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ScienceDirect

Rice Science, 2018, 25(4): 197−207

QTL Analysis for Grain Iron and Zinc Concentrations in Two *O. nivara* Derived Backcross Populations

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Abstract: Identification of quantitative trait loci (QTLs) for grain mineral elements can assist in faster and more precise development of micronutrient dense rice varieties through marker-assisted breeding. In the present study, QTLs were mapped for Fe and Zn concentrations in two BC_2F_3 mapping populations derived from the crosses of *O. sativa* cv Swarna with two different accessions of *O. nivara*. In all, 10 and 8 QTLs were identified for grain Fe and Zn concentrations in population 1, and 7 and 5 QTLs were identified in population 2, respectively. Eighty percent of the QTLs detected in both populations were derived from *O. nivara*. Five QTLs for Fe and three QTLs for Zn explained more than 15% phenotypic variance either in interval or composite interval mapping. The locations of *O. nivara* derived QTLs such as *qFe2.1*, *qFe3.1*, *qFe8.2* and *qZn12.1* were consistently identified in both the populations. Epistatic interaction was observed only between RM106 and RM6 on chromosome 2 and between RM22 and RM7 on chromosome 3 for Fe concentration in population 1. Sixteen candidate genes for metal homeostasis were found to co-locate with 10 QTLs for Fe and Zn concentrations in both the populations. Most of the Fe and Zn QTLs were found to co-locate with QTLs for grain yield and grain quality traits. Some of the major effect QTLs identified can be used to improve rice grain Fe and Zn concentrations.

Key words: biofortification; micronutrient; Fe concentration; Zn concentration; rice; wild rice; quantitative trait locus; gene; marker assisted breeding

Iron (Fe) and zinc (Zn) are highly essential for the normal growth and development of human beings. Fe is an important component of hemoglobin, while Zn is a major co-factor for more than 300 enzymes involved in major biological functions (Black et al, 2013; Stevens et al, 2013). 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, mental lethargy, etc (Hotz and Brown, 2004; Prasad, 2004). Globally more than two billion people, particularly children, pregnant and lactating women, suffer from Fe and Zn deficiencies (Wessells and Brown, 2012). Therefore, it is urgent to address these micronutrients malnutrition. Breeding crop varieties with higher mineral densities also called as 'biofortification' is found to be effective in addressing micronutrient malnutrition (Graham et al, 1999; Bouis et al, 2003, 2010, 2013; Swamy et al, 2016).

Rice is the major staple food and source of energy for more than 90% of Asian population, but the milled rice is a poor source of Fe and Zn. Breeding rice varieties with higher mineral densities can help in tackling hidden hunger in most of the Asian countries (Bouis et al, 2013; Swamy et al, 2016). There is a wider

Received: 15 October 2017; **Accepted:** 12 February 2018

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Peer review under responsibility of China National Rice Research Institute

http://dx.doi.org/10.1016/j.rsci.2018.06.003

variation for grain Fe and Zn in the rice germaplsm, which can be exploited in rice biofortification breeding programs (Zhang et al, 2004; Heinemann et al, 2005; White and Broadley, 2005).

High Zn rice varieties have been developed and released in Bangladesh, India and the Philippines (HarvestPlus, 2014). However, lack of cheaper and field based phenotyping methods for the estimation of grain Fe and Zn concentrations, and significant genotype by environment interactions are some of the major landraces in developing high Fe and Zn rice varieties (Wissuwa et al, 2008; Chandel et al, 2010; Du et al, 2013; Swamy et al, 2016). The recent advances in rice genomics technologies such as genome sequence information of several rice accessions and genome wide molecular markers can be used to identify major QTLs and genes for complex traits (Li et al, 2011; Swamy et al, 2011a). The major effect QTLs/genes for grain Zn concentration can be used in marker-assisted breeding programs for the faster and more precise development of high Zn rice varieties.

The A-genome wild progenitors of cultivated rice such as *O. nivara* and *O. rufipogon* are the rich source of natural allelic variations for several agronomic, grain quality and grain micronutrient traits (Joshi et al, 2000; Sarla et al, 2003; Juneja et al, 2006; Kovach and McCouch, 2008; McCouch et al, 2008; Swamy et al, 2008a, 2011a, b, 2014; Ma et al, 2016). *O. nivara* has contributed several genes for disease resistance, yield and yield components, and grain quality traits for the improvement of cultivated rice (Brar and Khush, 2004; Martinez et al, 2004; Juneja et al, 2006; Kaladhar et al, 2008; Swamy et al, 2012, 2014; Sanchez et al, 2014; Ma et al, 2016; Haritha et al, 2018). An inter-specific hybrid derived from *O. nivara* shows higher levels of grain Fe, Zn and protein (Ramaiah and Rao, 1953; Mahmoud et al, 2008; Swamy et al, 2011a). There are efforts to map QTLs for Fe and Zn concentrations, however, wild rice rich in Fe and Zn concentrations has been less explored in QTL mapping.

In this study, we used two *O. nivara* derived mapping populations (Kaladhar et al, 2008; Swamy et al, 2011a, b, 2014) to detect QTLs for Fe and Zn concentrations in rice grains. The main objectives were to study the variations for grain Fe and Zn concentrations in the two mapping populations, and to identify QTLs for grain Fe and Zn concentrations, and co-localized QTLs for grain Fe and Zn concentrations, as well as grain yield and grain quality traits.

MATERIALS AND METHODS

Rice materials

O. nivara accessions with red kernel and higher grain Fe and Zn concentrations as the donor parents were collected from Bihar (IRGC81832) and Uttar Pradesh (IRGC81848), two northern states of India. Swarna, one of the most popular rainfed lowland and high-yielding rice varieties was used as a recipient parent, and grown on more than 3×10^6 hm^2 in South and Southeast Asia. Crosses were made between Swarna and each *O. nivara* accession, the F_1 and BC_1 progenies were backcrossed to recipient parents and the resulting BC_2 populations were selfed to develop BC_2F_2 populations, following the methods of Thomson et al (2003).

Two mapping populations consisting of 227 (population 1) and 245 (population 2) BC_2F_2 families were grown in two replications in an augmented block design at the Indian Institute of Rice Research, Hyderabad, India, during 2005 and 2006, respectively. Each of the backcross families and the control accession consisted of 30 plants (planted in 3 rows) with the space of 20 cm × 15 cm. Standard agronomic practices and protection measures were adopted. Leaf samples were collected from five plants in the middle row and bulked for DNA isolation. BC_2F_3 seeds of 140 families from the population 1 and 146 families from the population 2 were analyzed for the grain Fe and Zn concentrations and used for QTL mapping.

Phenotyping for quality traits

All the rice samples from the two replications were dried to a uniform moisture content of 14% and 100 g seeds were dehusked using the Satake Dehuller (THU35A, California, USA). Seeds from every lot were divided into three parts and analyzed as three replicates to make sure the accuracy and consistency. Briefly, brown rice samples were dried in hot air oven at 80 ºC. The whole grains (1 g) each with embryo intact were digested in a triacid mixture (nitric acid : sulphuric acid : perchloric acid = 5 : 2 : 1) until clear white residue was obtained. The above mixture was then filtered through Whatman filter paper No. 42. Required volume (50 mL) was made after the completion of digestion process, and digests were analyzed using an atomic absorption spectrophotometer (Varian Model-AA240, California, USA). Mean, range and trait

correlations among Fe and Zn concentrations in rice grains were estimated using PB Tools v1.4.

Genotyping

A set of 250 randomly selected simple sequence repeat (SSR) markers on all the 12 chromosomes were screened for polymorphism between the parental lines Swarna and *O. nivara* accessions. A total of 100 polymorphic SSR markers with an average density of 4.0 Mb were used for whole population genotyping of 227 BC_2F_2 families of population 1, whereas 75 markers with an average density of 5.3 Mb were used for genotyping of 245 BC_2F_2 families of population 2. Among the SSR markers used for genotyping, 45 were common between the two populations. Leaf samples were collected from 60-day-old seedlings, and DNA was extracted using the protocol of Zheng et al (1995). The PCR reaction for SSR primers was performed with a final volume of 15 μL, containing 45 ng genomic DNA, 10× buffer, 0.125 mmol/L final concentration of each dNTPs, 0.2 μmol/L each of forward and reverse primers, 2% formamide and 1 U Biogene *Taq* DNA polymerase. PCR amplification was performed under the following conditions: initial denaturation at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min, extension at 72 ºC for 2 min, followed by the final extension at 72 ºC for 5 min. The amplified products were checked for polymorphism or marker segregation on an agarose gel (3%) and scored for segregating bands.

Linkage and QTL analysis

Two linkage maps were constructed using MapMaker v.3.0 (Lander et al, 1987) following Kosambi function (Kosambi, 1944). Linkage groups were determined with a LOD score of 2.5 and a recombination fraction of 0.5. Assignment of linkage groups to the respective chromosomes was carried out based on the rice maps developed by Temnykh et al (2001) and Thomson et al (2003). A consensus linkage map for both the populations was developed using Biomercator v2.0 (Arcade et al, 2004). QTLs were identified using QTL Cartographer v.2.5, LOD thresholds for each trait were fixed based on 1000 permutations, and QTLs were analyzed using a minimum LOD threshold of 2.5. Digenic QTL interactions were identified using QTL network v.2.0.

Candidate gene analysis

QTL analysis was carried out using Single Marker Analysis (SMA), Interval Mapping (IM) or Composite Interval Mapping (CIM) methods in both the populations. The physical positions of the QTLs on respective chromosomes were obtained from Gramene (www.gramene.org). The nucleotide sequences underlying all the QTLs were downloaded as bacterial artificial chromosome (BAC) clones and contigs from International Rice Genome Sequencing Project (IRGSP) (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html). All genes within and close to QTL regions were downloaded from the Rice Annotation Project Data Base (http://rapdb.dna.affrc.go.jp).

RESULTS

Phenotypic variation

The mean, range and correlations for grain Fe and Zn concentrations in both the populations and the parents are presented in Table 1. The correlations between Fe and Zn were highly significant and positive in both the populations.

QTL analysis for grain Fe and Zn concentrations

QTLs identified by IM and CIM are presented in Table 2. In all, 10 and 7 QTLs were identified for Fe and Zn concentrations in population 1, and 8 and 5 QTLs in population 2, respectively. And 14 QTLs in population 1 and 11 QTLs in population 2 were derived from *O. nivara*.

QTLs for Fe concentration

Ten QTLs for Fe concentration were identified on chromosomes 1, 2, 3, 6, 8 and 11, explaining a phenotypic variance (PV) of 4% to 25% with an additive effect of 1.28 to 6.60 mg/kg in population 1

Table 1. Variation for grain Fe and Zn concentrations in *O. nivara* derived populations.

Statistics	Population 1		Population 2	
	Fe	Zn	Fe	Zn
Swarna (mg/kg)	10.3	25.4	11.8	23.5
IRGC81832 (mg/kg)	–	–	28.5	62.1
IRGC81848 (mg/kg)	26.2	65.2	–	–
Mean (mg/kg)	7.4	14.5	6.1	15.2
Range (mg/kg)	2.2–22.2	7.1–64.7	1.6–19.4	10.0–25.6
CV (%)	12.9	16.2	12.7	20.5
h^2 (%)	52.3	85.6	49.6	79.5
GA (%)	19.5	26.3	16.3	27.2
Correlation	0.685**		0.240**	

CV, Coefficient of variation; h^2, Heritability; GA, Genetic advancement.

** means significant differences at the 0.01 level by the Pearson test.

Table 2. QTLs for Fe and Zn concentrations in two *O. nivara* derived populations.

QTL	Chr	Marker interval	Allelic effect	IM			CIM			Population	Reference
				LOD	R^2 (%)	A [a] (mg/kg)	LOD	R^2 (%)	A [a] (mg/kg)		
qFe1.1	1	RM499–RM428	*O. nivara*	5.5	10	-6.55	5.0	8	-0.76	P2	Lu et al, 2008
qFe1.2	1	RM243–RM81A	*O. nivara*	4.2	14	-3.17	5.6	17	-3.80	P1	
qFe1.3	1	RM24–RM595	*O. nivara*	3.1	12	-2.20				P1	
qFe2.1	2	RM324–RM475	*O. nivara*	3.1	12	-3.04				P1	
qFe2.1	2	RM324–RM475	*O. nivara*	4.1	8	-0.16	3.7	12	-0.12	P2	
qFe2.2	2	RM6–RM250	Swarna	3.1	8	2.39	2.7	5	2.00	P1	
qFe3.1	3	RM517–RM156	*O. nivara*	3.1	12	-2.70				P1	Anuradha et al, 2012b
qFe3.1	3	RM517–RM7	*O. nivara*	5.7	5	-0.62	4.9	4	-1.07	P2	Anuradha et al, 2012b; Kumar et al, 2014
qFe3.2	3	RM520–RM514	*O. nivara*	4.2	16	-1.64	4.4	19	-2.86	P2	
qFe4.1	4	RM241–RM348	*O. nivara*	5.2	12	-5.57	9.0	15	-5.10	P2	Norton et al, 2010
qFe6.1	6	RM204–RM314	*O. nivara*	3.9	10	-1.72				P1	Norton et al, 2010; Zhang et al, 2014
qFe8.1	8	RM337–RM152	*O. nivara*	3.2	6	-1.60				P2	Garcia-Oliviera et al, 2009
qFe8.2	8	RM152–RM38	*O. nivara*	3.6	4	-1.28				P1	Garcia-Oliviera et al, 2009
qFe8.2	8	RM38–RM223	Swarna	5.1	6	0.24	4.0	3	0.81	P2	Garcia-Oliviera et al, 2009
qFe11.1	11	RM332–RM287	*O. nivara*	3.6	18	-6.60	3.2	19	-6.50	P1	
qFe11.2	11	RM209–RM21	*O. nivara*	5.1	25	-4.68	2.5	9	-2.90	P1	
qFe11.3	11	RM254–RM224	*O. nivara*	3.1	7	-1.70	2.7	6	-1.70	P1	Nawaz et al, 2015
qFe12.1	12	RM19–RM247	*O. nivara*	5.3	5	-0.75	5.0	4	-0.39	P2	Nawaz et al, 2015
qZn1.1	1	RM488–RM431	Swarna	2.6	4	0.56				P1	Nawaz et al, 2015
qZn2.1	2	RM250–RM535	*O. nivara*	4.3	13	-2.10	3.4	8	-1.83	P1	
qZn3.1	3	RM517–RM16	*O. nivara*	3.2	23	-2.36	2.9	15	-1.34	P2	
qZn3.2	3	RM55–RM520	*O. nivara*	3.5	10	-1.56	2.9	7	-1.54	P1	Anuradha et al, 2012
qZn5.1	5	RM153–RM413	*O. nivara*	8.6	16	-1.82	12.4	36	-2.07	P2	
qZn6.1	6	RM30–RM439	*O. nivara*	2.7	10	-0.77				P2	
qZn8.1	8	RM152–RM223	Swarna	2.8	13	1.87				P2	
qZn8.2	8	RM256–RM264	*O. nivara*	5.1	3	-6.60	4.8	3	-0.26	P1	Garcia-Oliviera et al, 2009
qZn9.1	9	RM215–RM189	*O. nivara*	2.5	4	-0.79				P1	Lu et al, 2008
qZn10.1	10	RM216–RM467	*O. nivara*	6.8	6	-1.34	5.1	3	-0.21	P1	
qZn12.1	12	RM415–RM19	Swarna				7.1	7	1.13	P1	
qZn12.1	12	RM415–RM19	*O. nivara*	8.5	14	-1.38	8.9	21	-2.28	P2	

Chr, Chromosome; IM, Interval mapping; CIM, Composite interval mapping; *A*, Additive effect; P1, Population 1; P2, Population 2.
[a] The negative values indicate trait increasing allele from *O. nivara*.

(Table 2). All the QTLs were derived from *O. nivara* except *qFe2.2*. Eight QTLs for Fe concentration were identified on chromosomes 1, 2, 3, 4, 8 and 12, explaining a PV of 3% to 19% with an additive effect of 0.12 to 6.55 mg/kg in population 2. Among them, seven of these QTLs were derived from *O. nivara.* In all, seven QTLs in population 1 and three QTLs in population 2 explained a PV of no less than 10%.

QTLs for Zn concentration

In population 1, seven QTLs for Zn concentration were identified on chromosomes 1, 2, 3, 8, 9, 10 and 12, explaining a PV of 3% to 13% with an additive effect of 0.21 to 6.60 mg/kg in population 1 (Table 2). All the QTLs were derived from *O. nivara* except *qZn1.1* and *qZn12.1*. In population 2, five QTLs for Zn concentration were identified on chromosomes 3, 5, 6, 8 and 12, explaining a PV of 10% to 36% with an additive effect of 0.77 to 2.36 mg/kg in population 2.

Co-localized QTLs for Fe and Zn concentrations

There was a very strong correlation between Fe and Zn concentrations, and in general, the highly correlated traits were found to be highly co-located. Four QTLs *qFe2.1*, *qFe3.1*, *qFe8.2* and *qZn12.1* were consistently identified in the same chromosomal locations in both the two populations (Table 2). *qFe2.2* and *qZn2.1*, *qFe3.2* and *qZn3.1*, *qFe8.1*/*qFe8.2* and *qZn8.1*, and *qFe12.1* and *qZn12.1* were co-located in either of the populations.

Table 3. Co-located QTLs for Fe and Zn concentrations.

Chromosome	Marker interval	Co-located QTLs
2	RM6–RM535	*qFe2.2* and *qZn2.1*
3	RM517–RM16	*qFe3.2* and *qZn3.1*
8	RM337–RM223	*qFe8.1*, *qFe8.2* and *qZn8.1*
12	RM415–RM247	*qFe12.1* and *qZn12.1*

One pair of QTLs for Fe and Zn concentrations on chromosome 2 was co-located in population 1 (Tables 2 and 3). Three QTLs for Fe and Zn concentrations were identified on same chromosomal locations on chromosomes 3, 8 and 12 in population 2. Three QTLs for Fe concentration on chromosomes 2, 3 and 8, and one QTL for Zn concentration on chromosome 12 were consistently identified in both the two populations.

Table 4. Epistasis for grain iron concentrations in *O. nivara* derived populations.

Trait	Marker interval	Chromosome	AA	AD	DA	DD
Fe content	RM106–RM6	2	-1.59	-6.70	0.48	-0.68
	RM22–RM7	3				

AA, Additive × additive; AD, Additive × dominance; DA, Dominance × additive; DD, Dominance × dominance.

Epistatic QTLs for grain Fe and Zn concentrations

Epistasis for grain Fe and Zn concentrations was analyzed in both the populations using QTL network v.2.0. There was only one significant interaction identified for Fe concentration in population 1. These loci were located between RM106–RM6 on chromosome 2 and RM22–RM7 on chromosome 3. All the interactions except dominance × additive were highly significant. It is interesting that the interacting alleles associated with these loci were derived from *O. nivara* (Table 4).

Co-localized QTLs for Fe and Zn concentrations with QTLs for yield and grain quality traits

In this study, 27 of the 30 QTLs identified for Fe and Zn concentrations were co-located with the QTLs for yield and yield-related traits or QTLs for grain quality traits identified and reported from the same two *O. nivara* derived populations (Table 5). In the marker interval RM488–RM431 on chromosome 1, QTLs for seven traits (plant height, number of spikelets, number of filled grains, grain weight, etc.) were co-located. Also, most of the alleles in these co-located QTL regions were derived from *O. nivara*.

Candidate gene analysis

QTLs for Fe and Zn concentrations were analyzed using RAP-DB, and important genes known to be involved in Fe and Zn homeostasis were shortlisted in Table 6. A total of 16 metal homeostasis-related genes were identified (Table 6). The enrichment of Fe and Zn QTLs with metal homeostasis-related genes showed the accuracy and consistency of our QTL mapping.

Table 5. Co-localized QTLs for Fe, Zn, yield, yield components and grain quality traits.

QTL	Chromosome	Marker interval	Yield and yield-related QTL	Grain quality QTL
qFe1.1	1	RM243–RM81A	*gw1.1*	*ac1.1*, *ac1.1*
qFe1.2	1	RM24–RM595	*kw1.2*	*mp1.2*, *ver1.2*
qFe1.1	1	RM499–RM428		*mp1.1*, *lwr1.1*, *kl1.1*, *ac1.1*
qFe2.1	2	RM324–RM475	*gw2.2*	*ac2.1*
qFe2.2	2	RM6–RM250	*nsp2.1*, *nfg2.1*, *bm2.1*, *yld2.1*	
qFe3.1	3	RM517–RM156	*dtm3.1*, *nsp3.1*	*mp3.1*
qFe3.2	3	RM520–RM514	*nt3.1*, *yld3.1*	*er3.1*
qFe4.1	4	RM241–RM348	*dtm4.1*, *gw4.2*	*mp4.2*
qFe6.1	6	RM204–RM314	*kw6.1*	
qFe8.1	8	RM152–RM38	*nfg8.1*, *yldp8.1*, *kw8.1*	*mp8.1*
qFe11.2	11	RM209–RM21	*nt11.1*, *bm11.1*, *yldp11.1*	*mp11.1*, *asv11.1*, *gc11.1*
qFe11.3	11	RM254–RM224	*npt11.1*	
qFe12.1	12	RM19–RM247	*nt12.1*, *npt12.1*, *npt12.2*, *nfg12.1*, *nsp12.1*, *yldp12.1*	
qZn1.1	1	RM488–RM431	*ph1.1*, *npt1.1*, *nsp1.1*, *nfg1.1*, *kw1.2*, *kw1.4*	*mp1.2*, *ver1.2*
qZn2.1	2	RM250–RM535	*ph2.1*, *nsp2.1*, *nfg2.1*, *bm2.1*, *yldp2.1*, *wup2.1*	*gc2.1*
qZn3.1	3	RM55–RM520	*nt3.1*, *yldp3.1*	*er3.1*
qZn3.2	3	RM517–RM16	*nsp3.1*, *gw3.1*	*mp3.1*
qZn5.1	5	RM153–RM413	*bm5.1*	*klac5.1*, *ac5.1*, *ac5.2*
qZn6.1	6	RM30–RM439	*npt6.1*	
qZn8.1	8	RM256–RM264		*klac8.1*
qZn8.2	8	RM152–RM223	*nfg8.1*	
qZn9.1	9	RM215–RM189	*bm9.1*	
qZn12.1	12	RM415–RM19	*nt12.1*	*mp12.1*, *klac12.1*, *lwr12.1*

ac, Amylose content; *asv*, Alkali spreading value; *bm*, Vegetative biomass; *dtm*, Days to maturity; *er*, Elongation ratio; *gc*, Gel consistency; *kl*, Grain length; *gw*(*kw*), Grain weight; *klac*, Kernel elongation after cooking; *lwr*, Grain length and width ratio; *mp*, Milling potential; *nt*, Number of tillers; *npt*, Number of productive tillers; *nsp*, Number of spikelets; *nfg*, Number of filled grains; *ph*, Plant height; *ver*, Volume expansion ratio; *wup*, Water uptake; *yldp*, Plot yield; *yld*, Yield.

Table 6. Candidate genes underlying or close to Fe and Zn QTLs.

QTL	Chromosome	Marker interval	Gene	Gene ID	Site (Mb) [a]
qFe1.1	1	**RM243**–RM81A	*OsNRAMP6*	Os01g0503400	9.5
			OsYSL1	Os01g0238700	0.3
qFe2.1	2	**RM324**–RM475	*OsNAAT1*	Os02g0306401	0.6
qFe3.1	3	RM517–**RM156**	*OsNAS2*	Os03g0307200	4.8
			OsNAS1	Os03g0307300	4.8
qFe4.1	4	**RM241**–RM348	*OsFRO1* [b]	Os04g0444800	4.1
			OsFRO2	Os04t0578600-02	3.5
			OsYSL16	Os04g0542800	0.3
qFe8.2	8	**RM38**–RM223	*OsZIP4*	Os08g0207500	4.1
			OsYSL17	Os08g0290300	8.8
qFe11.1	11	**RM332**–RM287	*OsNAC5*	Os11g0184900	1.4
qZn1.1	1	**RM488**–RM431	*OsNAC4*	Os01g0816100	4.2
			OsHAP3	Os01g0834400	3.1
qZn5.1	5	**RM153**–RM413	*Metallothionein* [b]	Os05g0111300	1.6
qZn6.1	6	RM30–**RM439**	*OsNRAMP3*	Os06g0676000	1.6
qZn12.1	12	**RM415**–RM19	*Myb* transcription factor	OJ1085_G07.8	1.1

[a] Approximate physical distance between the candidate gene location and the nearest flanking marker (in bold) of the QTL; [b] Genes close to the QTL but not within.

DISCUSSION

Several previous studies have reported wider variations for grain Fe and Zn concentrations within rice gene pool, especially in landraces and wild species (Chandel et al, 2010; Anuradha et al, 2012a; Sarla et al, 2012). *O. nivara*, *O. rufipogon*, *O. officinalis* and *O. latifolia* were reported to have 4–5 times higher mineral concentrations in comparison to *O. sativa* (Chandel et al, 2010; Anuradha et al, 2012a; Sarla et al, 2012). This provides huge opportunities for exploitation of rice germplasm to develop mineral rich rice varieties through breeding.

Wild rice species have been less utilized in rice breeding due to their poor phenotypic appearance, crossing barriers, seed-setting rate and segregation distortions (McCouch et al, 2008; Swamy et al, 2008a). However, advanced backcross QTL analysis (AB-QTL) approach has been successfully used in exploiting wild progenitors of rice (Tanksley and Nelson, 1996; Septinignsih et al, 2003; Thomson et al, 2003; Ma et al, 2016). This approach helps to restore seed-setting rate and get rid of undesirable traits through successive backcrosses with cultivated rice. This process helps in simultaneous QTL identification and introgression to develop backcross inbred lines (Tanksley and Nelson, 1996). Thus, it is possible to explore the wild rice genetic sources in breeding programs to combine important agronomic traits and grain mineral elements (Swamy et al, 2011b, 2012, 2014).

O. nivara accessions IRGC81848 and IRGC81832 were very diverse, and they had 2–3 folds higher Fe and Zn concentrations compared to the recipient parent Swarna. The two BC_2F_3 mapping populations showed transgressive segregants for both Fe and Zn concentrations. Such transgressive segregants have been frequently reported in the progenies of wide crosses, and this phenomenon is mainly attributed to accumulation of superior alleles and positive allelic interactions in the segregating progenies (Septiningsih et al, 2003; Thomson et al, 2003). Transgressive segregants have been reported for grain quality traits in populations derived from *O. rufipogon* and *O. glaberrima* (Septiningsih et al, 2003; Aluko et al, 2004; Li et al, 2004).

Fe and Zn concentrations showed normal distribution pattern in both the populations, indicating their polygenic inheritance. Correlations between Fe and Zn concentrations were highly significant and positive in both the two populations. Previous studies also reported positive correlation between these two elements in brown rice (Anandan et al, 2011; Gande et al, 2013; Sathisha, 2013). This indicates that uptake, translocation and loading mechanisms of Fe and Zn may involve common genes and gene networks and pathways in rice.

The QTLs identified in this study were distributed on all chromosomes except chromosome 7. The PV explained by each of the QTLs varied from 3% to 36%. Most of the QTLs identified for Fe and Zn concentrations in both populations were derived from *O. nivara*. The results substantiate the significant contributions of *O. nivara* compared to *O. sativa* allele to increase the Fe and Zn concentrations in rice grains.

QTLs for Fe and Zn concentrations detected in our study were compared with those previously reported. In all, 14 of the 30 QTLs shared similar chromosomal locations with the QTLs reported for Fe and Zn concentrations from previous reports (Table 2). *qFe1.1* and *qZn9.1* were also reported by Lu et al (2008); *qFe3.1* and *qZn3.2* were also reported by Anuradha et al (2012b); *qFe4.1* was also reported by Norton et al (2010); *qFe6.1* was reported by Norton et al (2010) and Zhang et al (2014); *qFe8.1*, *qFe8.2* and *qZn8.2* were also reported by Garcia-Oliviera et al (2009). A previous study reported QTLs for Fe concentration on chromosomes 7, 8 and 9, respectively (Gregorio, 2002). These results clearly show the accuracy and consistency of QTLs identified for grain Fe and Zn.

Three QTLs for Fe concentration (*qFe2.1*, *qFe3.1* and *qFe8.2*) were common between the two populations. Similarly, *qZn12.1* has been identified on chromosome 12 between RM415 and RM19 in both the two populations (Table 2). This clearly indicates the conservation of some alleles for Fe and Zn concentrations in two different accessions of *O. nivara*. Co-location of Fe and Zn QTLs on chromosome 12 has also been reported previously (Stangoulis et al, 2007; Anuradha et al, 2012b). Recently, Xu et al (2015) reported a QTL region between RM19 and RM247 on chromosome 7 for both Fe and Zn concentrations. However, we found no QTLs on chromosome 7 in this study. The QTLs identified for Fe and Zn concentrations were also co-located with QTLs for other micronutrients. For example, *qFe1.1* identified in population 1 is located with QTL for Mn (Stangoulis et al, 2007), and *qFe4.1* identified in the population 2 is co-located with QTL for Ca (Lu et al, 2008).

QTLs for Fe and Zn concentrations co-localized with QTLs for grain quality traits

All the QTLs identified for Fe and Zn concentrations in both populations were associated with one or more QTLs for yield, yield components and grain quality traits except *qFe8.2*, *qFe11.1* and *qZn10.1*. A clear understanding of the co-location of QTLs and their effect on grain Fe, Zn concentrations and yield is very important for using major effect QTLs in marker assisted breeding. Studies have clearly shown negative relationship between yield and grain Zn concentration. Hence, in biofortification breeding programs, more consideration should be given to grain size, weight and grain yield when selecting for accessions with higher grain Zn concentration (Zeng et al, 2005, 2010; Anandan et al, 2011; Swamy et al, 2016). The epistatic analysis detected only one significant interaction in population 1. The interacting loci were RM106–RM6 on chromosome 2 and RM22–RM7 on chromosome 3. Lu et al (2008) reported 28 genome wide additive × additive interactions for mineral elements in rice. Anuradha et al (2012a) also reported epistatic interactions between loci on chromosomes 1 and 5 for grain Zn concentration.

We shortlisted metal homeostasis genes underlying QTLs for Fe and Zn concentrations using RAP-DB (Table 6). *OsNAC5* underlying *qFe11.1* overexpresses in flag leaf and panicle, and it is positively correlated with grain Fe and Zn concentrations (Sperotto et al, 2010). A senescence controlling *OsNAC5* gene NAM-B1 was reported to be associated with grain protein, Fe and Zn concentrations in wheat (Uauy et al, 2006). Therefore, this gene may be required for effective Fe and Zn mobilization in rice.

Nicotianamine synthase is required for the biosynthesis of nicotianamine, a co-substrate of the yellow stripe like proteins involved in metal homeostasis (Schaaf et al, 2004). Recently, overexpression of *OsNAS1*, *OsNAS2* and *OsNAS3* genes in rice shows about two to three folds increase in Fe and Zn concentrations in unpolished rice (Lee et al, 2009b; Johnson et al, 2011). *OsNAS1* and *OsNAS2* are found to be overexpressed in roots under Fe deficiency conditions (Agarwal et al, 2014). *OsNAS2* overexpression shows 2.7-fold increase in Zn and 20-fold increase in nicotianamine levels. It has been suggested that higher nicotianamine leads to greater exudation of phytosiderophores from the roots, and it stimulates higher Zn uptake, translocation and seed-loading as well (Lee et al, 2011).

OsYSL family is major metal transporters in rice. *OsYSL16* and *OsYSL17* were co-located within Fe QTLs. Activation of *OsYSL16* have been reported to improve Fe use efficiency by facilitating Fe distribution within a plant (Lee et al, 2009a; Kakei et al, 2012). *MYB10* and *MYB72* are the major transcription factors found to be overexpressed under both Fe and Zn toxicity and deficiency conditions, and they drive the expression of *OsNAS4*, which is essential for the plant survival under Fe deficiency (Palmer et al, 2013). MYB transcription factors were detected within *qZn12.1* in population 2. The OsFRO2 family genes help in absorption of metal ions during early growth stages of the plants, hence, essential for crop establishment (Robinson et al, 1999; Banerjee

and Chandel, 2011). The co-location of metal homeostasis genes with Fe and Zn QTLs provides a positive evidence for the accuracy of the mapping and usefulness of these QTLs in marker assisted breeding. The identified candidate genes can be functionally analyzed for their roles in Fe and Zn homeostasis.

CONCLUSIONS

Development of micronutrient dense rice varieties is one of the sustainable and cost effective approaches to tackle micronutrient malnutrition. *O. nivara* accessions IRGC81848 and IRGC81832 have 2–3 times higher Fe and Zn levels than popular cultivated rice variety Swarna. Most of the trait-increasing QTL alleles were derived from *O. nivara*. Eight QTLs contributed more than 15% phenotypic variance. *qFe2.1*, *qFe3.1*, *qFe8.2* and *qZn12.1* were consistently identified in both the two populations. Epistatic interaction was observed only for Fe concentration in population 1. A total of 16 metal homeostasis genes were found to co-locate with 10 QTLs for Fe and Zn concentrations in both the populations. Comparison of Fe and Zn QTLs were found to co-locate with QTLs for grain yield and grain quality traits. Major effect QTLs are useful in marker assisted breeding, fine mapping and candidate gene identification. The introgression lines with high Fe and Zn concentrations produced in this study clearly show that mineral dense rice varieties can be developed using closely-related wild species of rice in conventional breeding.

ACKNOWLEDGEMENTS

The authors thank University Grant Commission-Council of Scientific and Industrial Research for fellowship and Mr G. ASHOK REDDY for help in field work and Ms Gowthami CHANDU for help in revision. The work was financially supported by Network Project on Transgenics and Functional Genomics of Crops-Project 3019 of the Indian Council for Agricultural Research, India (NPTC/FG/05/2672/33).

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(Managing Editor: LI Guan)