



Mapping QTLs for grain iron, zinc, and yield traits in advanced backcross inbred lines of Samba mahsuri (BPT5204)/*Oryza rufipogon*

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Abstract

Iron and zinc deficiency is a major problem among large populations in rice-consuming countries. Development micronutrient dense rice varieties with high yield is a key target area in breeding programmes and QTL mapping studies using backcross inbred lines to transfer beneficial genes from wild relatives is one of the potential strategy. In this study, 136 BC₄F₁₀ backcross inbred lines (BILs) from BPT5204 x *Oryza rufipogon* WR119 were field evaluated for 3 years for nine yield related traits. Grain Fe and Zn were estimated using ED-XRF. In all, 11 major QTLs with phenotypic variance from 10 to 16.8% were identified for Fe, Zn, and 5 yield related traits. *O. rufipogon* alleles were trait-enhancing in 18% of all QTLs and an allele at *qFe2.1* increased iron concentration. Major effect QTLs *qFe1.1* for grain Fe and *qZn5.1*, *qZn8.1*, and *qZn10.1* for grain Zn explained 11 to 16% PVE, *qZn8.1* and *qZn10.1* were co-located with QTLs for grain yield related traits. Seven chromosomal regions showed QTLs for more than two traits. QTLs were associated with several high priority candidate genes for grain Fe, Zn and yield. One elite BIL [IET 24775 RP4920-Bio51B] was tested in AICRIP bio fortification trials for 4 years [2014–2017], and three BILs [IET 28715 RP4920-Bio61-1B], [IET28706 RP4920-Bio83B] and [IET28695 RP4920-Bio88B] are evaluated for 2 years of trials. The significant BILs and QTLs are useful in rice bio fortification and for gene discovery.

Keywords Bio fortification · Grain Fe and Zn concentration · *Oryza rufipogon* · QTL mapping · Wild introgression

Abbreviations

BM	Biomass
BY	Bulk yield
DFF	Days to 50% flowering
EAR	Estimated average requirement
GWAS	Genome-wide association studies
MAGIC	Multi-parent advanced generation inter-cross
NPN	Number of productive tillers per plant
PH	Plant height
PW	Panicle weight
RCBD	Randomized complete block design
TN	Number of tillers per plant

QTL	Quantitative trait loci
YLDP	Yield per plant

Introduction

Rice (*Oryza sativa*) is the most important staple food and the major dietary carbohydrate source for more than half of the world's population. It is cultivated over 165.25 million hectares accounting for about 502.98 million tons milled rice production worldwide (Statista 2023). In rice, micronutrients are largely stored in the husk, aleurone layer, and embryo, of which a large portion is lost during milling and polishing. Brown rice is rich in mineral content; however, rice is mostly consumed in the polished form that contains low mineral levels. Polished rice is a poor source of many essential micronutrients and vitamins leading to malnutrition in developing countries where it constitutes the primary diet. Polished rice contains an average of only 2 parts per million (ppm) iron and 12 ppm of zinc whereas nutritional targets for 30% estimated average requirement (EAR) in rice grains is 13 ppm iron and 28 ppm zinc (FAO Stats 2018)

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to meet the daily requirement in diet. Globally more than two billion people, particularly children and pregnant and lactating women suffer from Fe and Zn deficiencies (Young et al. 2023). Under nutrition is the main cause of 45% of deaths in children aged under 5 years of age and recent estimates indicate that 155 million children suffer from stunting of which more than 50% live in Asia and more than 25% live in Africa where there is an alarmingly high incidence of malnutrition (WHO 2021). Iron (Fe) and zinc (Zn) are highly essential for the normal growth and development of both plants and human beings. Iron (Fe) plays a crucial role in electron transfer reactions within mitochondria and chloroplasts, and is also involved in heme and chlorophyll biosynthesis and serves as cofactor for various enzymes (Briat et al. 2015). It is mainly essential for oxygen transport as it is the functional component of haemoglobin, myoglobin and also involved in vitamin D metabolism (Singh et al. 2016; Toxqui and Vaquero 2015). Zinc (Zn) is a major co-factor for more than 300 enzymes and 2000 transcription factors involved in major biological functions (Ozyildirim and Baltaci 2022). It also aids in nucleic acid metabolism, apoptosis, immune function and reproductive processes (Wairich et al. 2022). Fe deficiency causes anemia, reduced growth and poor cognitive development, whereas Zn deficiency causes stunting, reduced immunity, diarrhea, lesions on eyes and skin, delayed healing of wounds and mental lethargy, etc. (Hotz and Brown 2004). Approximately 33% of the global population are suffering from iron deficiency anaemia (IDA) and 20% with zinc deficiency, making hidden hunger a widespread global challenge. Iron (Fe) and zinc (Zn) deficiencies are prevalent nutritional concerns affecting a substantial portion of the human population (Garcia-Oliveira et al. 2018; United Nations Environment Programme 2021). Therefore, increasing the nutrient concentration of Fe and Zn in the grain through biofortification is a high-priority research area. Many efforts are being made around the world to breed Fe/ Zn dense grain crops as the most cost-effective and sustainable strategy to solve micronutrient malnutrition for people living in developing countries that cannot afford Fe and Zn fortified foods in their staple diets. The Bio-fortification Priority Index, clearly highlighted the urgent need for iron (Fe) and zinc (Zn) bio fortification in several Asian countries (Harvest Plus 2020). The International Rice Research Institute (IRRI) and its collaborating national partners in the target countries are actively engaged in developing rice varieties biofortified with iron and zinc to address nutritional deficiencies. So far, 29 high Zn rice varieties have been developed and released for commercial cultivation in Bangladesh, India, Indonesia and Philippines of which a maximum of 16 are from India (Senguttuvel et al. 2023). ICAR-Indian Institute of Rice Research in Hyderabad has developed six high zinc rice varieties to date. Recently, DRR Dhan67 and DRR Dhan69, with respective zinc

concentration of 27.6 ppm and 25.7 ppm in polished grain, were released in 2022. There is a wide variation for grain Fe and Zn in rice germplasm, which can be exploited in rice biofortification breeding programs.

Wild rice accessions of *O. nivara* and *O. rufipogon* have the highest concentration of grain Fe and Zn compared to the cultivars (Anuradha et al. 2012a). The grain Zn concentration in brown rice of the A-genome wild rice species, *O. rufipogon*, *O. meridionalis*, *O. glumaepatula*, and *O. barthii* was reported to be higher compared to the cultivars of *O. sativa* Japonica ‘Nipponbare’ and Indica ‘IR36’ (Ishikawa et al. 2017). It is known that the bioavailability of mineral nutrients in plant foods is low because of the presence of anti-nutrients such as polyphenols and phytic acid in plants (Samtiya et al. 2020). Phytic acid is a potent inhibitor of Fe and Zn absorption while ascorbic acid and nicotianamine (NA) are enhancers of Fe absorption (Zhang et al. 2021). The chelation of Fe and Zn with phytic acid has a strong negative effect on the absorption of these minerals in humans and other monogastric animals that largely lack the phytase enzyme, which is required to degrade phytate. Therefore, it is important to reduce the phytate levels, which in turn increases Fe and Zn bioavailability. The regulation of myo-inositol 3-phosphate synthase 1 (*INO1*) gene expression at early developmental stages is important in determining PA content in rice grain (Perera et al. 2019). Rice Mutants with low phytic acid concentration (54–64%) were developed through XS- LPA mutation in *OsMRP5* gene in basmati rice and the germination rate, yield was improved through back-cross breeding (Qamar et al. 2019). Efforts are being made to reduce the phytate level by transgenesis or overexpressing the gene for phytase which could be a cost-effective process for improving the bioavailability of micronutrients in rice.

Breeding of rice genotypes with enriched micronutrients can be accelerated through germplasm screening of landraces, traditional varieties and wild species to harness natural allelic variations. The A-genome wild progenitors (*O. nivara* and *O. rufipogon*) of cultivated rice are a rich source of natural allelic variations for several agronomic, grain quality and grain micronutrient traits (Swamy and Sarla 2008, 2011; Swamy et al. 2014). *O. rufipogon* has contributed several QTLs and genes for disease resistance, cold tolerance, fertility restoration, grain yield, and grain quality traits for the improvement of cultivated rice. 11 consistent QTLs from 3 consecutive generations derived from *O. rufipogon* for yield and photosynthesis traits and 145 CSSLs in BC₂F₂ generation representing 87% of *O. rufipogon* genome were identified (Yadavalli et al. 2022). Haritha et al. (2017) studied *O. rufipogon* ILs with enhanced photosynthetic rate (*Pn*) and showed these ILs can be used as varieties and to develop hybrids with higher biomass and yield. Hussain et al. (2020) identified SNPs/QTLs and determined the candidate genes influencing grain size in a mapping

population derived from *O. rufipogon*. McCouch et al. (2007) used the AB-QTL method to simultaneously detect and transfer yield-related QTLs from *O. rufipogon*. Garcia-Oliveira et al. (2009) identified 2QTLs for Fe and 4 QTLs for Zn from *O. rufipogon*. *Oryza rufipogon* showed higher spatial distribution of iron in the embryo region, endosperm, and aleurone layer. It also showed a higher expression of the ferritin gene and low levels of phytate concentration (Mishra et al. 2020). QTLs *qFe10* for grain iron and *qZn10* grain zinc were identified from 96 introgression lines derived from *O. rufipogon* (Adeva et al. 2023). QTL for grain Zn *qGZn9* was identified from *O. meridionalis* (Ishikawa et al. 2017). Liu et al. (2020) mapped 13 QTLs for Fe, Zn and Se in brown rice using 127 BILs derived from the African wild rice *Oryza longistaminata*. However, other wild rices have been less explored in QTL mapping for Fe and Zn concentration. QTLs *qFe2.1*, *qFe3.1*, *qFe8.2* and *qZn12.1* were consistently identified in two mapping populations of Swarna x *O. nivara* using two accessions IRGC81832 and IRGC81848 of *O. nivara* (Swamy et al. 2018a, b). Hu et al. (2016) identified 6 QTLs for grain Zn and 3 QTLs for grain Fe including both brown and milled rice from 202 BILs derived from *O. rufipogon*. In other studies, at IIRR, 14 QTLs for grain Fe and Zn were identified from a RIL mapping population of Madhukar x Swarna. Ten candidate genes known for Fe and Zn homeostasis were located in these QTL regions whereas another six candidate genes were close to QTLs on chromosomes 3, 5, and 7 respectively and the importance of the candidate genes *OsYSL1* and *OsMTP1* for Fe; *OsARD2*, *OsIRT1*, *OsNAS1*, and *OsNAS2* for Zn; and *OsNAS3*, *OsN-RAMP1*, heavy metal ion transport, and *APRT* for both Fe and Zn in rice bio-fortification was emphasized (Anuradha et al. 2012b). Two SSR markers RM3322 and RM7488 were found significantly associated with grain Fe and Zn in RILs of Jalmagna / Swarna (Kiranmayi et al. 2014). Two alleles of a *Gramineae*-specific heavy metal transporter gene *OsHMA7* were identified by analyzing the expression patterns and sequences of genes within QTLs for high Fe and Zn in Madhukar x Swarna recombinant inbred lines (RILs) (Kappara et al. 2018).

INGR20003185M (IET 23814), which is an F10 recombinant inbred line (RIL) derived from Madhukar x Swarna, showed high grain Zn (32 ppm overall mean) in polished rice across 15 locations in India (Agarwal et al. 2018). Eight major effect QTLs for grain Zn were identified on chromosomes 2, 3, 6, 8, 11 and 12 with a PVE of up to 22% in a double haploid mapping population of rice (Swamy et al. 2018b). Genome-wide association studies (GWAS) were conducted using a Multi-parent Advanced Generation Inter-Cross (MAGIC) Plus population to identify QTLs and SNP markers that could potentially be integrated in biofortification and disease resistance breeding (Descalsota et al. 2018). Consistent QTLs for grain Zn in polished rice (*qZnPR.2.1*)

and Zn brown (*qZnBR.2.2*) were identified on chromosome 2 in RILs which are associated to the key genes related to transporter activity (Uttam et al. 2022). Dixit et al. (2019) identified four consistent QTLs for Fe (*qFe1.1*, *qFe1.2*, *qFe6.1* and *qFe6.2*), two QTLs for Zn (*qZn1.1* and *qZn6.2*) and three meta-QTLs for grain Fe (*MFeQTL7.1*, *MFeQTL12.1*, *MFeQTL12.2*) and two (*MZnQTL7.1* and *MZnQTL12.1*) for grain Zn. Calayugan et al. (2020) identified five QTLs for Fe and Zn that harbor several candidate genes, e.g. *OsZIP6* on QTL *qZn5.1* and also reported that a number of QTLs were associated with a combination of greater yield and increased grain Zn levels in a double haploid population of Japonica rice. Integration of omics tools and breeding methods is more efficient for enhancing rice nutritional quality and maintaining food security for the growing global population (Rana et al. 2019). In this study, we used *O. rufipogon* derived backcross mapping population to identify consistent and stable QTLs for grain Fe, Zn and yield-related traits.

Materials and methods

Plant material

An interspecific cross was made using an elite fine grain indica rice cultivar BPT5204 (*O. sativa*) also known as Samba Mahsuri as a recurrent parent and a wild accession *O. rufipogon* (*WR119*) (Fig. 1) as a donor parent and a set of 136 BC₄F₁₀ BILs was raised after four consecutive backcrosses. These 136 BILs were used in the present study to identify QTLs for grain Fe, Zn and yield related traits.

Phenotyping

Yield trait estimation

136 BC₄F₁₀ lines along with recurrent parent BPT5204 were grown at Indian Institute of Rice Research (IIRR), Hyderabad for 3 years with 2 replicates of each season, which includes one wet season—Kharif 2015 and two dry seasons -Rabi2016 and Rabi2017. The following yield related traits were evaluated first and then grain Fe and Zn concentration was estimated for each of the 3 years: days to fifty percent flowering (DFF; number of days from sowing to that of 50% flowering), plant height (PH; height of the plant in centimeters from the soil surface to the tip of the primary panicle at the time of harvest), number of tillers per plant (TN; average number of tillers per plant at the time of harvest), number of productive tillers per plant (NPN; Total number of panicle-bearing tillers per each plant at the time of harvest), panicle weight (PW; average weight of five panicles per plant), yield per plant (YLDP; weight of the well dried and cleaned seeds per plant), bulk yield (BY; weight



Fig. 1 Plants, Seeds and field view of BPT5204 and *O. rufipogon* and BILs. **A** Plants of parents BPT5204 and *O. rufipogon* (WR119). **B** Seeds of parents BPT5204 and *O. rufipogon* (WR119). **C** Variation of grain type in both paddy and de-husked seed of BILs. **D** Field view

of BILs at different flowering and maturity stages. **E** Panicles of elite BILs 51B, 61-1B and 83B with BPT5204. **F** Polished seed of elite BILs 51B, 61-1B and 83B with BPT5204

of dried and cleaned seeds from 33 plants per square meter of the plot), biomass (BM; dry weight of mature harvested plants without panicles). Randomized Complete Block Design (RCBD) with two replications was followed to conduct the experiments.

Fe and Zn estimation

Fe and Zn were analyzed in rice grains using non-destructive, energy-dispersive X-ray fluorescence spectrometry (EDXRF) instrument (model X-Supreme 8000; Oxford Instruments plc, Abingdon, UK) at IIRR, Hyderabad. 10 g of single plant paddy was de-husked using metal free dehusker (H-750, Krishi International, India). Whole grains were separated from the broken ones and used for analysis. 5 g of this brown rice was cleaned with tissue wipes and

analyzed for grain Fe and Zn using XRF spectrometry. In case of all the phenotypic data, least significant means were calculated from replicated data using PB tools (Version 1.4) and were used in further statistical analysis and QTL mapping.

Trait correlation

Correlation among the phenotypic traits was analyzed based on two sample *t*-test with equal variances. The data was imported to MS excel and the CORREL function in the data analysis tool pack to find the correlation coefficient between two variables. Correlation among all the phenotypic traits was computed at $P < 0.05$ and $P < 0.01$, respectively.

Genotyping

Fresh leaf samples of the 136 BILs along with the parents were collected and the total genomic DNA was isolated following CTAB method (Doyle and Doyle 1987). In all, 800 randomly selected microsatellite markers including 50 gene specific markers from all 12 chromosomes were used to identify polymorphism between the parents BPT5204 and *O. rufipogon* (WR119). 166 Simple Sequence Repeats (SSRs) (Supplementary Table 1) markers including 18 gene specific markers were polymorphic and also segregated in BC₄F₁₀ BILs. Polymerase Chain Reaction was carried out in thermal cycler (T100 Bio Rad U.S.A) with a final reaction mixture volume of 10 µl consisting 20 ng of genomic DNA, 1X assay buffer, 200 µM of dNTPs, 1.5 mM MgCl₂, 10 pmol of forward and reverse primers and 1 unit of Taq DNA polymerase (Thermo Scientific, USA). PCR cycles were programmed as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension of 10 min at 72 °C. The amplified PCR products were resolved in 3% agarose gel prepared in 0.5×TBE buffer with 0.5 µg/ml of ethidium bromide added and electrophoresed for 2 h at 140 V. These gels were documented using gel documentation system (InGenius 3, Syngene, USA).

Construction of linkage map and QTL analysis

The genotypic data of 136 BILs with 166 polymorphic markers (Supplementary Table 1) on all the 12 chromosomes was used for the construction of linkage map using the mapping function Kosambi (Kosambi 1944) of QTL IciMapping v4.1. QTLs were identified using Inclusive Composite Interval Mapping (ICIM). Analysis was undertaken using automatic

parameter setting and controlling marker forward stepwise. The threshold of LOD for declaring the presence of significant QTL for each trait was determined using 1000 permutations at $\alpha = 0.05$, the LOD threshold values ranged from 0.9 to 13.6. Marker trait associations were detected using Single marker analysis in ICIM.

Candidate gene analysis

All the major effect and the consistent minor effect QTLs were analyzed in silico for the identification of candidate genes. The physical positions of the flanking markers of QTLs were obtained from Gramene (www.gramene.org). All the genes present within and close (≤ 5 Mb) to major effect and consistent minor QTL regions were downloaded from the Rice Annotation Project Data Base (<http://rapdb.dna.affrc.go.jp>).

Results

Phenotypic evaluation

The grain Fe, Zn in brown rice samples and nine yield traits of BILs were analyzed each year. Year wise mean values for the all the phenotypic traits evaluated with the recurrent parent BPT5204 are presented in Table 1. The highest range of variation 7.8–17.8 ppm for Fe and 7.3–32.7 ppm for Zn was observed in Rabi2017 in the brown rice samples. BILs 72B and 105B showed the highest mean Fe and Zn concentration of 13.3 ppm and 25.5 ppm respectively across the years. BIL 11B had the highest grain Fe and 61-1B had the highest grain Zn concentration in polished rice samples. Positive transgressive

Table 1 Variation for grain Fe, Zn and yield related traits in BPT5204/*O. rufipogon* mapping population

Trait	Kharif2015		Rabi2016		Rabi2017	
	BPT5204	BILs (range)	BPT5204	BILs (range)	BPT5204	BILs (range)
DFP (days)	120	92–139	105	90–138	112	86–138
PH (cm)	75	50–100	77	52–104	78	58–100
TN	17	7–25	19	7–28	15	11–28
NPN	14	6–24	17	6–26	14	10–30
BM (g)	32.5	5.37–72.25	25.2	11.6–71.6	26.7	8.1–60.46
YLDP (g)	25	6.1–43.7	18	5.2–45.6	18.71	8–37.2
BY (g)	857	202–1444	618	171–1507	618	264–1227
PW (g)	1.9	0.3–4.0	1.6	0.5–3.8	1.4	0.7–3.1
TGW (g)	14.08	11.55–33.95	14.25	11.50–28.65	13.8	10.93–28.82
Fe (ppm)	7.8	7.5–13.2	6.3	4.5–17.2	9.4	8.2–14.2
Zn (ppm)	16.6	6.3–28.5	18.5	7.3–32.7	17.2	12.6–28

DFP: days to fifty percent flowering; PH: plant height, TN: number of tillers, NPN: number of productive tillers, BM: vegetative biomass, YLDP: yield per plant; BY: Bulk yield, PW: panicle weight; TGW: Thousand grain weight; Fe: grain Fe concentration in brown rice; Zn: grain Zn concentration in brown rice

segregants with more than 10% increase over BPT5204 for the 12 traits in all the 3 years were detected. Significant pair-wise mean comparison (at $P=0.05$) of the 136 BILs with BPT5204 as control using the phenotypic data of all the 12 traits with two replicates each showed that 101 BILs had significantly higher grain Fe. Among them 17 lines had significantly higher TGW also. IL 161B had significantly higher grain Fe, DFF, and TGW. BIL 142B was significantly taller while 191B was significantly shorter than BPT5204. Three lines 114B, 115B and 48B had significantly higher panicle weight. Five BILs 110B, 161B, 191B, 31B and 57B showed significantly higher and 100B significantly lower values for DFF. Overall, BILs 80B, 19B, 74B and 27B had highest grain yield with 3 years mean of 27–30 g per plant. Four lines 61-1B, 83B, 88B and 16-1B with high grain Fe, Zn, yield with low phytate concentrations were identified for multi-location trials.

Trait correlations

Significant correlations were observed among the traits across 3 years at $P < 0.05$ and $P < 0.01$ (Table 2). Correlation analysis of all the 3 years mean data indicated that plant height had significant correlation with biomass, single plant yield, panicle weight and bulk yield. TGW showed significant positive correlation with panicle weight. Grain Fe concentration had significant positive correlation with tiller number and number of productive tillers. Grain Zn had significant positive correlation with grain Fe in brown rice samples and was negatively correlated with thousand grain weight and panicle weight.

Genotyping of BILs

Allelic variations were detected at all 166 loci covering all chromosomes across 136 BILs. BPT5204 homozygous alleles accounted for 83%, *O. rufipogon* homozygous alleles for 10% and heterozygous alleles for 3.2% of all the loci in BILs. The highest number of *O. rufipogon* alleles were detected on chromosome 2 (82 alleles) and chromosome 12 (72 alleles) while the lowest number of *O. rufipogon* alleles were identified on chromosome 8 and 10 (1 allele at some loci). The *O. rufipogon* introgressions among the 136 BILs ranged from 5 to 46 with a mean value of 17. The number of heterozygotes at any locus varied from 0 to 48 (3.2%). RM210 on chromosome 8 followed by RM429 on chromosome 7 showed the highest number of heterozygotes. BIL 119B had the highest number of 33 heterozygotic loci.

Linkage map and QTL analysis

Linkage map was constructed based on the genotypic data of 136 BILs using 166 polymorphic SSR markers including 18 gene specific markers on all chromosomes (Fig. 2). The QTLs identified in at least two out of 3 years were considered as consistent QTLs. Further, QTLs showing $> 10\%$ PVE were considered as major QTLs. In all, 11 major QTLs were identified for Fe, Zn, DFF, PH, TN, NPN and PW. PVE explained by these 11 QTLs ranged from 10 to 16.8%. *O. rufipogon* alleles were trait enhancing in 18% of all QTLs.

Single marker analysis

Four markers were significantly associated with grain Fe and Zn concentration. RM316 on chromosome 9 showed

Table 2 Correlation of the Fe and Zn content with yield and related traits in BPT5204 \times *O. rufipogon* mapping population

	PH	TN	NPN	BM	YLDP	PW	BY	TGW	DFF	Fe	Zn
PH	1.00										
TN	-0.07	1.00									
NPN	-0.03	0.95***	1.00								
BM	0.19*	0.23	0.22*	1.00							
YLDP	0.09	0.23	0.23*	0.43***	1.00						
PW	0.25**	-0.15	-0.14	0.15	0.22*	1.00					
BY	0.09	0.23*	0.23*	0.43***	1.00***	0.22*	1.00				
TGW	0.11	-0.17	-0.17	-0.03	0.02	0.37***	0.02	1.00			
DFF	-0.08	-0.03	-0.08	0.00	0.01	-0.02	0.01	-0.16	1.00		
Fe	-0.19	0.25**	0.25**	-0.01	-0.06	-0.13	-0.06	-0.19	-0.07	1.00	
Zn	-0.16	0.16	0.14	-0.06	-0.06	-0.28	-0.06	-0.32**	0.07	0.34***	1.00

DFF: days to fifty percent flowering; PH: plant height, TN: number of tillers, NPN: number of productive tillers, BM: vegetative biomass, YLDP: yield per plant; BY: Bulk yield, PW: panicle weight; TGW: Thousand grain weight; Fe: grain Fe concentration in brown rice; Zn: grain Zn concentration in brown rice

*Significant at the 0.05 level (2-tailed). **Significant at the 0.01 level (2-tailed). ***Significant at the 0.001 level (2-tailed). For the correlation analysis mean data of 3 seasons for the all the traits was used

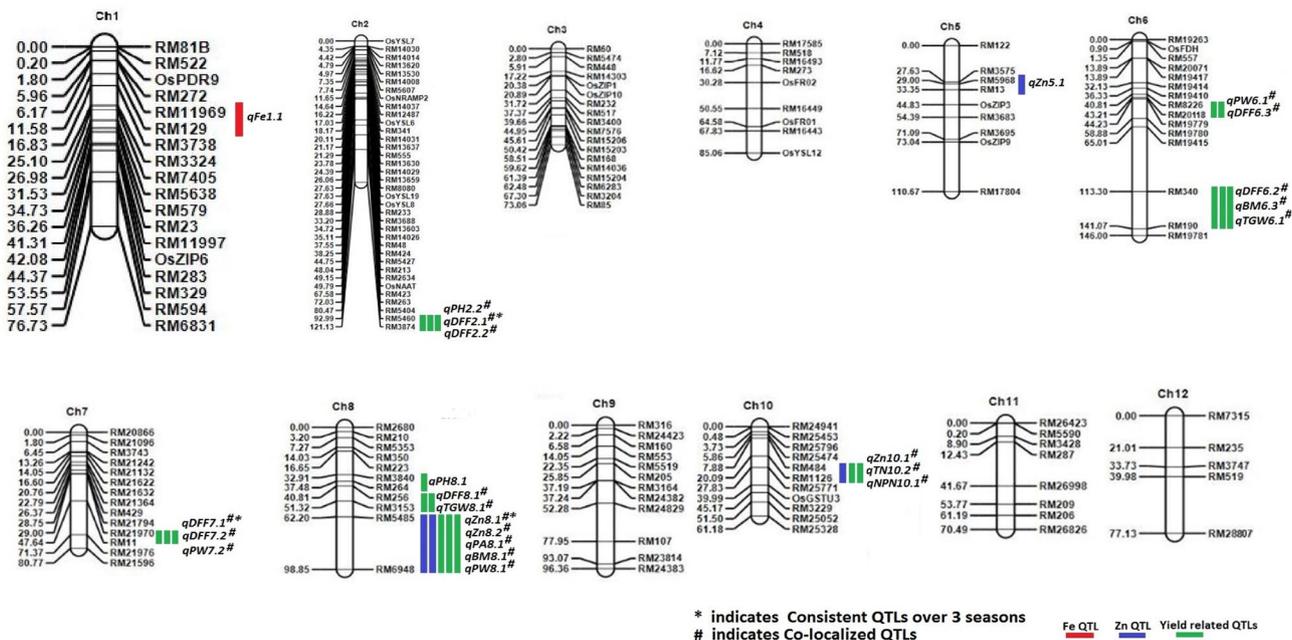


Fig. 2 Chromosomal locations of QTLs identified for grain concentration of Fe, Zn and yield related traits. *Ch: Chromosome number; Distances among markers are indicated in cM to the left of the linkage groups; names of the markers are shown on the right; *qFe*: QTL of iron; *qZn*: QTL of zinc; *qDFF*: QTL of days to fifty percent flowering; *qPH*: QTL of plant height; *qTN*: QTL of tiller number; *qNPN*: QTL of number of productive tillers; *qBM*: QTL of vegetative biomass; *qYLDP*: QTL of yield per plant; *qBY*: QTL of Bulk

yield, *qPW*: QTL for panicle weight; *qTGW*: QTL for thousand grain weight; Year 2015 indicates QTLs identified in kharif 2015; Year 2016 indicates QTLs identified in rabi 2016; Year 2017 indicates QTLs identified in 2017; QTLs underlined with red represents QTLs of grain Fe concentration; QTLs underlined with blue represents QTLs of grain Zn concentration; QTLs underlined with green represents major and consistent yield related QTL

significant association with grain Fe concentration in both 2016 and 2017. RM5968 and RM8226 correspond to the major QTLs *qZn5.1* and *qPW6.1* with PVE 12.1% and 12.6% respectively. RM 1126 on chromosome 10 was significantly associated with 5 traits PH, TN, NPN, TGW and Zn in all the three years. Markers RM3688, RM7576, RM19417, RM8226, RM21976, RM25052, RM1126 and RM519 were linked to TGW consistently across all the 3 years and also corresponded to the flanking markers of the minor QTLs associated with TGW. Of these TGW linked markers RM7576 and RM1126 were also significantly linked to Zn. RM3688 on chromosome 2 for TGW showed highest LOD value followed by RM1126 on chromosome 10 for Zn and TGW and RM519 on chromosome 12 for TGW (Supplementary Table 2).

QTL analysis for grain Fe and Zn concentrations

QTLs identified using ICIM are presented in Table 3 and Fig. 2. One major QTL for Fe and 3 major QTLs for Zn were identified and BPT5204 contributed the trait enhancing allele. In all, 13 QTLs were identified for grain iron on chromosomes 1, 2, 6, 8, 9 and 12 of which one is a major QTL (*qFe1.1*) with PVE 11% and the remaining 12 are minor

QTLs explaining PVE ranging from 0.4 to 5.6% and LOD from 2.6 to 13.4. All the QTLs for grain Fe concentration in brown rice samples which includes a major effect QTL *qFe7.1* of 2016 and two minor QTLs *qFe9.1* and *qFe12.1* of 2017 were from the recurrent parent BPT5204. A total of 10 QTLs were identified for grain zinc on chromosomes 1, 5, 6, 8, 10, and 12 of which 3 QTLs *qZn5.1*, *qZn8.1* and *qZn10.1* were major effect QTLs explaining PVE of 11%, 13% and 16% respectively with an additive effect of 1.8 ppm, 1.6 ppm and 1.9 ppm. BPT5204 allele was trait enhancing in all the major effect QTLs and a minor QTL *qZn1.1* of 2017. *O. rufipogon* alleles were trait enhancing in all the other minor QTLs.

QTLs for yield and related traits

In all, 7 major effect QTLs for grain yield and related traits were identified. Two major effect QTLs *qDFF7.1* and *qDFF7.2* with PVE of 16.8%, 13.9% and LOD of 4.3, 4.4 respectively were identified at the same chromosomal region RM21970–RM11 in both Rabi 2016 and Rabi 2017. For plant height, *qPH2.2* and *qPH8.1* were identified with PVE 12.4% and 10.1%, respectively in Rabi 2016. *qTN10.2* for tiller number and *qNPN10.1* for number of productive

Table 3 Major Effect QTLs for grain Fe, Zn and yield related traits detected using QTL IciMapping v4.1

Trait	QTL	Ch	Position (cM)	Left Marker	Right Marker	LOD	PVE (%)	Add	Trait enhancing allele	Season
Fe	<i>qFe1.1</i>	1	7	RM11969	RM129	3.1275	11.2855	1.0302	BPT5204	Rabi2016
Zn	<i>qZn5.1</i>	5	30	RM5968	RM13	5.5283	11.7959	1.9286	BPT5204	Rabi2017
	<i>qZn8.1</i>	8	89	RM5485	RM6948	3.745	16.1256	1.8513	BPT5204	Rabi2016
	<i>qZn10.1</i>	10	20	RM484	RM1126	6.5644	13.5482	1.5998	BPT5204	Rabi2016
DFF	<i>qDFF7.1</i>	7	39	RM21970	RM11	4.3971	16.8907	8.2327	BPT5204	Rabi2016
	<i>qDFF7.2</i>	7	39	RM21970	RM11	4.4042	13.9487	8.8881	BPT5204	Rabi2017
PH	<i>qPH2.2</i>	2	104	RM5460	RM3874	7.6205	12.4692	4.8965	BPT5204	Rabi2016
	<i>qPH 8.1</i>	8	37	RM3840	RM264	8.6065	10.1791	-3.7379	<i>O.rufipogon</i>	Rabi2016
TN	<i>qTN 10.2</i>	10	20	RM484	RM1126	4.8266	13.1978	1.9478	BPT5204	Rabi2017
NPN	<i>qNPN10.1</i>	10	20	RM484	RM1126	4.9866	14.8353	1.9247	BPT5204	Rabi2017
PW	<i>qPW6.1</i>	6	41	RM8226	RM20118	4.0035	12.6188	-0.3882	<i>O.rufipogon</i>	Kharif2015

*QTLs mentioned in the above table are the major effect QTLs with PVE > 10%; Fe: grain Fe concentration in brown rice; Zn: grain Zn concentration in brown rice; DFF: days to fifty percent flowering; PH: plant height; TN: number of tillers; NPN: number of productive tillers; PW: panicle weight; *q*: QTL; Ch: chromosome number; Position: QTL position in centi Morgan; Left Marker: Left flanking marker of the QTL; Right Marker: Right flanking marker of the QTL; LOD: Logarithm of odds value of the QTL; PVE: Phenotypic variance percentage; Add: Additive effect

tillers were identified in 2017 in the same marker interval of RM484–RM1126 on chromosome 10 with PVE 13.1% and 14.8% and LOD 4.8 and 4.9 respectively. One QTL for panicle weight *qPW6.1* was identified in Kharif 2015 with PVE 12.6% and LOD 4.0. *O.rufipogon* allele was trait enhancing for QTLs *qPW6.1* and *qPH8.1*.

Co-localized QTLs for Zn and yield traits

Grain Zn concentration and some yield related traits were significantly correlated and their QTLs were also co-located (Table 4). Two QTLs, *qZn8.1*, *qZn8.2* were co-located with the QTLs of yield related traits *qBM8.1*, *qBY8.1* and *qPW8.1* at the same loci RM5485–RM6948 on chromosome 8. The trait-increasing alleles in the co-located QTLs *qBM8.1*, *qBY8.1* and *qPW8.1* were derived from *O. rufipogon*. 2 QTLs *qTN10.2* and *qNPN10.1* were co-located with *qZn10.1*. *qDFF8.1* and *qTGW8.1* were co-located at the same chromosomal region RM256 and RM3153.

Candidate genes associated with major and consistent minor QTLs

The candidate genes underlying or close to (≤ 5 Mb) the major effect and the consistent minor QTL regions were identified and the details of these genes are presented (Table 5). In all, 19 candidate genes are present within the QTL region with the physical distance of 0.01–4.8 Mb from the flanking markers of the respective QTL. Two candidate genes *OsYSL18* (Iron (III)-deoxy mugineic acid transporter) and *OsHMP7* (heavy metal transport/detoxification protein) were identified within the QTL *qFe1.1* associated with grain Fe. *OsPDR3*, *OsSPL14* (*WFP*), *GW8* and a zinc iron permease protein were within a major QTL for grain zinc concentration *qZN8.1*. Gene for heading date *OsHDL1* and *OsZIP10* were identified within the QTL *qPW6.1*. Remorin was identified within *qDFF2.1*. *OsHd1*, *GW6* and *OsHd3a* were within *qTGW6.2*. Five genes *RAG2*, *OsZIP8*, *OsNAC3*, *OsNRAMP1* and *Ghd7* were within *qTGW7.3* associated with thousand grain weight.

Table 4 Chromosomal regions/Marker intervals harboring co-localized QTLs for Fe, Zn and yield traits

Ch	Marker interval	QTLs for Fe and Zn	QTLs for yield traits
2	RM5460 – RM3874	–	<i>qDFF2.2</i> , <i>qDFF2.1</i> , <i>qPH2.2</i>
6	RM340 – RM190	<i>qZn6.1</i>	<i>qDFF6.2</i> , <i>qBM6.3</i> , <i>qTGW6.1</i>
6	RM8226 – RM20118	–	<i>qPW6.1</i> , <i>qDFF6.3</i>
8	RM5485 – RM6948	<i>qZn8.1</i> , <i>qZn8.2</i>	<i>qBM8.1</i> , <i>qBY 8.1</i> , <i>qPW8.1</i>
8	RM256 – RM3153	–	<i>qDFF8.1</i> , <i>qTGW8.1</i>
10	RM484 – RM1126	<i>qZn10.1</i>	<i>qTN10.2</i> , <i>qNPN10.1</i>

*QTLs mentioned in the above table includes both major and minor QTLs co-located in the same chromosomal region; Ch: chromosome number; Marker interval: Right and Left markers flanking the respective QTL region

Table 5 List of Candidate genes associated with the major and consistent minor QTLs

QTL	Ch	Marker Interval	Gene	Gene ID	Site (Mb)
<i>qFe1.1</i>	1	RM11969–RM129	<i>OsHMP7*</i>	Os01t0826000-00	2.9a
			<i>OsYSL18*</i>	Os01t0829900-01	2.7a
<i>qZn5.1</i>	5	RM5968–RM13	<i>OsGATA15*</i>	Os05t0564300-01	0.05a
			<i>OsISC5*</i>	Os05t0568100-01	0.1a
			<i>OsZIP9</i>	Os05t0472400-01	5a
			<i>OsZIP5</i>	Os05t0472700-01	5a
<i>qZn8.1</i>	8	RM5485–RM6948	<i>OsPDR3*</i>	Os01t0609200	0.06a
			<i>OsSLG</i>	Os08t0562500-01	0.8b
			<i>WFP/IPAI/OsSPL14*</i>	Os08g0509600	1.2a
			<i>OsSPL16 (GW8)*</i>	Os08t0531600-01	0.8b
			<i>Zincfiron permease family protein*</i>	Os08t0467400-01	1.1a
<i>qZn 10.1</i>	10	RM484–RM1126	<i>OsMT-IIA</i>	Os10t0542100-01	0.1a
<i>qDFF7.1</i>	7	RM21970–RM11	<i>OsERF65</i>	Os07t0617000-02	0.04a
<i>qDFF7.2</i>	7	RM21970–RM11	<i>OsHMA7</i>	Os07t0623200-01	0.3a
<i>qPH2.2</i>	2	RM5460–RM3874	<i>MGDG synthase</i>	Os02t0802700-03	0.5a
			<i>YSL15</i>	Os02t0650300-02	2.3b
			<i>YSL2</i>	Os02t0649900-01	2.4b
<i>qPH 8.1</i>	8	RM3840–RM264	<i>OsSLG</i>	Os08t0562500-01	0.2a
			<i>OsPT6</i>	Os08t0564000-01	0.3a
<i>qTN 10.2</i>	10	RM484–RM1126	<i>OsMTA4</i>	Os10t0542100-01	0.1a
<i>qNPNI0.1</i>	10	RM484–RM1126	<i>OsGATA7</i>	Os10t0557600-01	0.9a
<i>qPW6.1</i>	6	RM8226–RM20118	<i>OsHdl *</i>	Os06t0275000-01	0.01a
			<i>OsZIP10*</i>	Os06t0566300-00	3.7b
<i>qDFF2.1</i>	2	RM5460–RM3874	<i>OsIpa1</i>	Os02t0819400-01	2.0a
			<i>Remorin*</i>	Os02t0602000-01	0.3b
<i>qTGW2.1</i>	2	RM3688–RM13603	<i>OsHMP15</i>	Os02g0584800	0.2a
			<i>OsYSL2</i>	Os02t0649900-01	1.6b
			<i>OsYSL15</i>	Os02t0650300-02	1.7b
			<i>GS2</i>	Os02t0701300-01	4.3b
<i>qTGW6.2</i>	6	RM190–19781	<i>OsHdl *</i>	Os06t0157500-01	0.02b
			<i>GW6*</i>	Os06t0266800-02	0.5b
			<i>OsHd3a*</i>	Os06t0157700-01	1.2a
			<i>RAG2*</i>	Os07t0214300-01	1.9a
<i>qTGW7.2</i>	7	RM21096–RM3743	<i>OsZIP8*</i>	Os07t0232800-00	3.0a
			<i>OsNAC3*</i>	Os07t0225300-01	2.6a
			<i>OsNRAMP1 *</i>	Os07t0258400-01	4.6a

Table 5 (continued)

QTL	Ch	Marker Interval	Gene	Gene ID	Site (Mb)
			<i>OsGhd7*</i>	Os07t0261200-01	4.8a

QTLs in bold represent major effect QTLs and not in bold are consistent minor QTLs; a in the site indicates distance from the left flanking marker; b is the distance from the right flanking marker of the respective QTL; Genes with * are within the QTL and the remaining genes are close to flanking markers of QTL but not within the QTL

Another 18 genes identified were 0.04–5.0 Mb close to the left and right flanking markers of the QTLs. *OsZIP9* and *OsZIP5* were close to *qZn5.1*. *OsMTII-1a* a plant EC metallothionein-like protein was closer to *qZn10.1* along with several zinc finger type domain proteins. *OsERF65* an Ethylene response factor gene and *OsHMA7* a heavy metal transport gene and were close to *qDFF7.1*. Three genes *OsMGD2*, *YSL15* and *YSL2* were close to *qPH2.2*. *OsSLG* (Slender grain), *OsPT6* (Phosphate ion transporter) were closer to *qPH8.1* and *Oslpa1* (*Low phytic acid-1*) was associated with the consistent minor QTL *qDFF2.1*. *OsHMP15*, *OsYSL2*, *OsYSL15* and *GS2* were associated with *qTGW2.1*. All these candidate genes are reported to be involved in the regulation of metal homeostasis and yield related traits in rice.

Discussion

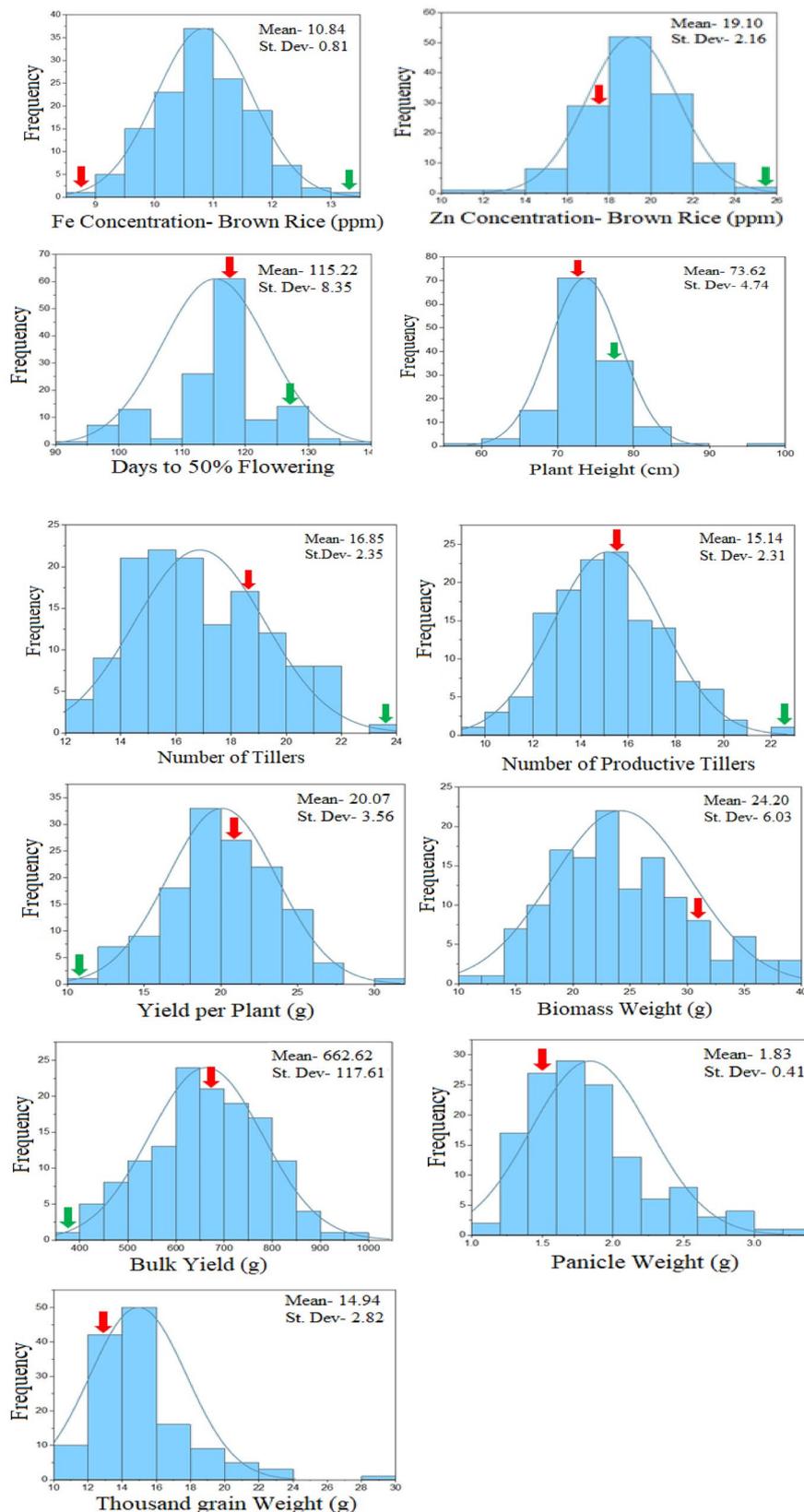
Phenotypic analysis

In the present study, transgressive segregants were obtained for grain Fe, Zn and yield related traits which indicate that there were favourable alleles from *O. rufipogon* in the genetic background of BPT5204 in BC₄F₁₀ generation also. The frequency distribution (Fig. 3) for Fe and Zn concentration showed normal distribution indicating that they are under complex genetic control and are suitable for QTL mapping studies. The trait correlations between Fe and Zn were highly significant and positive in the mapping population. Several studies have reported the positive correlation between these two elements in brown rice (Naik et al. 2020; Anusha et al. 2021). TN and NPN were also correlated with each other and also with grain Fe across 3 years (mean) and the QTLs of these traits were also co-located. Grain Fe and Zn have been reported to be positively correlated with tiller number and number of productive tillers in rice (Akinwale et al. 2011). Thousand grain weight was negatively correlated with the grain zinc concentration in brown rice and was also reported in previous studies (Calayugan et al. 2020).

Marker-trait associations

Single marker analysis showed 59 significant markers trait associations for grain Fe, Zn and yield traits in all the three years. Thousand grain weight (TGW) showed the maximum number of 36 marker associations. 12 markers showed distinct association with more than one trait. RM5968 associated with grain zinc concentration and thousand grain weight in our study was reported to be linked to a QTL cluster for Nitrogen use efficiency and recent studies suggest that N management also plays a key role in zinc bio fortification and their synergic effect could increase grain yield in cereal crops (Dai et al. 2015; Montoya et al. 2020).

Fig. 3 Frequency distribution of grain concentration of Fe, Zn and yield traits in BILs. *Red arrow represents BPT5204; Green arrow represents *O.rufipogon*



RM8226 associated with panicle weight and thousand grain weight in our study was linked to the QTLs of grain Fe, Zn and days to flowering in earlier reports (Dixit et al. 2019). RM1126 which was associated with the highest number of traits PH, TN, NPN, TGW and ZN in the present study was reported to flank the major effect QTLs of grain width (*qGW10*), grain thickness (*qGT10*), grain length/width ratio (*qLWR10*), thousand grain weight (*qTGW10*) and number of secondary branches per panicle (*qNSB10.1*) (Yan et al. 2014; Jia et al. 2019). RM7576 linked to grain Zn and TGW in the present work was reported to be associated with a novel wax crystal sparse leaf 3 gene which regulates the epidermal wax formation in rice (Bing Gong et al. 2017). It is significant that two TGW linked markers RM7576 and RM1126 were linked with Zn concentration which was not been reported previously. RM3688 linked to TGW in our study showed significant association with grain yield traits under different field conditions in N22 mutants (Yugandhar et al. 2017). The sub QTL region of the yield QTL *yld 2.1* from *O. rufipogon* linked with RM3688 was fine mapped and the introgression lines with this locus showed significant increase in grain yield related traits (Babu et al. 2017). RM19417 linked to TGW and DFF in our study is a marker associated for the rice flowering locus T gene - *Hd3a*, total number of spikelets/ panicles, number of filled grains /panicle and thousand grain weight (Zhu et al. 2017; Cheng et al. 2007). RM21976 linked to TGW in our study flanks the QTL for soil surface rooting in rice which is a homologue of *DRO1* (Deeper rooting - 1) (Kitomi et al. 2020). RM519 linked to NPN and TGW in our study was also linked to the QTLs of number of productive tillers, number of filled grains, number of spikelets /panicles, grain yield and grain length (Swamy et al. 2014; Qiao et al. 2016; Surapaneni et al. 2017). 29 marker-trait associations (MTAs) with significant associations for traits viz., FeBR (brown rice) (6 MTAs), FeMR (milled rice) (7 MTAs), ZnBR (brown rice) (11 MTAs), and ZnMR (milled rice) (5 MTAs) were identified in a genome wide association study in 132 Indian rice germplasm signifying the possibility of their simultaneous improvement (Bollinedi et al. 2020).

QTLs identified for Fe, Zn and yield traits and their comparison with the previous reports

In our study, 4 major QTLs were detected for Fe and Zn, and 7 major QTLs for yield related traits. Most of these QTLs showed a positive additive effect and thus BPT5204 allele was trait-enhancing in these QTLs. Two QTLs *qPH8.1* and *qPW6.1* had trait-enhancing allele from *O. rufipogon*. *qFe1.1*, *qZn5.1*, *qZn8.1*, *qZn10.1* are novel QTLs for grain Fe and Zn concentration. However, these loci also correspond to other agronomic traits and disease resistance in earlier reports. For example, flanking markers of QTL

qZn8.1 RM5485-RM6948 were reported linked to nitrogen absorption ability, grain length, and amylose content (Dai et al. 2015; Yin et al. 2015; Lou et al. 2009). RM129, the flanking marker of *qFe1.1* was linked with cold tolerance, non-photochemical quenching, seedling height, and nitrogen concentration (Andaya and Mackill 2003; Dufey et al. 2015). Further dissection of these genetic regions rich in loci for agronomic ally important traits would be crucial for rice functional genomics and traits improvement. QTLs for Fe and Zn were co-located as a continuous locus on chromosome 12 at RM235–RM3747 and RM3747–RM519. This may be due to sharing of overlapping region of QTLs or pleiotropic effects. This indicates that Fe and Zn uptake, translocation and loading mechanisms share common gene networks and pathways, so both of these mineral elements can be improved simultaneously. Co-location of Zn QTL with Fe QTL on chromosome 12 was reported previously (Anuradha et al. 2012b; Swamy et al. 2018a, b; Calayugan et al. 2020). Several QTL clusters were identified in this study.

Co-localized QTLs

Two QTLs identified for Zn were co-located with the QTLs for yield and related traits. QTLs *qBM8.1*, *qBY8.1*, *qPW8.1* were co-located with *qZn8.1* and *qZn8.2* at RM5485-RM6948 on chromosome 8. *qTN10.2* and *qNPN10.1* were co-located with *qZn10.1* (Table 4). Correspondingly, the correlation of grain Zn with TN and NPN was also significant. The relationship between grain Zn and yield is well known and consideration should also be given to grain size, grain weight while selecting for high grain Zn (Swamy et al. 2016). Likewise, TGW showed significant positive correlation with the panicle weight. These QTLs which are found to be in strong and consistent association with grain yield can be assessed for simultaneous improvement of different traits through marker assisted breeding methods.

Consistent QTLs

Two major effect QTL *qZn8.1*, *qDFF7.1* with the highest PVE of 16.8% and 16.1% respectively were identified in both *Rabi 2016* and *Rabi 2017* when analyzed year wise showing that these QTLs were more stable. The chromosomal region RM5460-RM3874 corresponding to a minor QTL *qDFF2.1* was consistent in *Kharif 2015*, *Rabi 2016* and *Rabi 2017* indicating its stable contribution to phenotypic expression across the three years. A QTL for biomass *qBM6.2* was found to be consistent in *Rabi 2016* and *Rabi 2017*. Five minor QTLs for thousand grain weight *qTGW2.1*, *qTGW6.2*, *qTGW7.1*, *qTGW7.2* and *qTGW10.2* were consistently identified in both *Rabi 2016* and *Rabi 2017* with a total PVE of 44.2% of these five QTLs in each year (Supplementary

Table 3 & 4). The consistent QTL *qTGW2.1* with the flanking markers RM3688–RM13603 on chromosome 2 harbors *Ubiquitin* associated protein (*Os02t0593700-03*) with a physical distance of 0.5 Mb from the left flanking marker RM3688 which plays a vital role in zinc ion binding. Single marker analysis showed RM3688 was significantly associated with TGW. This QTL with trait-enhancing allele from *O. rufipogon*, has a physical distance of 2 Mb between the two flanking markers and is worth fine mapping. RM3688 was reported to be associated with yield and related traits within the sub QTL-3 region of a yield QTL *yl2.1* derived from *O. rufipogon* (Thalapati et al. 2014). Among all the identified QTLs, few were consistent across the years and are stable and this could be ascribed to small population size. This points to the need to consider minor QTLs which are consistent for use in MAS. The novel QTLs *qFe1.1*, *qZn5.1*, *qZn8.1*, *qZn10.1* and the consistent minor QTLs *qTGW2.1*, *qTGW6.2*, *qTGW7.1*, *qTGW7.2* and *qTGW10.2* identified in our study will help to develop grain Fe and Zn rich lines with high yield.

The major effect QTLs and the consistent QTLs identified in our study harbor genes known for grain iron, zinc and yield related traits. In all, 19 candidate genes present within the QTL regions are identified on chromosomes 1, 2, 5, 6, 7 and 8. For example, the genes *OsYSL18* and *OsHMP7* were located 2.7 Mb and 2.9 Mb upstream of the left flanking marker within the QTL *qFe1.1*. *OsYSL18* is a transporter of the iron (III)-2'-deoxymugineic acid complex in reproductive organs and phloem of lamina joints (Aoyama et al. 2009). Heavy metal-associated proteins (HMPs) are the metallo-proteins having heavy metal associated domains and are involved in heavy metal transport and detoxification in plant cells. A gene expression profile analysis among 46 *OsHMPs* in rice showed differential expression in various tissues of the plant and their biological functions in rice are elucidated (Li et al. 2020). The introgression of *OsSPL14^{WFP}* allele significantly increased grain number/panicle by 10.6–59.3% in elite indica cultivars and *OsSPL16/GW8* positively regulates grain weight, size and shape in rice (Kim et al. 2016; Liu et al. 2016). *GW6* (*GRAIN WIDTH 6*) encodes a GA regulated GAST family protein and are involved in the positive regulation of grain width, weight and yield in rice (Shi et al. 2020). Interaction of *Hd1* with other flowering genes such as *Ghd7*, *Hd3a* delays heading date in some rice varieties under long-day (LD) which results in increased plant height and grain yield (Zhang et al. 2017). Simultaneous increase in grain size, yield and improved quality was observed in rice with the over expression of the 16-kDa α -amylase/trypsin inhibitor *RAG2* (Zhou et al. 2017). *Ghd7* which encodes a CCT domain protein regulates the heading date, grain yield and drought tolerance in rice (Weng et al. 2014).

Apart from the genes within the QTL regions, 18 other candidate genes were identified close to the major and

consistent minor QTLs on chromosomes 2, 5, 7, 8 and 10 and are of 0.04–5 Mb close to the right and left flanking markers of the QTLs. Two ZIP (Zinc/Iron regulated transporter protein) family proteins *OsZIP5* and *OsZIP9* closer to the left flanking marker of *qZn5.1* encodes the plasma membrane proteins with the influx transporter activity synergistically function in the uptake of Zn and Cd (Tan et al. 2020). *OsMT-II-1a* was located 0.1 Mb downstream the left flanking marker of the QTL *qZn10.1* and is responsible for zinc homeostasis and drought tolerance in rice (Yang et al. 2009). *OsHMA7* was positioned 0.3 Mb downstream the left flanking marker of the consistent major effect QTL *qDFF7.1* (RM21970–RM11) on chromosome 7 and is involved in regulation of grain Fe, Zn concentration and yield. Kappara et al. (2018) observed the influence of transcript levels of iron responsive genes by altering the expression of *OsHMA7* gene which indicated cellular Fe-Zn homeostasis and also several domestication-related genes in rice. Under Fe deficiency conditions *YSL2* up regulated in roots and *YSL15* in shoots of a high Fe, Zn line compared to a low Fe, Zn line (Agarwal et al. 2014). *GRAIN SIZE 2* encodes a transcription factor *OsGRF4* which can enhance grain weight significantly and increase the grain yield in rice (Hu et al. 2015). *OsSLG* controls grain size and leaf angle by modulating brassinosteroid homeostasis in rice (Feng et al. 2016). *OsPT6* plays a vital role in Pi acquisition and mobilization in rice (Zhang et al. 2014). The association of these candidate genes responsible for regulation of grain Fe, Zn and yield with the major effect and the consistent minor QTLs in our study clearly emphasize the importance of these QTLs in biofortification breeding programs. The candidate genes associated with the QTLs are worth for further analysis to know their actual function in metal homeostasis and yield improvement.

An elite BIL 51B [IET 24775] was tested for 4 years (2014–2017) in All India Coordinated Rice Improvement Project biofortification trials, it showed 21 ppm grain Zn in AVT-1 2015 was promoted to AVT-2 in 2016 and it showed high grain yield (5.1 kg/ha, 8% more than BPT-5204) in Zone-7 (Southern India) with highest yield in Tamil Nadu and ranked 3rd among 11 entries with 19 ppm of grain zinc concentration in polished rice (Supplementary Table 5). Zinc enriched high yielding bio fortified rice BRRIDhan84 with 27.6 ppm grain zinc concentration in polished rice suitable for dry season was released in Bangladesh in 2017 (Kader et al. 2020). In a study conducted with 102 rice genotypes in two wet seasons 2016, 2017 at ICAR NRRI, India, 51B showed high grain Fe of 4.3 ppm in polished rice and high yield of 4623 kg/ha compared to yield check BPT5204 (4431 kg/ha) and IR64 (4250 kg/ha) (Pradhan et al. 2020). In another study, a set of 170 genotypes were analyzed for grain zinc concentration using ED-XRF and they reported that in BIL 51B it was in the range

of 20.1–25.0 mg/kg while in 28B and 117B zinc was in the range of 15.1–20.0 mg/kg in brown rice samples (Sanjeeva Rao et al. 2020). In the same study among 7 ILs derived from Swarna x *O. nivara* mapping population BILs 14S, 236 K, 65S, 142S and 14-3S showed grain zinc concentration was in the similar range of BPT5204 x *O. rufipogon* ILs whereas 233 K and 24 K were in the highest range of 25–30 mg/kg. These studies with backcross inbred lines derived from wild relatives validate previous reports that wild accessions *O. nivara* and *O. rufipogon* which have high concentration of Fe and Zn in brown rice can serve as a valuable resource in rice biofortification. The three elite BILs 61-1B [IET28715], 83B [IET28706] and 88B [IET28695] with an overall mean (3 years data) of 22 ppm grain zinc concentration and 25 g of mean grain yield/plant were nominated to All India Coordinated Rice Improvement Project multi-location trials for bio fortification and showed 0.06% to 9.6% higher yield than parent BPT5204 (4484 kg/ha) and overall mean of 4913 kg/ha, 4897 kg/ha and 4487 kg/ha respectively, from the data of 20 locations tested all over India in 2019 were promoted to Advanced Varietal Trial-1, 2020. The mean Zn concentration was 18.71 ppm, 20.19 ppm and 19.6 ppm respectively while that of BPT5204 was 18.6 ppm. These 4 elite lines including 51B have major effect QTL alleles from BPT5204 which is the trait enhancing allele in BILs in 9 out of 11 major QTLs identified in our study. The introgression of chromosomal segments from *O. rufipogon* likely enhances native gene expression to give higher zinc. This has been shown for yield increase in rice introgression line KMR3 with introgressions from *O. rufipogon* (Thalapati et al. 2012). It is not necessary that an allele from *O. rufipogon* increases yield or Fe or Zn, a strong promoter/ activator/ enhancer from wild rice can help increase expression of native gene and thus trait value as shown in previous studies (Kaur et al. 2017, 2020; Duan et al. 2017).

Conclusion

Considerable genetic variations for grain Fe, Zn and yield related traits were observed among BILs. Comparative analysis revealed a major QTL *qFe1.1* for Fe and three major QTLs *qZn5.1*, *qZn8.1*, *qZn10.1* for Zn. In all, 11 major QTLs were identified for different traits. Analyzing QTLs for Fe, Zn and yield components together revealed co-localized QTLs influencing different traits on chromosomes 2, 6, 7, 8, 10, and 12. The 4 major effect QTLs associated with grain iron and zinc in this study are novel and the candidate genes associated with Fe, Zn homeostasis were co-located with these QTLs. Major effect QTLs can be assessed for use in marker assisted breeding and causal gene identification. The potential candidate genes *GS₂*, *GW₆*, *OsGhd₇*, *RAG₂*, *OsZIP1*, *OsYSL2*, and *OsYSL15* involved in the increase of

grain weight and Fe, Zn accumulation in rice were co localized with the consistent minor QTLs *qTGW2.1*, *qTGW6.2* and *qTGW7.3* and are worth fine mapping and further functional analysis. Though the Zn enhancing alleles are from BPT5204 the elite introgression lines have higher yield and zinc than parent which demonstrate the usefulness of *O. rufipogon* introgressions in rice improvement. The elite ILs with high grain Fe, Zn and yield with low phytate will be useful in gene discovery and in bio fortification breeding programs.

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Author contributions GC: Investigation; Methodology; Writing—original draft. DB: Methodology; Data curation; Software; Formal analysis; Validation; Writing—review & editing. SBM: Investigation. SKM: Project administration; Writing—review & editing. SRD, CN: Investigation, methodology, writing- review and editing, supervision, validation. RMS: supervision, writing- review and editing, validation. Sarla Neelamraju: Conceptualization; Funding acquisition; Resources; Supervision; Project administration; Validation; Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Data availability Authors declare data and materials of this study are available as supplementary material.

Declarations

Conflict of interest The authors declare no conflict of interest.

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