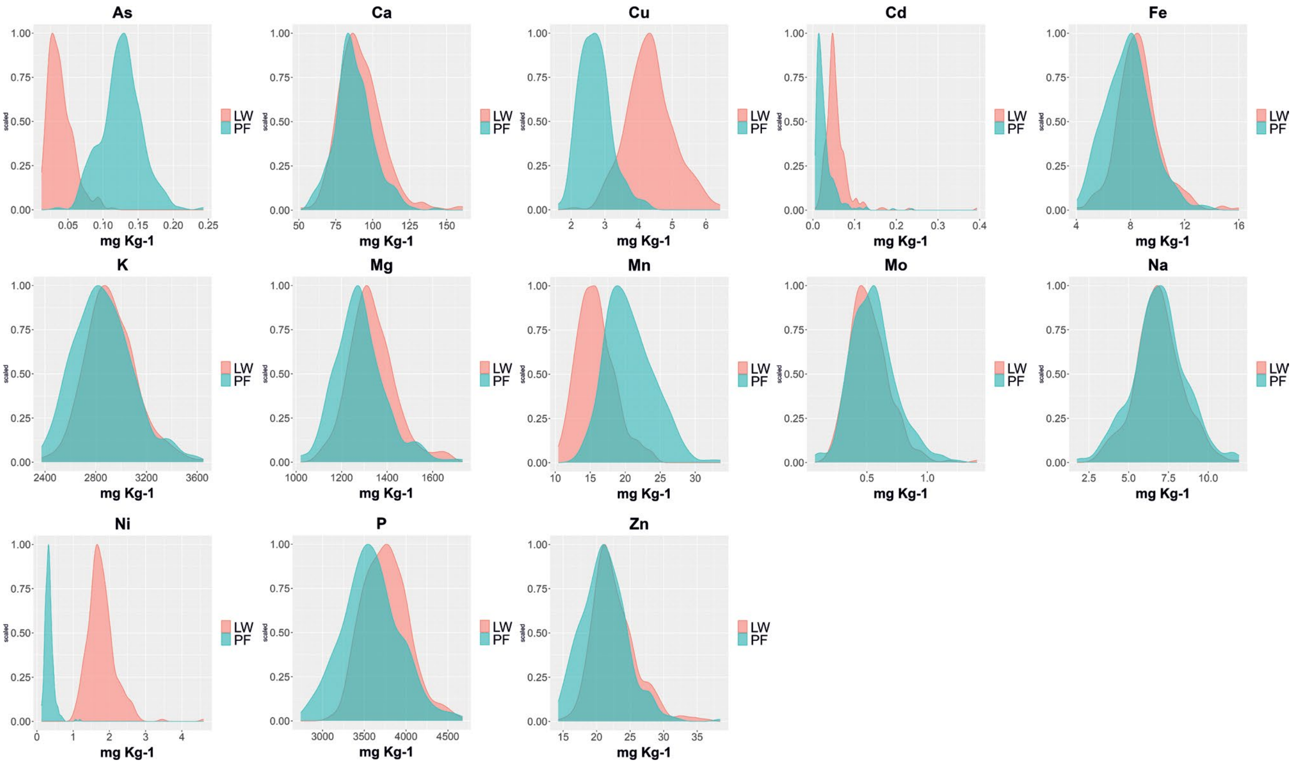

Fig. 1 Frequency distribution of the phenotypic data expressed as mg kg^{-1}. The average values, of the three biological replicates and two technical replicates for the two years, are shown, for each of the 13 elements evaluated, and for both watering conditions (PF and LW). Y axis: scaled density to a maximum of 1. X axis: average value of ion concentration in mg kg^{-1}

Considering the data distribution, the rice panel showed a wide variation in ionomic composition, depending on the considered element and the watering system, as shown in Table 1. As previously observed (Yang et al. 2018), the most abundant macronutrient (Mg, K, P), with an average of more than 1,000 mg kg^{-1} of dry weight, showed the lowest variation, with a coefficient of variation (CV) ranging from 6.89 to 9.57% while for other macro-elements and the micronutrients (Fe, Mn, Zn, Mo, Cu) a CV ranging from 14.44 to 33.84%, with Mo showing the highest variation, was observed. Non-essential and toxic elements, namely As, Cd and Ni, showed the highest diversity in the panel (CV from 21.82 to 89.95%). In particular, we note that the concentrations of inorganic arsenic in the samples with superior haplotypes never exceeded the Codex Alimentarius limit for brown rice (0.35 mg kg^{-1}). Similarly, cadmium concentrations were always below both the Codex limit (0.4 mg kg^{-1}) and the more stringent threshold established by the European Union for rice (0.15 mg kg^{-1}), as defined in Regulation (EU) 2023/915.

The broad sense heritability (H^2, Table 1) ranged from 0.33 to 0.75 under LW and from 0.51 to 0.88 under PF. These values are consistent with previous observations, showing that the genetic background strongly affects element concentration in grains (Pinson et al. 2015; Yang et al. 2018; Cu et al. 2021). Interestingly, $H^2$was usually higher in PF than in LW conditions, except for Mo, that showed a slight reduction of H^2 in PF. Arsenic showed the highest fluctuation in $H^2$with 0.33 in LW and 0.83 in PF conditions.

PF conditions generally allowed a higher variability of the ionomic composition in the rice panel, especially for Cd and Ni, while As is much more variable in the LW system (Table 1). Therefore, within a given water management (PF or LW), a good phenotypic variability for element concentration in the germplasm collection was highlighted, suitable for GWA analyses (Table 1; Fig. 2). This behavior is also illustrated in the circle plots (Supplementary Fig. 1), in which it is evident that even under a single water management, the accessions showed a range of variation useful for carrying out association mapping of these complex traits. Moreover, it was observed that some accessions showed a deeply contrasting phenotype under the two water managements (Supplementary Fig. 1); as a few examples, from PF to LW conditions, King and Thaiperla accumulated 90% less As (0.016 vs. 0.169 mg kg^{-1} for the former 0.013 vs. 0.121 mg kg^{-1} for the latter); Adelaide Chiappelli accumulated 1203% more Cd in LW than in PF (0.120 vs. 0.009 mg kg^{-1}); Bengal accumulated 103% more Fe (8.6 vs. 4.2 mg kg^{-1}) and Teqing 74% more Zn (30.3vs 17.3 mg kg^{-1}) in LW than

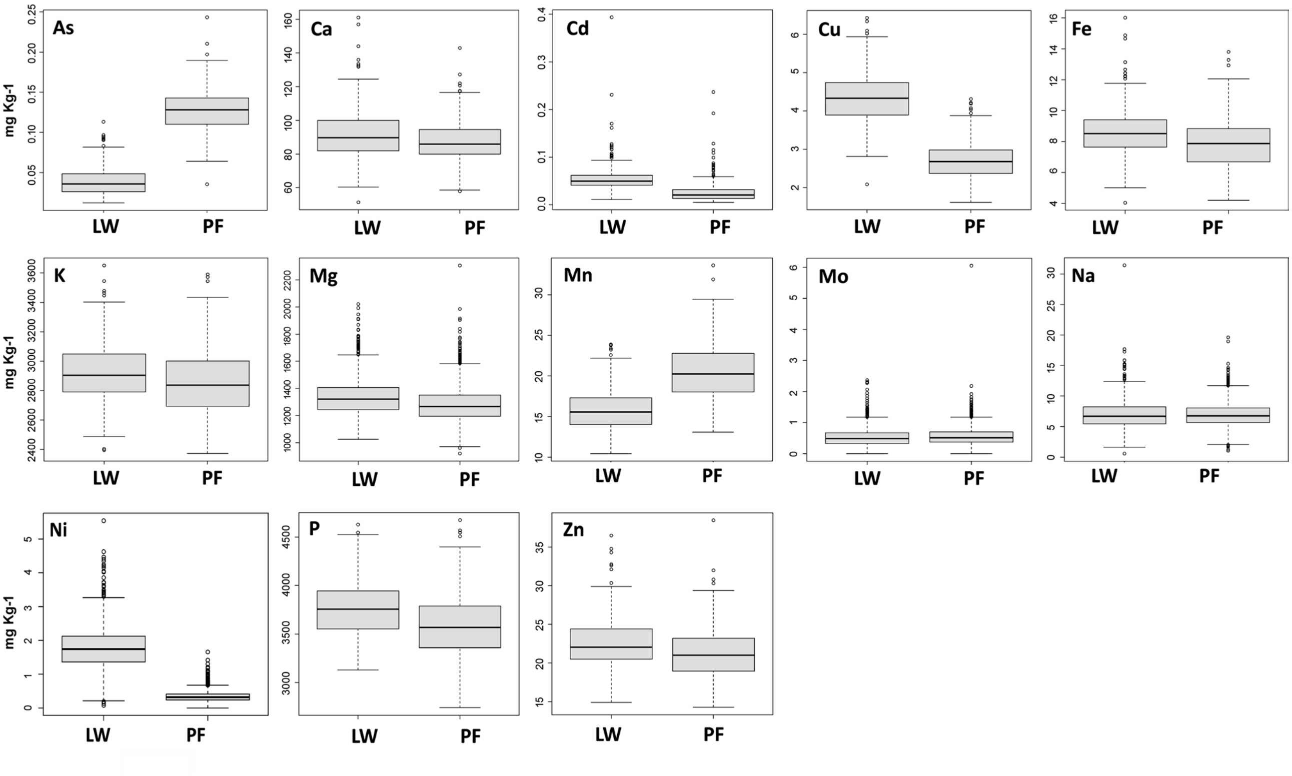

Fig. 2 Boxplots showing distribution of grain elements content in brown rice in the two watering conditions. Data are the average of the three biological replicates and two technical replicates for the two years. The medians are shown in the middle of each box by the bold lines. The whiskers extend 1.5 times the inter-quantile range from the top (75th percentile) and bottom (25th percentile) of the box. Data on the Y-axis are ion concentration in mg kg^{-1}

PF, respectively. Therefore, these results could help to address varietal choices in relation to water management and the foreseen heavy metal handling and/or useful element enrichment in brown rice.

Correlation of Different Traits

A Pearson correlation matrix was built for each water management system to evaluate the relationship among the analysed elements. Under PF conditions (Fig. 3A), Cd was negatively and significantly correlated ($p < 0.001$) with most of the elements, except Ca and Ni, which resulted positively correlated to it ($p < 0.001$). Cd and As were the most strongly negatively correlated elements. Fe, K, Cu, Zn, Mo, and P showed a strong positive correlation among each other ($p < 0.001$) and a less strong correlation with As, Na, Mg, and Mn ($p < 0.001$). Under LW conditions (Fig. 3B), the negative correlation between As and Cd was lost, as it was the negative correlation of Cd with all the other elements, except for Na, to which it remains negatively correlated ($p < 0.001$). On the contrary, Fe, K, Cu, Zn, Mo, P, Mn, and Mg retained a strong positive correlation regardless of the water management system used.

GWAS Analysis for Grain Element Concentrations in PF and LW

Phenotypic data on element concentrations (i.e., the average values obtained from the three biological replicates and the two technical replicates for the two years) and genotypic data were used to perform GWAS on the rice panel, separately for each watering system (LW and PF), using a Mixed Linear Model (MLM). For each marker, p-values were calculated and corrected for multiple testing according to Benjamini and Hochberg (1995), with an FDR threshold of 0.05.

A total of 234 significant MTAs, which were distributed on all 12 chromosomes, were identified for brown grain element concentrations. For each MTA, the marker with the lowest p-value was considered as the peak-marker (Supplementary Table S3). The Manhattan plots and the corresponding Q-Q plots for each element/condition are reported in Fig. 4. The Q-Q plots generally show a good match between the observed and expected distributions of p-values. In a few cases, e.g. Ca_LW and Cd_LW, the observed inflation can be explained by the large number of significant SNP markers mapping at the same loci. The highest number of MTAs was detected for Cd (25 in LW and 39 in PF), followed by Ca (14 in LW and nine in PF), while, for the other elements, the number of MTAs ranged from 22 for P (11 in LW and 11 in PF) to

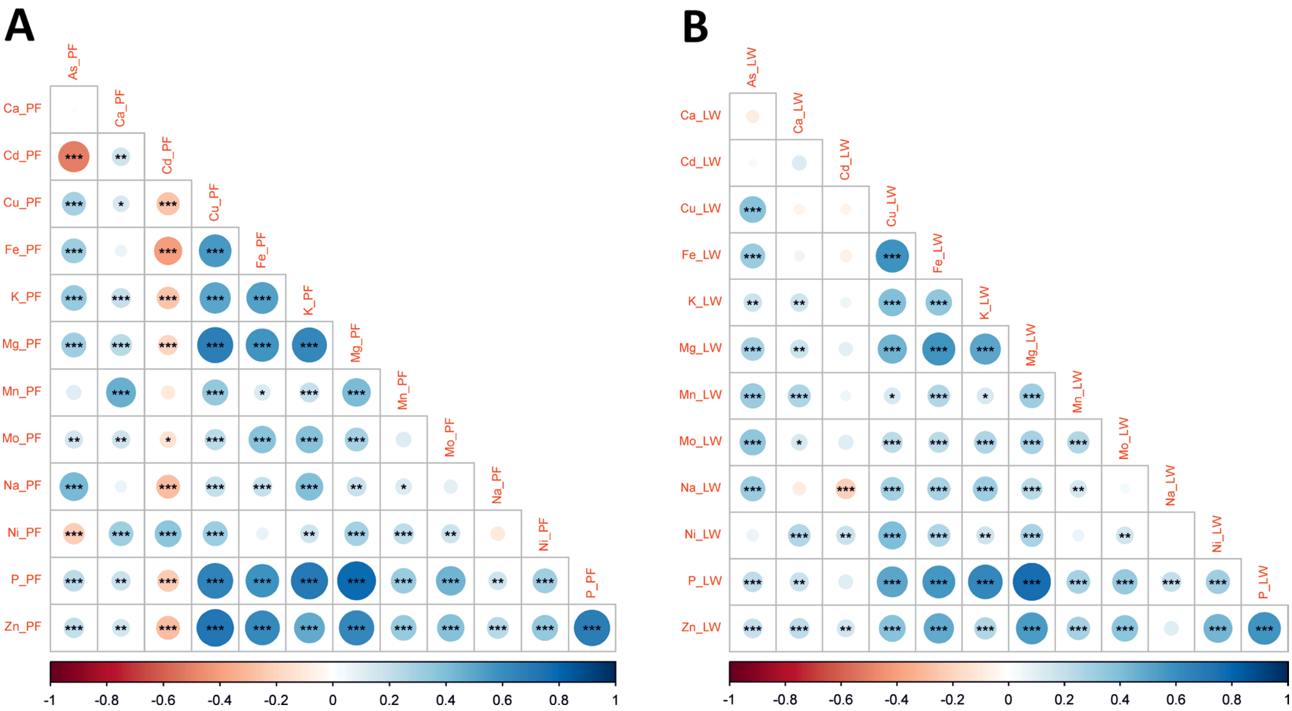

Fig. 3 Pearson correlations among the element concentrations in PF (A) and LW (B) water management systems. Asterisks show the significance level (***: 0.001; **:0.01; *:0.05). The area of circles shows the absolute value of the corresponding correlation coefficients. Colors scale indicating the correlation coefficient is reported below

five for Zn (two in LW and three in PF) (Supplementary Table S3). Environment-wise, 125 and 109 MTAs were detected under PF and LW conditions, respectively. The explained variance (R^2) for these 234 MTAs ranged from 4.48 to 14.25%. The 10 MTAs with the largest R^2 value in each environment were: qCd2.32 (14.25%), under LW on chromosome 2, qCd8.73 (9.64%), qCd8.76 (9.36%) and qCd8.78 (9.15%) under PF on chromosome 8, qCd7.43 (8.75%) under LW on chromosome 7, qCd1.56 (8.69%) under PF on chromosome 1, qMg4.128 (8.77%) for Mg under LW on chromosome 4, qNi1.172 (8.39%) for Ni under LW on chromosome 8 and qAs10.2 (8.01%) for As under LW on chromosome 10, qMn3.148 (13.26%) for Mn under PF on chromosome 3 (Supplementary Table S3; Fig. 4).

A list of 87 MTAs with high R^2 and low p-value was selected based on the following criteria: (i) where possible, MTAs with R^2 higher than 6 were selected; (ii) for those elements that did not have at least four MTAs with R^2 higher than 6, the four with the highest R^2 value were selected to allow subsequent investigations on an appropriate number of MTAs; (iii) if one MTA, present in one condition (LW or PF) and following the two above-mentioned criteria co-localized with a MTA in the other condition, also the second MTA was included. Following these criteria, 48 and 39 MTAs were considered for the PF and LW conditions, respectively (Supplementary Table S4; Fig. 5). Supplementary Table S4 also reported the average grain concentration (mg kg^{-1}) of the elements in the accessions carrying alternate SNP alleles at the peak marker.

To provide an index about the percent contribution of the allele that increased the concentration, in Supplementary Table S4, a specific index was calculated, named Contribution %(1), using the following formula:

(mg kg^{-1} in the presence of the allele that provides higher concentration – mg kg^{-1} in the presence of the allele that provides the lower concentration) / (mg kg^{-1} in the presence of the allele that provides the lower concentration).

Furthermore, the Contribution %(2), (Supplementary Table S4) represents the comparison between the presence of the minor or the major allele on the average concentration of the elements given by the minor allele. The most interesting examples have been described in Fig. 6, where the phenotypic distribution for the minor and major alleles are shown. Considering the allelic effect of Cd concentration as an example, it was observed that the G minor allele at MTA qCd2.32(LW) and the C minor allele at the MTA qCd8.76(PF) increased Cd concentration by more than 100% (0.052 vs. 0,116 mg kg^{-1} and 0.025 vs. 0.058 mg kg^{-1}, respectively), while the T minor allele at MTA qCd2.64(PF) increased the element concentration by more than 200%. (0.025 vs. 0.078 mg kg^{-1}). Regarding Fe, the average Fe concentration of the accession was 21% higher in accessions with the C minor

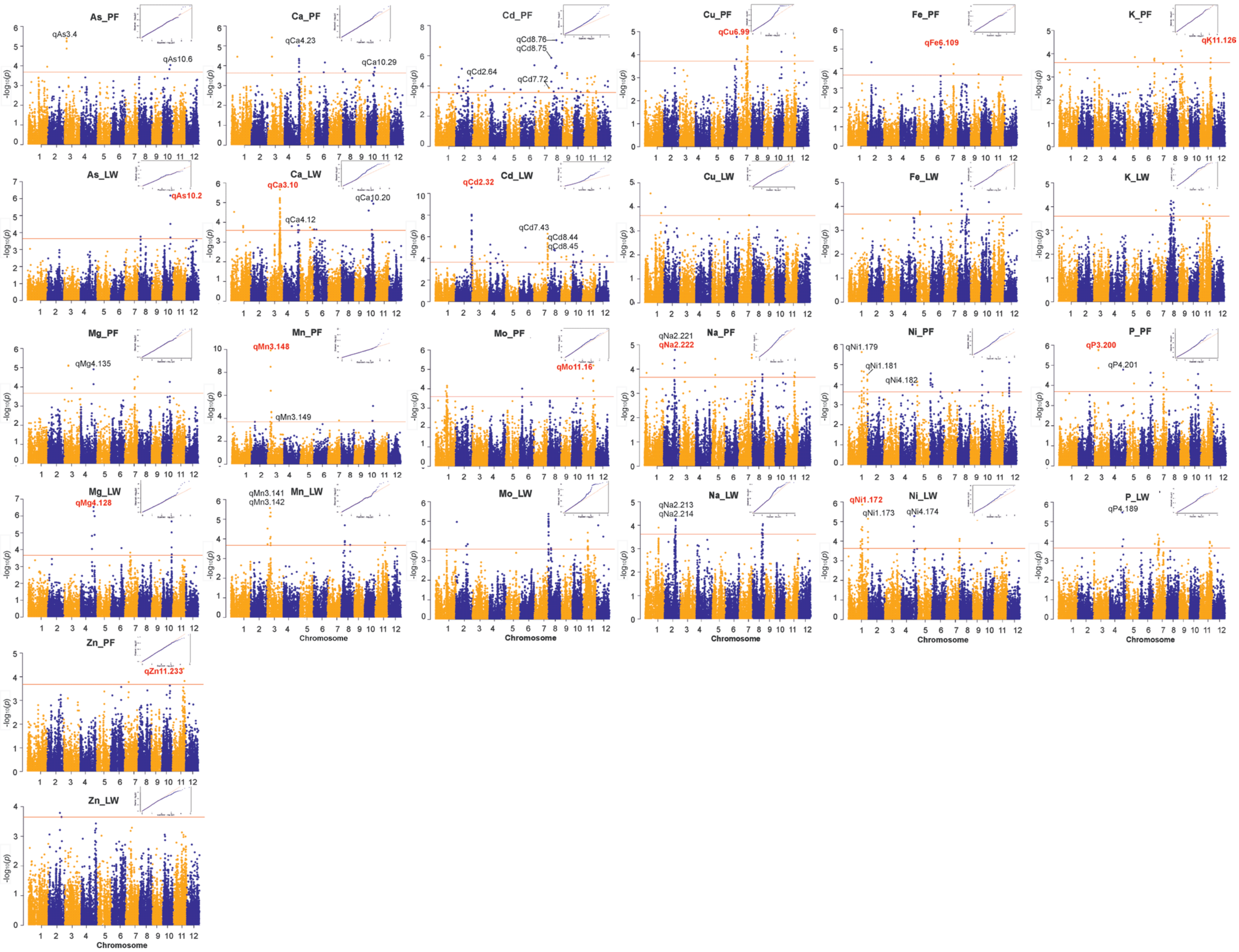

Fig. 4 Genome-wide associations of 13 micronutrients from the diverse panel of *Oryza sativa* ssp. *japonica* accessions. The Manhattan plots reflect the results of genome-wide association studies for each of the 13 micronutrients from the panel showing significant single nucleotide polymorphism (SNPs) passing the false discovery rate (FDR) cutoff (red line) according to Benjamini and Hochberg (1995). MTAs conserved under the two watering conditions are indicated at the top of the peak, while in red bold are indicated the MTAs with highest R^2 and lowest p-value for each element. Also shown in each Manhattan figure are the quantile-quantile (QQ) plots for each mineral

allele at the peak marker of the MTA qFe8.105, and the C minor allele at the peak marker of qFe6.109 was present in rice accessions with 28% higher Fe content (9.91 vs. 7.73 mg kg^{-1}). For qAs10.2, where the G minor allele was present in rice accessions showing 45% higher As content on average (0.053 vs. 0.036 mg kg^{-1}). As for Mn, the G minor allele of qMn3.148 was in rice accessions showing an average 13% lower Mn content (18.99 vs. 21.52 mg kg^{-1}), and the minor allele G of the MTA qMn3.141 is accompanied by a 11% lower average Mn concentration (16.37 vs. 14.73 mg kg^{-1}). Relevant differences were highlighted in other MTAs, including qCa10.19, where the G minor allele was present in accessions with 23% higher Ca (111.08 vs. 90.60 mg kg^{-1}), and qNi1.179, where accessions with the C minor allele showed an average 64% higher Ni content (0.54 vs. 0.33 mg kg^{-1}).

In conclusion, for several elements, a relevant effect on the concentration was observed for the different alleles at the marker peak (see Supplementary Table S4 for additional information). This observation supports the usefulness of these SNPs as markers discriminating between accessions with higher or lower tendency to accumulate a specific element in brown rice grains.

Interestingly, 28 selected MTAs were identified in both water management conditions (Table 2). The elements that showed overlapping MTAs in PF and LW were As, Ca, Cd, Mg, Mn, Ni, P, and Na, suggesting that the traits of these QTLs were not influenced by water availability. Conversely, only environment-specific MTAs were identified for Cu, Fe, K, Mo, and Zn.

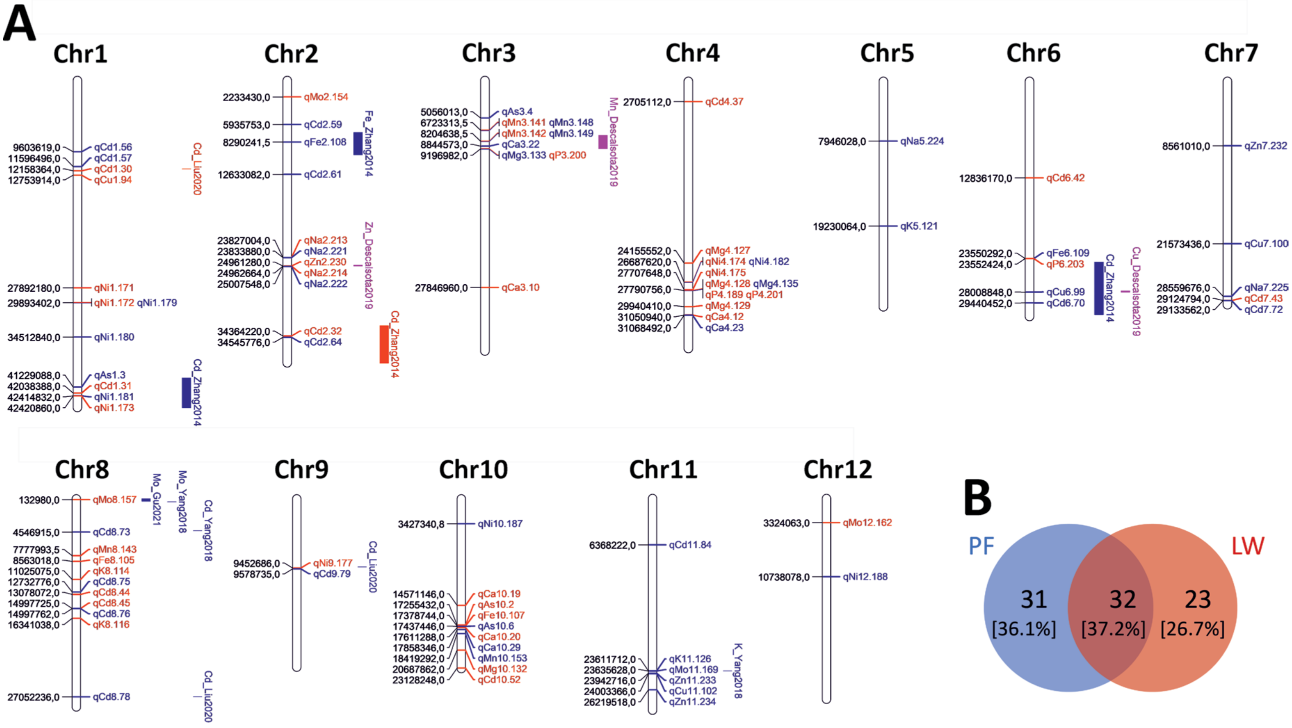

Fig. 5 MTAs of ionomic traits of the 13 elements and positional relationships with previously mapped QTLs. (A) Distribution of the 87 selected MTAs with highest R^2 and lowest p-values across the 12 chromosomes under PF (blue) and LW (red) conditions. The physical location (Mb) of the MTA-leading SNPs were reported on the left side of each chromosome. The name of the MTA is related to the element. Bars on the right side indicate the position of previously mapped QTLs for grain elements under flooded (blue), unflooded (red) or unspecified conditions (violet). (B) The Venn diagram indicates the number of condition-specific or overlapping loci between the different conditions (PF or LW)

Associations for Multiple Traits Under the Two Watering Conditions

Significant associations with different elements were identified in several genomic regions, including a total of 60 MTAs, 23 in LW and 37 in PF (Table 3; Fig. 5). These co-localizations were detected on all chromosomes except for chromosomes 5 and 12. Interestingly, three co-localizations related to more than two elements were observed.

A colocalization was observed in the region from bp 27,607,649 to bp 27,890,754 (therefore spanning 283,105 bp) of chromosome 4 for MTAs related to Ni, Mg, and P under LW. Another colocalization was observed in the region from bp 17,079,400 to bp 17,990,472 (spanning 911,072 bp) of chromosome 10 for MTAs affecting the concentration of four elements (Mg, Mn, P, Cu) under PF. Finally, a co-positional relationship was detected for Mo, K, and P concentration from bp 23,430,659 to bp 23,942,716 (a region spanning 512,057 bp) on chromosome 11.

Mining Candidate Genes

A search for candidate genes associated with the 39 MTAs with $-\log_{10}(p) > 5$ and $R^2 > 6.0$ (Supplementary Table S4) was performed considering the genes annotated on Os-Nipponbare-Reference-IRGSP-1.03 (Supplementary Table S5). Genes, whose function is consistent with the phenotypic traits under consideration, were identified for 12 MTAs (eight for LW and four for PF; Table 4). Seven of these genes (*OsVPE1*, *OsACA.8*, *OsMTP8.1*, *OsMOT1;1*, *OsLCD*, *OsCAX1c*, *OsHMA2*) encode transporters previously reported to be involved in root uptake of specific elements, root-to-shoot translocation or accumulation in different parts of the shoot. One gene (*OsWOX10*) encodes a protein previously reported to be involved in the development of root architecture and metal binding, while three genes (*OsCESA7*, *OsF-box131 and OsCLT1*) encode proteins previously indicated as involved in detoxification/tolerance mechanisms related to the undesirable elements As and Cd and one gene is for a miRNA precursor, namely OsmiR396.

Discussion

Phenotypic Variation for Grain Element Concentration Under Different Water Management

Three essential macronutrients (K, Mg, P) showed relatively low levels of variation for their concentration in the grain under the two water management conditions (CV values below 10%); similarly, another macronutrient (Ca) and three essential micronutrients (Mn, Cu, Zn) displayed CV below 20% (Table 1; Supplementary

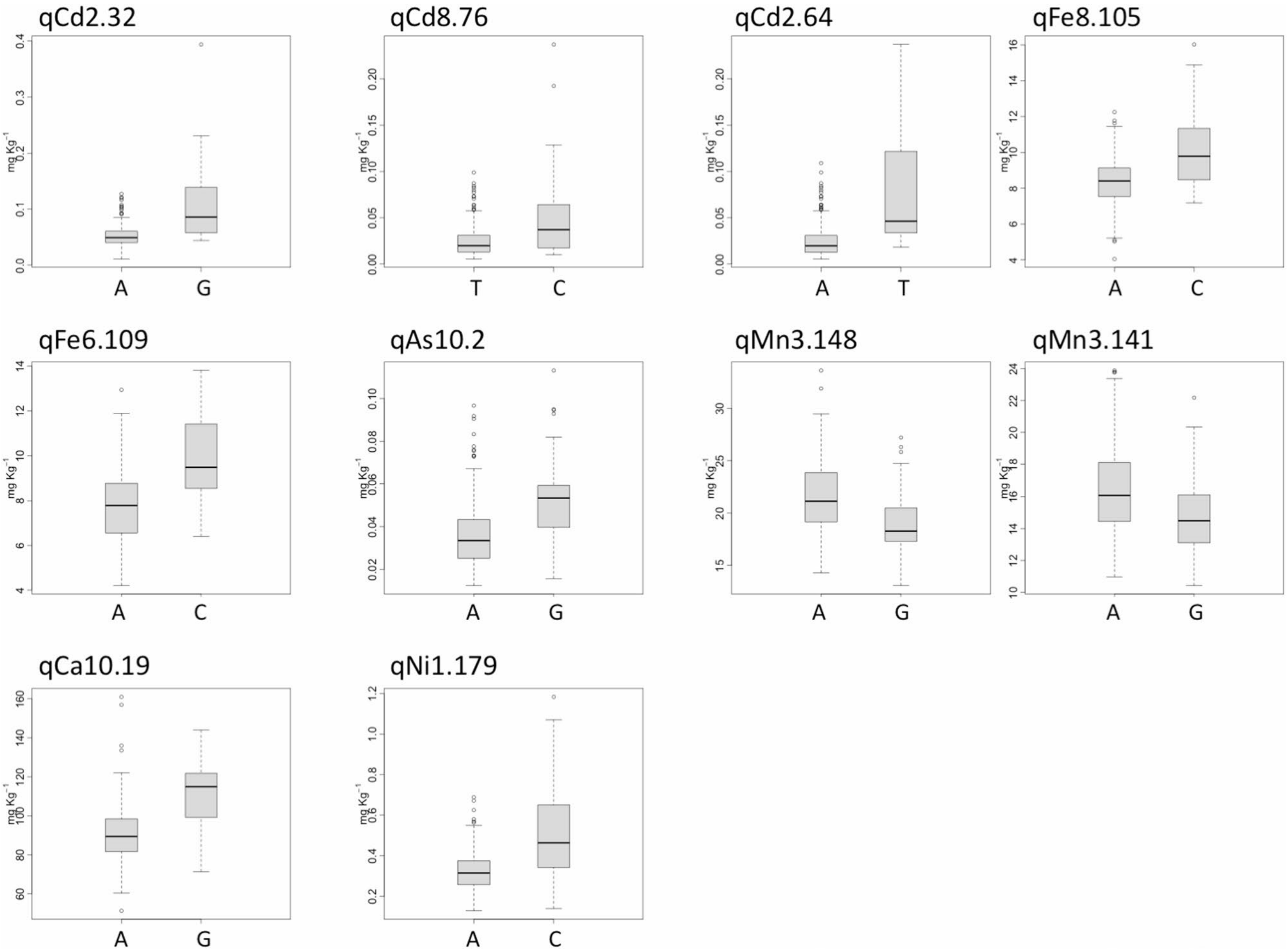

Fig. 6 Boxplots showing phenotypic distribution of grain elements (ion concentration in mg kg^{-1}) for the two alleles at each peak marker. The first allele to be shown is always the major allele. The medians are shown in the middle of each box by the bold lines. The whiskers extend 1.5 times the interquantile range from the top (75th percentile) and bottom (25th percentile) of the box

Fig. 1). These observations support the presence of a tight regulation in the homeostasis of these elements and are in agreement with previous studies that highlighted the presence of regulatory mechanisms in plants to maintain the concentrations of essential nutrients within narrow ranges to allow optimal physiological processes for growth, development and seed production (Castro et al. 2018; Cu et al. 2021). Larger variations, with CV above 20% in both watering conditions, were observed for the concentrations of the other six elements, represented by the micronutrients Fe, Ni, Mo, As, Cd, and Na (where Na is a functional but nonessential element; Subbarao et al. 2003). This result supports the hypothesis of a lower pressure for the regulation of the concentrations of these elements (unless they approach toxicity levels), resulting in less stringent control mechanisms. Considering the differences in CV between the two watering systems, significant differences were observed for three elements (Ni, Cd, As), with Ni and Cd showing higher CV under PF and As under LW. Alternation from anaerobic to aerobic soil, like in the LW condition, determines shifts of Ni^{2+} and Cd^{2+} from their insoluble sulphide salts to the soluble sulphate ones, thus increasing their bioavailability (Rinklebe and Shaheen 2017; Hu et al. 2013). Conversely, anaerobic conditions, like under PF, favour the presence of the mobile and water soluble arsenite (As(III), which is bioavailable for plant roots, since it hampers its transformation to arsenate (As(V), which is strongly adsorbed on soil (hydr)oxides, poorly mobile and consequently less bioavailable (Takahashi et al. 2004; Hu et al. 2013). Since the bioavailability of Ni and Cd is lower under PF conditions, while the bioavailability of As is lower under LW, our CV results (Table 1) suggest the occurrence of a higher variability of the concentration of these elements when their bioavailability is reduced. It can be hypothesized that at lower bioavailability, even small changes in environmental conditions (like soil pH and even microbial activity) or the presence of other substances (like the presence of other metals or organic matter) can significantly affect how much Cd, Ni, As are accessible for

Table 2 MTAs for the same element showing overlapping positions in the two water management conditions (LW and PF)

Element	Env.	MTA ID	Peak Marker/Region (bp)	Chr	Associated Overlapping Region			# SNP	-log$_{10}$(p)	Peak marker R^2%
					start	end	size (bp)			
As	LW	qAs10.2	S10_17255433	10	17,155,433	17,355,433	115,751	3	6.18	8.01
	PF	qAs10.6	S10_17437446	10	17,337,446	17,537,446	-	1	4.03	5.17
Ca	LW	qCa4.12	S4_31050939	4	30,950,939	31,150,939	17,593	4	3.86	4.89
	PF	qCa4.23	S4_31068491	4	31,050,899	31,495,347	444,449	8	5.00	6.56
Cd	LW	qCd2.32	S2_34364220	2	34,355,303	35,118,443	763,141	23	10.55	14.25
		qCd7.43	S7_29124793	7	29,082,918	29,639,849	556,932	39	6.28	8.75
		qCd8.45	S8_14997724	8	14,897,724	15,097,724	-	1	4.73	6.14
	PF	qCd2.64	S2_34545778	2	34,445,778	34,645,778	-	1	4.52	5.81
		qCd7.72	S7_29133562	7	29,033,562	29,233,562	-	1	3.86	4.87
		qCd8.76	S8_14997761	8	14,997,724	14,997,761	38	2	7.03	9.36
Mg	LW	qMg4.128	S4_27790754	4	27,790,754	27,790,806	53	2	6.53	8.77
	PF	qMg4.135	S4_27790754	4	27,790,754	27,790,806	53	2	4.92	6.44
Mn	LW	qMn3.141	S3_6723314	3	6,580,184	7,023,285	443,102	3	5.56	7.37
		qMn3.142	S3_8204638	3	8,104,638	8,304,638	22,310	5	5.33	7.04
	PF	qMn3.148	S3_6723314	3	6,580,184	7,023,285	443,102	3	9.92	13.26
		qMn3.149	S3_8204638	3	8,104,638	8,304,638	11,033	2	4.57	5.86
Ni	LW	qNi1.172	S1_29893402	1	29,532,694	30,055,721	523,028	8	5.86	8.39
		qNi1.173	S1_42420861	1	42,320,861	42,520,861	6,031	5	5.14	6.78
		qNi4.174	S4_26687620	4	26,587,620	26,787,620	67,802	2	4.74	6.25
	PF	qNi1.179	S1_29893402	1	29,793,402	29,993,402	196,468	4	5.62	7.91
		qNi1.181	S1_42414831	1	42,314,831	42,514,831	39,633	4	4.50	5.84
		qNi4.182	S4_26687620	4	26,587,620	26,787,620	-	1	4.00	5.15
P	LW	qP4.189	S4_27790754	4	27,690,754	27,890,754	53	2	5.48	7.23
	PF	qP4.201	S4_27790754	4	27,690,754	27,890,754	-	1	4.78	6.18
Na	LW	qNa2.213	S2_23827004	2	23,827,004	24,272,048	445,045	3	3.80	4.67
		qNa2.214	S2_24962665	2	24,745,485	25,060,656	315,172	9	4.26	5.29
	PF	qNa2.221	S2_23833880	2	23,733,880	23,933,880	15,116	2	4.37	5.47
		qNa2.222	S2_25007547	2	24,907,547	25,107,547	-	1	4.79	5.97

For each MTA the region of co-positional relationships, the number of SNPs, the -log$_{10}$(p) and the R^2 are indicated

uptake. In essence, when these elements are readily available, their variability is constrained by high bioavailability. However, when bioavailability is low, the small fraction that is available becomes highly susceptible to various factors, leading to a greater range in uptake and effects. A higher CV value would allow a better differentiation among genotypes and should therefore facilitate the selection of accession with higher or lower accumulation, therefore supporting their value in breeding.

In this work, a high heritability value (ranging from 33 to 88%, with most of the traits showing values above 60%) was observed for the grain concentration of the 13 elements, indicating a significant genotypic contribution to the total phenotypic variance for these traits, as observed in previous studies (Descalsota-Empleo et al. 2019; Descalsota et al. 2018; Nawaz et al. 2015; Cu et al. 2021). These data support the idea that a direct selection for these elements may be a practical approach for trait improvement (see below).

MTAs with Strong R^2 and their Overlap with Previously Detected QTL

This work detected 234 MTAs for brown grain elements under two watering conditions. Among them, 125 MTAs were highlighted under PF and 109 under LW. Consistently, in other studies a higher number of QTLs was highlighted under flooded than under unflooded conditions (Zhang et al. 2014; Liu et al. 2020), suggesting a lower microenvironmental variability under flooded conditions.

Considering MTAs with the highest values of explained variance (R^2) for all the 13 elements, a total of 87 loci were selected (Supplementary Table S4; Fig. 5). Frequently, the allelic variation of the peak SNPs is associated with a strong effect on the element concentration, as shown in Fig. 6 and Supplementary Table S4 and highlighted in the Results section GWAS analysis for grain element concentrations in PF and LW. Taken together, these observations support the possibility that the exploitation of SNPs

Table 3 MTAs for different elements showing overlapping positions in the two water management conditions

Env	Element	MTA ID	Peak Marker/Region (bp)	Chr	Associated Region			# SNP	$-\log_{10}(p)$	Peak marker R^2%
					start*	end*	Size (bp)			
LW	Ca	qCa1.8	S1_26446550	1	26,346,550	26,546,550	17,377	3	3.82	4.83
	Ni	qNi1.170	S1_26542124		26,442,124	26,642,124	76,226	9	3.98	5.12
	Ni	qNi1.172	S1_29893402	1	29,532,694	30,055,721	523,028	8	5.86	8.39
	Na	qNa1.212	S1_29879401		29,779,401	29,979,401	-	1	3.90	4.88
	Na	qNa2.214	S2_24962665	2	24,745,485	25,060,656	315,172	9	4.26	5.29
	Zn	qZn2.230	S2_24961280		24,861,280	25,061,280	-	1	3.79	5.26
	Ca	qCa3.9	S3_27054692	3	26,816,542	27,054,692	238,151	2	3.88	5.21
	Cd	qCd3.35	S3_27021886		26,921,886	27,121,886	-	1	4.26	5.51
	Ni	qNi4.175	S4_27707649	4	27,607,649	27,807,649	-	1	5.28	6.97
	Mg	qMg4.128	S4_27790754		27,690,754	27,890,754	53	2	6.53	8.77
	P	qP4.189	S4_27790754		27,690,754	27,890,754	53	2	5.48	7.23
	P	qP4.190	S4_29940409	4	29,840,409	30,040,409	-	1	4.09	5.27
	Mg	qMg4.129	S4_29940409		29,840,409	30,040,409	18,584	2	5.99	8.01
	P	qP7.193	S7_11136086	7	11,084,380	11,344,118	259,739	3	3.84	4.90
	Mg	qMg7.131	S7_11344081		11,244,081	11,444,081	-	1	3.83	4.89
	P	qP7.196	S7_22592963	7	22,492,963	22,692,963	-	1	3.97	5.10
	Cu	qCu7.97	S7_22677602		22,577,602	22,777,602	130,410	2	3.65	4.60
	Cd	qCd7.43	S7_29124793	7	29,082,918	29,639,849	556,932	39	6.28	8.75
	Ni	qNi7.176	S7_29183199		29,083,199	29,283,199	152,802	2	4.12	5.81
	As	qAs10.2	S10_17255433	10	17,155,433	17,355,433	115,751	3	6.18	8.01
	Fe	qFe10.107	S10_17378745		17,278,745	17,478,745	-	1	4.18	5.85
	Cd	qCd10.51	S10_20659308	10	20,559,308	20,759,308	104,923	2	3.77	4.78
	Mg	qMg10.132	S10_21133925		20,687,862	21,133,925	446,064	4	3.72	4.74
PF	As	qAs1.3	S1_41229086	1	41,129,086	41,329,086	-	1	3.95	5.36
	Cd	qCd1.58	S1_41309302		41,209,302	41,409,302	-	1	4.09	5.61
	Mn	qMn3.150	S3_8679586	3	8,579,586	8,779,586	-	1	4.08	5.18
	Ca	qCa3.22	S3_8844573		8,744,573	8,944,573	176,301	3	5.42	7.17
	Mg	qMg3.133	S3_9196982	3	9,096,982	9,296,982	1,556	2	5.12	6.70
	P	qP3.200	S3_9196982		9,096,982	9,296,982	1,556	3	5.77	7.54
	Mg	qMg4.135	S4_27790754	4	27,690,754	27,890,754	53	2	4.92	6.44
	P	qP4.201	S4_27790754		27,690,754	27,890,754	-	1	4.78	6.18
	Ni	qNi6.185	S6_5635622	6	5,535,622	5,735,622	20	2	4.01	5.16
	Mo	qMo6.167	S6_5703916		5,603,916	5,803,916	-	1	4.01	5.12
	Fe	qFe6.109	S6_23550291	6	23,450,291	23,650,291	-	1	5.08	6.98
	P	qP6.203	S6_23552423		23,452,423	23,652,423	151,148	2	4.63	6.44
	P	qP6.204	S6_27396916	6	27,296,916	27,496,916	-	1	4.13	5.62
	Cu	qCu6.98	S6_27396916		27,296,916	27,496,916	-	1	3.80	5.16
	Cu	qCu7.100	S7_21573436	7	20,440,415	21,718,129	1,277,715	32	4.77	6.17
	Fe	qFe7.110	S7_20749813		20,449,450	20,749,813	300,364	2	4.22	5.10
	Mg	qMg7.136	S7_20915752	7	20,815,752	21,015,752	21	3	4.40	5.67
	P	qP7.205	S7_20915752		20,815,752	21,015,752	21	3	4.61	5.92
	Mg	qMg7.137	S7_21509141	7	21,409,141	21,609,141	-	1	3.99	5.09
	P	qP7.206	S7_21573436		21,473,436	21,673,436	-	1	3.90	4.91
	Mg	qMg7.138	S7_27784632	7	27,684,632	27,884,632	-	1	4.53	5.87
	P	qP7.207	S7_27784632		27,684,632	27,884,632	-	1	3.80	4.77
	Ni	qNi8.186	S8_4361481	8	4,261,481	4,461,481	-	1	3.72	4.76
	Cd	qCd8.73	S8_4546915		4,278,372	4,546,975	268,604	4	7.25	9.64
	Fe	qFe9.111	S9_17512380	9	17,412,380	17,612,380	-	1	3.70	4.48
	P	qP9.208	S9_17512380		17,412,380	17,612,380	-	1	4.27	5.44
	As	qAs10.5	S10_15368426	10	15,268,426	15,468,426	-	1	3.83	4.86
	Cd	qCd10.83	S10_15440815		15,340,815	15,540,815	-	1	3.60	4.81
	Mg	qMg10.139	S10_17179400	10	17,079,400	17,279,400	-	1	4.26	5.52
	Mn	qMn10.152	S10_17179400		17,079,400	17,279,400	-	1	3.78	4.78
	P	qP10.210	S10_17179400		17,179,400	17,990,472	811,073	3	4.57	5.91
	Cu	qCu10.101	S10_17323647		17,223,647	17,423,647	-	1	4.13	5.28
	Cd	qCd11.89	S11_20941386	11	20,841,386	21,041,386	-	1	4.26	5.44
	Na	qNa11.229	S11_21026921		20,926,921	21,126,921	21,560	2	3.85	4.77
	Mo	qMo11.169	S11_23635627	11	23,430,659	23,635,627	204,969	4	5.20	7.38
	K	qK11.126	S11_23611713		23,611,713	24,267,310	655,598	5	4.43	6.06
	P	qP11.211	S11_23611948		23,611,948	23,942,716	330,769	2	4.02	5.09

For each MTA the region of co-positional relationships, the number of SNPs, the $-\log_{10}(p)$ and the R^2 are indicated. MTAs and SNPs in red indicates that have also overlapping positions in PF and LW condition for the same element (Table 2) at the MTAs identified here could be used in breeding approaches to reduce or increase the concentration of toxic and valuable elements, respectively.

Considering the 87 MTAs with the highest R^2 and lowest p-value listed in Supplementary Table S4, a search was conducted to identify positional relationships with QTLs previously identified (Fig. 5). For As, Ca, Mg, Ni, P, and Na, no overlapping of mapping positions among MTAs identified in the present work with those currently present in the literature were observed, supporting that the MTAs here identified represent unexplored regions containing new loci that affect the concentration of these elements in *japonica* rice grains, offering new breeding targets to reduce As, Ni and Na uptake while enriching for useful minerals. Differently, co-localizations between the MTAs identified in this work and previously identified QTLs were observed for Cd, Cu, Fe, K, Mn, Mo, and Zn (Fig. 5).

For Cd concentration, several MTAs co-localize with previously identified QTLs. The MTAs qCd1.30 (on chromosome 1, position 12,158,364 bp) related to LW is close to a QTL for Cd accumulation under unflooded conditions that was identified using the USDA Rice mini-core collection (11.91 Mb; Liu et al. 2020). The MTA qCd1.31 (on chromosome 1, position 42,038,389 – 42,050,647 bp), related to LW, co-localizes with a QTL for Cd in brown rice grain under flooded paddy conditions detected on the same chromosome in the region from 40 to 44 Mb using the Lemont × Teqing recombinant inbred lines (Zhang et al. 2014). On chromosome 2, the two Cd MTAs qCd2.32 (34,355,303 – 35,118,443 bp) and qCd2.64 (34,445,778 – 34,645,778 bp), that were identified under LW and PF, respectively, showed co-positional relationships with a QTL associated to Cd concentration under unflooded condition that was identified in the interval 33–38 Mb using the Teqing x Lemont backcross introgression lines (Zhang et al. 2014). Since qCd2.32 is the MTA with the highest R^2 and lowest p-value identified in this work, this region would represent an important and robust target for reducing Cd content in rice grain. The position of the PF-related MTA qCd6.70 on chromosome 6 (29,440,451 bp) is related to a grain Cd QTL identified under flooded conditions at 24–31 Mb using the TeqingxLemont backcross introgression lines (Zhang et al. 2014). On chromosome 8, the qCd8.73 (PF, 4,278,372-4,546,975 bp) overlaps with a Cd QTL at 4.35 Mb, that was identified in two locations under flooded conditions using a GWAS approach carried out with 529 rice accessions (Yang et al. 2018). On the same chromosome 8, the position of qCd8.78 (PF, 26,932,673 – 27,052,235 bp) overlapped with a Cd QTL detected at 27.04 Mb under flooded conditions using the USDA Rice mini-core collection (Liu et al. 2020). Finally, a co-positional relationship for Cd was detected on chromosome 9 for qCd9.79 (PF: 9,578,735-9,582,584 bp), which lies in a region adjacent to a Cd QTL positioned at 9.31 Mb in the USDA Rice mini-core collection under flooding conditions (Liu et al. 2020).

For the Cu element, qCu6.99 on chromosome 6 (PF: 28,008,847 bp) was mapped in the same region of a Cu QTL identified on this chromosome in the 27.85-28.00 Mb interval by using a doubled-haploid population derived from IR64 × IR69428 (Descalsota-Empleo et al. 2019).

The Fe MTA qFe2.108 on chromosome 2 (PF: 8,290,242 bp) mapped in a region where a Fe-related QTL was previously identified in the 7–10 Mb interval using two mapping populations under flooding conditions (Zhang et al. 2014), thus providing a robust locus for improving Fe content in rice grains.

A co-localization of the K-related MTA on chromosome 11, qK11.126 (PF: 23,611,713 – 24,267,310 bp), was found with a K-related QTL identified under flooded conditions at 23.63 Mb (Yang et al. 2018).

Furthermore, the two MTAs for Mn concentration detected on chromosome 3 (qMn3.142, LW: 8,193,361-8,215,670 bp; qMn3.149, PF: 8,204,638- 8,215,670 bp) were mapped in a genomic region from where a Mn QTL was positioned from 7.41 to 9.19 Mb (Descalsota-Empleo et al. 2019).

Another co-positional relationship was highlighted for the Mo-related MTA qMo8.157 on chromosome 8 (LW: 17,973 – 492,332 bp), which was related to one QTL detected under flooded conditions at 0.43 Mb by GWAS on 529 rice accessions (Yang et al. 2018) and to one QTL mapped under in at least three environments in the genomic interval from 0.00 to 0.33 Mb using GWAS with a diverse panel of *indica* accessions (Cu et al. 2021).

Finally, for the Zn MTA qZn2.230 on chromosome 2 (LW: 24,961,280 bp), a co-positional relationship was observed with a Zn QTL mapped in the interval 24.85–24.97 Mb using a doubled-haploid population derived from IR64 × IR69428 (Descalsota-Empleo et al. 2019).

Relationships of MTAs Shared Under PF and LW with Other Element MTAs

MTAs highly effective within breeding programs need to explain a significant proportion of the phenotypic variation and be stable across environments and populations (Collard and Mackill 2008).

The MTA identified in this work show an explained variance between 4.48% and 14.25%, not too high but typical for such complex traits. Interestingly, 4 MTA associated to Cd concentration MTA qCd2.32 (under LW condition), and qCd8.73, qCd8.76, qCd8.78 (under PF condition) have the R^2 above 9%. Also the qMn3.148 MTA show a very high R^2 (13.26%) and is stable in both water management condition. The stability of the MTAs

Table 4 Main candidate genes related to MTAs with -log$_{10}$(p) < 5.00

MTA ID	LOCUS ID	CGSNL Gene Symbol	GENE DESCRIPTION	GENE FUNCTIONAL CATEGORY	REFERENCE
qAs10.2 (LW)	*Os10g0467800*	***OsCesA 7***	**Cellulosesynthase andcelluloseSynthase-like (CesA/CSL)**	*Protein involved in As stress response in rice*	Shabab et al. (2025)
qAs3.4 (PF)	*Os03g0196500*	***OsFbox131***	**Cyclin-like F-box domain containing protein**	*Protein putatively involved in the regulation of As(V) stress response in A. thaliana*	Peña-Garcia et al. (2021)
qCa10.19 (LW)	*Os10g0418100*	***OsACA.8***	**Calcium-transporting ATPase**	*Transporter regulating Ca^{2+}homeostasis*	Singh et al. (2014)
qCd1.31 (LW)	*Os01g0955700* *Os01g0956700*	***OsCLT1*** ***OsLCD***	**CRT-like transporter 1** **Low Cadmium transporter**	*Transporter mediating the export of γ-glutamylcysteine from the plastid, ensuring homeostasis of glutathione* *Transporter involved in Cd^{2+}partitioning in the plant*	Yang et al. (2016) Shimo et al. (2011)
qCd2.32 (LW)	*Os02g080400*	***OsmiR396c***	**Primary transcript of miR396**	*miRNA targeting Growth Regulating Factors (GFR) and downregulated by Cd stress*	Ding et al. (2013) Tang et al. (2014)
qCd2.61 (PF)	*Os02g0314300*	***OsCAX1c***	**Cation/H + exchanger CAX**	*Transporter mediating Ca and Cd accumulation into the vacuole*	Zou et al. (2021)
qCd7.43 (LW)	*Os07g068200* *Os07g0684800* *Os07g0684900*	***OsHIPP9*** ***OsNAC15*** ***OsWOX11***	**Heavy Metal-associated Isoprenylated Plant Protein 9** **NAM/CUC2-like transcription factor** **WUSHEL-type homeobox (WOX) transcription factor**	*Metallochaperone involved in metal homeostasis and detoxification* *Transcription factor contributing to Zn deficiency tolerance and Cd stress tolerance in rice* *Transcription factor activating genes involved in Cd intake and transport in rice*	Xiong et al. (2023 Zhan et al. (2022) Xu et al. (2021)
qCd6.70 (PF)	*Os06g0700700*	***OsHMA2***	**Heavy metal ATPase 2**	*Plasma membrane P1B-type ATPase controlling Zn/Cd translocation from the root to the shoot in rice*	Nocito et al. (2011)
qCd8.78 (PF)	*Os08g0544400*	***OsPDR1***	**Member of pleiotropic drug resistance (PDR) subfamily of ATP-binding cassette (ABC) transporter**	*Transporter induced under Cd stress*	Ogawa et al. (2009)
qFe8.105 (LW)	*Os08g0242400*	***OsWOX10***	**WUSCHEL-related homeobox (WOX) transcription factor**	*Activator of genes involved in binding and translocation of Fe in rice*	Kawai et al. (2022)
qMn3.141 (LW) qMn3.148 (PF)	*Os03g0226400*	***OsMTP8.1***	**Manganese transporter**	*Vacuolar transporter facilitating Mn diffusion in the plant*	Chen et al. (2016)
qMo8.157 (LW)	*Os08g0101500*	***OsMOT1;1***	**Molybdate transporter**	*Transporter mediating uptake and translocation of Mo*	Huang et al. (2019)
qP4.189 (LW)	*Os04g0555300*	***OsVPE1***	**Vacuolar phosphate efflux transporter 1**	*Transporter involved in remobilization of Pi*	Julia et al. (2016) Xu et al. (2019)

in the present work was investigated under two environments. Twenty-eight MTAs, that are, related to eight elements (As, Ca, Cd, Mg, Mn, Ni, P, Na), resulted common to the two environments (Table 2), suggesting that the watering conditions did not affect these QTLs. Considering MTAs identified for different elements, we pointed out the presence of co-localized MTA (Table 3), suggesting the presence of genes with a pleiotropic effect governing common physiological processes or cluster of gene with similar functions for uptake and transport of different elements.

One of the most interesting region identified is on chromosome 10 (in the region 17.08–17.99 Mb) where two As-related MTAs (qAs10.2(LW) and qAs10.6(PF)) co-localize with one MTA for Fe (qFe10.107(LW)) and several additional MTAs for multiple elements under LW (As, Fe, Ca) and PF (As, Ca, Mn, Mg, P, Cu) were present (Fig. 5; Table 3), suggesting that this region can include some candidate genes involved in the accumulation of different elements in rice grain.

Identification of MTAs for Cd accumulation is for sure of outstanding interest, and in particular the region on chromosome 2 (spanning from about 34.3 to 35.1 Mb) where 2 MTA in common between the two watering conditions are present: qCd2.32(LW), and qCd2.64(PF). In this region, a QTL for Cd concentration was detected in the 33–38 Mb interval under unflooded conditions (Zhang et al. 2014), reinforcing the robustness of the MTAs identified here. Additionally, the MTAs for Cd on chromosome 7 (qCd7.43(LW) and qCd7.72(PF) co-localize with the Ni-related MTA detected under LW (qNi7.176; Table 3) in the genomic region 29-29.6 Mb. The identification of these stable MTAs for Cd accumulation, provides a breeding tool for decreasing Cd concentration in rice grains in particular under LW conditions that promote Cd bioavailability.

On chromosome 3, in the regions 6.5-7 Mb and 8.1–8.3 Mb, a perfect overlap was observed for Mn-related MTAs detected under LW and PF conditions. A Mn-related QTL was previously detected in the 7.41–9.19 Mb interval, thus allowing the identification of a stable effect for this element in this genomic region.

Six Ni-related MTAs were shared between the two environments on chromosome 1 and chromosome 4. The MTAs on the region of chromosome 1 from 29.5 to 30 Mb (qNi1.172(LW) and qNi1.179(PF)) reside in the same genomic region of an MTA for Na accumulation (qNa1.212, from 29.7 to 29.9 Mb), thus supporting an overlap in mechanisms of uptake and transport for these elements.

The chromosome 4 region from 27.6 to 27.8 Mb is characterized by a perfect overlapping of MTAs for P in the two watering conditions (qP4.189(LW) and qP4.201(PF)). In this region, two MTAs for Ni (qNi4.175) and Mg (qMg4.128) under LW and one MTA for Mg (qMg4.135) under PF were located, further supporting the presence of genes with a pleiotropic effect or with similar functions for uptake and transport of these elements. Four MTAs co-localized on chromosome 2 under LW and PF for the Na element (Table 2). The Na-related MTAs in the 24.7 to 25.1 Mb region of chromosome 2 (qNa2.214(LW) and qNa2.222(PF)) are localized in a region where also significant effects for Zn accumulation under LW were identified (qZn2.230).

Relationships of MTAs Shared by Diverse Elements with Trait Correlation

Under LW conditions, 23 MTAs were shared by several elements (Table 3). Some of these co-positional relationships were paralleled by medium to strong significant correlations between the element concentrations in the grains that included Ca/Ni (qCa1.8/qNi1.170), Ni/Mg/P (qNi4.175/qMg4.128/qP4.189), P/Mg (qP4.190/qMg4.129 and qP7.193/qMg7.131), P/Cu (qP7.196/qCu7.97), Cd/Ni (qCd7.43/qNi7.176), As/Fe (qAs10.2/qFe10.107). Similarly, under PF conditions, 37 MTAs were shared by two or more elements and, in several cases, accompanied by medium to strong significant correlations between the element concentrations in the rice grains. These situations included As/Cd, strongly negatively correlated (qAs1.3/qCd1.58 and qAs10.5/qCd10.83), Mn/Ca (qMn3.150/qCa3.22), Mg/P (qMg3.133/qP3.200, qMg4.135/qP4.201, qMg7.136/qP7.205, qMg7.137/qP7.206, qMg7.138/qP7.207), Ni/Mo (qNi6.185/qMo6.167), Fe/P (qFe6.109/qP6.203 and qFe9.111/qP9.208), P/Cu (qP6.204/qCu6.98), Cu/Fe (qCu7.100/qFe7.110), Ni/Cd (qNi8.186/qCd8.73), Mg/P/Cu (qMg10.139/qP10.210/qCu10.101), Cd/Na, negatively correlated (qCd11.89/qNa11.229), Mo/K/P (qMo11.169/qK11.126/qP11.211). These relationships could be explained by an overlap in uptake and transport mechanisms for these elements within the plant. Indeed, there have been several studies that reported correlations between different trace elements (Zhang et al. 2014; Nawaz et al. 2015; Yu et al. 2015; Cu et al. 2021) and between essential minerals and toxic elements (Zhang et al. 2014; Cu et al. 2021). These co-localisations of MTA for diverse elements, accompanied by their correlations in concentrations, support the possibility of simultaneous improvement of these elements in the rice grain. As an example, the co-localisations of Na/Zn (Chr 2 LW), Ni, Mg, K (Chr 4 LW), Fe/P (Chr 6 PF), Cu/Fe (Chr 7 PF), Fe/P (Chr 9 PF), Mo/K/P (Chr 11 PF) MTAs support the possibility of simultaneous improvement of these elements in the rice grain. However, the simultaneous presence of favourable alleles for P and Fe is not desirable since in mature grain, P is mainly stored as phytate (myo-inositol-1,2,3,4,5,6-hexakisphosphate, InsP6), which can complex Fe forming insoluble complexes that cannot be digested or absorbed by humans (Gibson et al. 2018).

Under LW condition, for qNi1.172/qNa1.212, qNa2.214/qZn2.230, qCa3.9/qCd3.35, qCd10.51/qMg10.132, despite their colocalization, no significant correlation among the element concentration was observed. This effect could be explained by the polygenic nature of the analyzed traits so that an MTA can be responsible for only a small fraction of the phenotypic variation, thus leading to the absence of relationships between MTA co-localization and correlations between element concentrations.

Key Candidate Genes Underlying the Most Robust MTAs

Concerning the 39 MTAs with $-\log_{10}(p) > 5$ and the $R^2 > 6$ (Supplementary Table S4), candidate genes were identified for 14 MTAs (Table 4)) and the haplotypes of these MTAs were also analyzed, taking into account the allele frequencies in the subsets of genotypes belonging to the 2nd and 98th percentiles for low and high content of each element, respectively (Table 5).

Interestingly, both the qAs10.2(LW) and qAs3.4(PF) MTAs contain genes involved in processes that control As stress. The qAs10.2 (LW) includes the *OsCesA 7* gene, which encodes a protein belonging to the rice Cellulose synthase and Cellulose Synthase-like (CesA/CSL) family. This protein is involved in the As stress response in rice through structural and regulatory mechanisms (Shabab et al. 2025). The qAs3.4(PF) contains the *OsFbox131* gene that belongs to the F-box protein family, some elements of which regulate As(V) (the molecular form dominant in flooded soils) stress responses in *A. thaliana* (Peña-Garcia et al. 2021). The accessions with the G haplotype (18.4% of total frequency) at the S10_17,255,433 peak marker of the qAs10.2(LW) MTA have 47% higher As concentrations in brown grain than the accessions with the A haplotype. In the subset of accessions with the grain As concentrations in the 98th percentile, the frequency of the G haplotype increases from 18% up to 66%, while none of the accessions in the 2nd percentile exhibit this allele. Thus, the A haplotype can be considered an interesting candidate for the preliminary identification of rice genotypes that can limit As accumulation in LW conditions. Similarly, the less frequent C haplotype (34% of the total frequency) at S3_5,056,013 peak marker of qAs3.4(PF) is associated with higher As accumulation (+18%). Accessions belonging to the 98th percentile show an 83% frequency of the minor allele C. In contrast, no accessions belonging to the 2nd percentile have this haplotype suggesting that the C haplotype may be associated with an increased risk of As accumulation in the grain when plants are grown under PF conditions.

Staple crops such as rice are important dietary sources of magnesium, phosphorus, manganese, selenium, iron, folic acid, thiamin and niacin (Fukagawa and Ziska 2019), but major staple food grains (i.e., rice, wheat, and maize) that make up the central part of diets within both developing and industrialized countries are relatively poor sources of Ca (Knez and Stangoulis 2021). Therefore, biofortification to increase Ca in grains of staple food crops is a sustainable approach to alleviate this deficiency. In qCa10.19(LW) the *OsACA.8* gene is located approximately 110 kb downstream of the peak marker (S10_14,571,146). This gene encodes a Ca^{2+}-ATPase pump that, together with other Ca^{2+}-transporters, controls cellular Ca^{2+} homeostasis (Singh et al. 2014). The presence of the minor allele G (6% of the total frequency) at position S10_14,571,146 is associated with a significantly higher Ca level (+23% compared to the major allele A) in the grain of the related accessions.

Our analysis identified several candidate genes involved in Cd accumulation. The Cd absorbed by the roots, and subsequently accumulated in the brown grains, depends on the balance of various transport and metabolic activities within the plant. The major processes include the vacuolar compartmentalization of Cd as a free cation and/or chelation to thiol-rich non-protein peptides (phytochelatins, PCs), the efflux of the free cation from root cells towards the xylem and its accumulation in the vacuoles of leaf cells or in enlarged vascular bundles (VBs) localized in each node, and finally, the loading into the phloem directed to the developing grains by membrane transporters at the VBs of the panicle node (Ma et al. 2021; Zhang et al. 2022; Chen at al. 2023). Considering the LW condition, in which Cd availability was enhanced by the aerobic soil condition (Husson 2013; Orasen et al. 2019), two interesting genes, *OsCLT1* and *OsLCD*, were identified within the considered region of qCd1.31(LW). The former is located approximately 48 kb downstream of the peak marker S1_42,038,389 and encodes a CRT-like transporter (CLT) which mediates the export of γ-glutamylcysteine (γ-EC) from the plastid and ensures Glutathione (GSH) homeostasis, precursor of PCs (Yang et al. 2016). The latter is located approximately 124 kb downstream of the peak marker and encodes the low Cd transporter OsLCD, which is expressed in the vascular tissues of roots and phloem companion cells in leaves and is involved in Cd partitioning within the plant, including the grains (Shimo et al. 2011). All the accessions showing the highest Cd accumulation in the grain (98th percentile) exhibited the minor haplotype G at S1_42,038,389. Furthermore, the average Cd concentration in brown grain of accessions with this haplotype (6% of the total frequency) were 80% higher than in accessions showing the major haplotype A (94% of the total frequency). A gene encoding the primary transcript of miR396 (OsmiR396c) was identified for qCd2.32(LW) MTA. This miRNA, which is highly conserved among different species, targets the GRF (Growth-Regulating Factors) transcription factors. The miR396-GRF module is known to influence rice yield and carbon/nitrogen allocation, as well as blast resistance (Li et al. 2018; Che et al. 2016; Duan et al. 2016; Chandran et al. 2019). Evidence from rice has shown that Cd stress downregulates miR396 (Ding et al. 2011, 2013; Tang et al. 2014). Similar observations have been made in maize, *Arabidopsis*, soybean and radish (Pegler et al. 2021; Gao et al. 2019; Xu et al. 2013; Fang et al. 2013), but the mechanism of action is yet to be elucidated. The accessions with the minor allele G (6% of the total frequency) exhibited a higher Cd concentration (+122%) in brown grains. The accessions in the 98th percentile showed a high frequency (67%) of G allele, which can be therefore considered a putative marker for Cd accumulation in rice brown grains.

Two genes, *OsNAC15* and *OsWOX11*, were present within a 100 kb region upstream and downstream of the S7_29,124,793 peak SNP of qCd7.43(LW). *OsNAC15* encodes a NAM/CUC2-like (no apical meristem/cup-shaped cotyledon) transcription factor, which is reported to contribute to Zn deficiency tolerance and Cd stress tolerance and whose expression is induced by Cd stress (Zhan et al. 2022). *WUSCHEL-related homeobox (WOX)* genes encode transcription factors that are involved in organogenesis, as well as responses to biotic and abiotic stresses (Rasheed et al. 2024; Khan et al. 2024). *OsWOX11* is expressed in root meristems, where it plays a role in crown root growth and in the response to abiotic stress. Its expression is also modulated by phytohormones (Cheng et al. 2014). The structure of the *OsWOX11* promoter has recently attracted particular attention due to the presence of interesting *cis*-regulatory elements (CREs), which could be exploited for biotechnological purposes to enhance Cd stress resistance in rice (Xu et al. 2024). A *OsHIPP9* (*Heavy Metal-associated*

Table 5 Haplotype of the peak marker of the MTAs with -$\log_{10}(p) < 5.00$ and $R^2 > 6$ containing the main candidate genes

Element	MTA ID	Peak Marker/Region (bp)	Haplotype	Element Concentration	Allele Frequency %		
				mg kg^{-1}	Total Panel	2nd perc.	98th perc
As	qAs10.2 (LW)	S10_17,255,433	A	0.036	82	100	33
			G	0.053	18	0	66
As	qAs3.4 (PF)	S3_5,056,013	G	0.119	66	100	17
			C	0.140	34	0	83
Ca	qCa10.19 (LW)	S10_14,571,146	A	90.604	94	100	66
			G	111.082	6	0	33
Cd	qCd1.31 (LW)	S1_42,038,389	A	0.053	93	100	33
			G	0.095	7	0	66
Cd	qCd2.32 (LW)	S2_34,364,220	A	0.052	95	100	33
			G	0.116	5	0	66
Cd	qCd7.43 (LW)	S7_29,124,793	T	0.050	80	100	20
			G	0.075	20	0	80
Cd	qCd2.61 (PF)	S2_12,633,082	C	0.027	94	100	83
			G	0.040	6	0	17
Cd	qCd6.70 (PF)	S6_29,440,451	C	0.028	94	100	83
			T	0.032	6	0	17
Cd	qCd8.78 (PF)	S8_27,052,235	G	0.027	95	100	66
			A	0.041	5	0	33
Fe	qFe8.105 (LW)	S8_8,563,018	A	8.334	84	100	0
			C	10.117	16	0	100
Mn	qMn3.141 (LW)	S3_6,723,314	A	16.371	68	17	100
			G	14.726	32	83	0
Mn	qMn3.148 (PF)	S3_6,723,314	A	21.522	68	50	100
			G	18.987	32	50	0
Mo	qMo8.157 (LW)	S8_132,980	T	0.550	80	17	100
			A	0.421	20	83	0
P	qP4.189 (LW)	S4_27,790,754	A	3731.253	92	100	17
			G	4077.175	8	0	83

The table also reports the mean concentration values (mg kg^{-1}) of each element measured in the panel genotypes that show the same haplotype for different MTAs. It shows also the allele frequencies in the total panel genotypes and in the genotypes belonging to the 2nd and 98th percentiles for the low and high content of each element, respectively (see also Supplementary Table S1)

Isoprenyl Plant Protein 9) gene was also present approximately 200 kb upstream the S7_29,124,793 peak SNP of qCd7.43(LW). This gene encodes a metallochaperone involved in metal homeostasis and detoxification. It plays a dual role in rice, chelating Cd in the xylem region of the EVB to retain it in the nodes, and chelating Cu in the roots to facilitate its absorption (Xiong et al. 2023). Accessions with the minor allele G (20% of frequency) exhibited a higher concentration of Cd (+49%) in brown grains. This allele was highly prevalent (80%) in accessions in the 98th percentile yet absent in those in the 2nd percentile.

Considering the PF condition, two genes encoding proteins putatively involved in Cd transport, OsCAX1c and OsHMA2, were identified in qCd2.61(PF) and qCd6.70(PF), respectively. The *OsCAX1c* gene, whose expression is specific to leaf, is located approximately 200 kb upstream of qCd2.61(PF) peak marker S2_12,633,082. This gene encodes a tonoplast cation/H^+ exchanger (CAX) that uses Ca^{2+} as substrate, but can also use Cd^{2+} when present, accumulating them into the vacuole (Zou et al. 2021). The *OsHMA2* gene, which encodes a plasma membrane P1B-type ATPase with a heavy metal-associated domain (HMA), is located within qCd.6.70(PF) MTA at approximately 33 kb from the peak marker (S6_29,440,451). *OsHMA2* has been widely described to control Zn/Cd translocation from root to shoot in rice by regulating Cd loading into the xylem (Nocito et al. 2011; Takahashi et al. 2012) and thus contributing to Cd accumulation into the grain (Maghrebi et al. 2021). Accessions with the minor allele at both S2_12,633,082 (G) and S6_29,440,451 (T) had higher Cd content in brown grain (+45% and +15%, respectively) than those with the major allele C. Consistent with this observation, all accessions in the 98th percentile for high Cd content showed the minor alleles at both the peak markers. The *OsPDR1* gene is located approximately 200 kb downstream of the S8_27052235 SNP leader of qCd8.78(PF). This gene is reported to be rapidly and strongly induced by Cd stress in rice. It encodes a member of the pleiotropic drug resistance (PDR) subfamily of ATP-binding cassette (ABC) transporters possibly involved in the active extrusion of Cd from the cytoplasm. A similar plasma membrane-located orthologue in *Arabidopsis* (AtPDR8), which is induced by Cd and lead (Pb), acts as Cd/Pb extrusion pump (Ogawa et al. 2009). Accessions with the minor allele A (5% of the total frequency) exhibited a higher concentration of Cd (+ 50%) in brown grains. This allele, which was absent in accessions in the 2nd percentile and present at a low frequency (33%) in those in the 98th percentile.

The *WOX10* gene, which encodes a transcription factor responsible for activating genes involved in Fe binding and translocation in rice (Kawai et al. 2022), is located 76,175 kb downstream of the S8_8,563,018 peak marker of qFe8.105(LW). On average, the accessions with the minor allele C accumulate higher levels of Fe in the grain (+ 21%) than those with the major allele A, and all the accessions in the 98th percentile have the C haplotype. This information can be used in breeding programs for biofortification of Fe in rice.

Mn is an essential trace element for humans and usually present at low concentrations in all diets. Nevertheless, groups of populations still suffer from subclinical Mn deficiency, therefore producing rice with adequate levels of Mn would benefit human health. The same MTA for Mn (qMn3.141(LW) or qMn3.148(PF) was found regardless of the two irrigation conditions, despite the fact that these conditions have different effects on the metal bioavailability in the soil and, thus, on its accumulation in the grain (higher in LW than in PF). Within this MTA was identified the *OsMTP8.1* gene, which encodes a tonoplast-localized Mn transporter belonging to the cation diffusion facilitator family and whose mutation can alter Mn transport and accumulation in plants (Chen et al. 2016). The presence of the minor allelic variant (G) at the peak marker (S3_6,723,314) is associated with a 11/13% lower Mn grain concentration than that measured in the presence of the A major allele.

Rice is one of the main dietary sources of Mo for humans (Hattori et al. 2004). The *OsMOT1;1* gene, which encodes a molybdate transporter expressed mainly in roots, has been identified in the qMo8.157(LW) MTA; this gene has been reported to be the causal gene for the qGMo8 QTL, with sequence variation in its promoter controlling the accumulation of this element in rice grain (Huang et al. 2019). On average, the accessions with the minor haplotype A at the peak marker S8_132,980 have a lower Mo grain concentration (approximately − 31%) than the accessions with the major haplotype T. This information could help to develop rice genotypes with Mo-enriched grains or to prevent excessive accumulation of the element in grain when plants are grown in Mo-rich soil.

More than 90% of the total P in rice grains is present as phytate, which is indigestible to humans and act as an antinutrient because phytic acid forms strong complexes with micronutrients such as Zn and Fe (Raboy 2020). For this reason, accumulation of P in the form of phytate in the grains must be limited. The *OsVPE1* (*Vacuolar Pi Efflux 1*) gene, which is very close to the peak marker S4_27,790,754 (approximately 7,174 kb upstream), has been identified in qP4.189(LW). *OsVPE1* encodes a tonoplast transporter that releases Pi from the vacuole under P starvation (Xu et al. 2019), as well as after flowering, when P is remobilized from leaf vacuole to the panicle (Julia et al. 2016). On average, the P content of grains with the G allelic variant at SNP S4_27790754 is approximately 10% higher than that of grains with the A allelic variant.

Conclusions

This work analysed the accumulation of 13 elements in 294 *japonica* rice accessions under two water management conditions, leading to several outputs. The used rice panel showed a wide variability in element concentration depending on the accession, with higher variability observed under PF. The large dataset produced could be very useful in identifying accessions that can provide good accumulation of useful elements or low accumulation of undesirable elements under PF or LW. The GWAS identified 87 MTAs with high R^2 and p-value, and the data on the effect of the alleles at the peak SNPs on grain element concentration support their usefulness as markers in rice breeding programs for increasing or decreasing the concentration of useful or toxic elements in rice grains, respectively. The robustness of several of these MTAs was confirmed by (i) their presence in both PF and LW, (ii) their overlapping with MTAs for multiple elements, and (iii) their co-positional relationships with previously mapped QTLs for the same elements. For 14 of these MTAs, robust candidate genes were identified, whose functions were consistent with an involvement in affecting grain concentration of the element underlying the MTA. Further functional studies involving gene knockout on selected candidates may help to find causal relationships between candidate genes and phenotypes. Genome editing approaches could be then used to lower heavy metals concentration while increasing essential minerals amount in rice. In conclusion, the presented results represent an important resource to potential gene-stacking for multiple micronutrients and rice biofortification, using a rational combination of conventional breeding and metabolic engineering strategies.

Abbreviations

ANOVA	Analysis of Variance
As	Arsenic
Ca	Calcium
Cd	Cadmium
CLT	CRT-like transporter
CRI	Collision-Reaction-Interface
Cu	Copper
CV	Coefficient of variation
FDR	False discovery rate
Fe	Iron
γ-EC	γ-glutamylcysteine
GBS	Genotyping-by-sequencing
Ge	Germanium
GSH	Glutathione
GRF	Growth-Regulating Factors
GWAS	Genome-wide association studies
G×E	Genotype × Environment
H^2	Broad sense heritability
H_2	Hydrogen
HMA	Heavy metal-associated domain
ICP-MS	Inductively Coupled Plasma Mass Spectrometer
Y	Yttrium
K	Potassium
K	Kinship matrix
kbp	Kilo base pairs
LD	Linkage disequilibrium
LW	Low watering
MAF	Minimum allele frequency
Mb	Mega base pairs
Mg	Magnesium
MLM	Mixed Linear Models
Mn	Manganese
Mo	Molybdenum
MTA	Marker-traits association
Na	Sodium
Ni	Nickel
P	Phosphorus
PCA	Principal Components Analysis
PCs	Phytochelatins
PF	Permanent flooding
QTL	Quantitative Trait Locus
R^2	Explained variance
SNP	Single Nucleotide Polymorphism
ssp	Subspecies
Tb	Terbium
USDA	U.S. Department of Agriculture
VB	Vascular bundles
VPE1	Vacuolar Pi Efflux 1
Zn	Zinc
σ^2	Variance

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

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Author Contributions

GV and GAS conceived the work and designed the experiment. GO collected all the raw data. GO, GL, FFN, EB collaborated in the ionomic analyses by ICP-MS. GO and EM analyzed experimental data and performed statistical analyses. EM and AT performed GWAS analysis. NN and GAS performed candidate genes search and analyses. NN, GAS, GV, EM wrote the paper with the contribution of all authors. All the authors read and approved the final manuscript.

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Availability of data and material.

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Data Availability

No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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