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Identification of QTLs and candidate genes for high grain Fe and Zn concentration in sorghum [*Sorghum bicolor* (L.)Moench]

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ABSTRACT

Sorghum is a major food crop in the semi-arid tropics of Africa and Asia. Enhancing the grain iron (Fe) and zinc (Zn) concentration in sorghum using genetic approaches would help alleviate micronutrient malnutrition in millions of poor people consuming sorghum as a staple food. To localize genomic regions associated with grain Fe and Zn, a sorghum F_6 recombinant inbred line (RIL) population (342 lines derived from cross 296B × PVK 801) was phenotyped in six environments, and genotyped with simple sequence repeat (SSR), DArT (Diversity Array Technology) and DArTSeq (Diversity Array Technology) markers. Highly significant genotype × environment interactions were observed for both micronutrients. Grain Fe showed greater variation than Zn. A sorghum genetic map was constructed with 2088 markers (1148 DArTs, 927 DArTSeqs and 13 SSRs) covering 1355.52 cM with an average marker interval of 0.6 cM. Eleven QTLs (individual) and 3 QTLs (across) environments for Fe and Zn were identified. We identified putative candidate genes from the QTL interval of *qfe7.1*, *qzn7.1*, and *qzn7.2* (across environments) located on SBI-07 involved in Fe and Zn metabolism. These were CYP71B34, and ZFP 8 (ZINC FINGER PROTEIN 8). After validation, the linked markers identified in this study can help in developing high grain Fe and Zn sorghum cultivars in sorghum improvement programs globally.

1. Introduction

Globally, more than three billion people suffer from micronutrient deficiency, particularly iron (Fe) and zinc (Zn) owing to consumption of poor quality diets that lack adequate micronutrients (Jin et al., 2013). Micronutrient deficiencies can impair the mental and physical development of children and adolescents and result in lower IQ. Stunting and blindness prevalence in women and children is disproportionately high in developing countries. Of the four billion people affected by Fe deficiency globally, more than two billion are in developing countries (WHO, 2011). According to Food and Agriculture Organization (FAO, 2017) two billion people still remain at the risk of Zinc deficiency, the most prone are infants, young children, pregnant and lactating women due to their elevated requirements of Zinc. Moreover Fe and Zn exist predominantly as phytate complexes within the aleurone layer and phytate is major inhibitor of Fe and Zn absorption, hence the bioavability of iron and zinc in sorghum grain is low due to presence of phytate (Zhao et al., 2019). Nutrient supplementation and fortification are the most widely used methods to address the micronutrient malnutrition globally. Biofortification is the process of increasing the density of vitamins and minerals in a crop, through plant breeding or agronomic practices so that when consumed regularly, will generate measurable improvement in vitamin and mineral nutritional status. It is considered a promising approach to overcome micronutrient

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malnutrition. Globally, sorghum is the fifth most consumed crop, and major staple for nearly 500 million people in Africa and Asia. The production and consumption of sorghum is significantly high in the semi-arid parts of Africa and India where in more than 70 percent of the sorghum grain is utilized for human consumption (FAO, 1996). It is a heat and drought tolerant C_4 plant with wider adaptation to a range of growing environments. Sorghum grain is gluten-free and safe for people with celiac disease and is a preferred dietary choice due to its low-glycemic index and high anti-oxidant levels (Ashok Kumar et al., 2015). Sorghum is good source of energy with sufficient amounts of Fe and Zn, also rich in potassium, phosphorus, calcium and sodium. Considering its importance as staple and rich nutrient status, sorghum is targeted for biofortification. Sorghum can be further improved to make it more nutrient-dense tobenefit the populations suffering from micronutrient malnutrition.

Genetic variation in the crop is critical for improving the trait of interest. Fe and Zn being quantitatively inherited, deciphering the genetic architecture of these traits is very essential for development of micronutrient-efficient genotypes. Earlier studies showed that there exists considerable genetic variability and high heritability for grain Fe and Zn concentration in sorghum. Largest variability for grain Fe and Zn in sorghum was found in the germplasm and it is feasible to exploit this variation by understanding the genetic control of grain Fe and Zn (Ashok Kumar et al., 2013). Further it was indicated that it is possible to breed sorghum with enhanced levels of micronutrients (Fe and Zn) in desirable maturity backgrounds (Ashok Kumar et al., 2013). An understanding of trait relationships helps to formulate an appropriate breeding strategy. Strong genotype × environment interaction was reported for grain Fe and Zn concentration in sorghum that can affect the heritability. However, high broad sense heritability for grain Fe (95.5%) and Zn concentrations (96.5%) was observed in diverse sorghum genotypes and in adapted cultivars (Hariprasanna et al., 2012). Highly significant and positive correlation was observed between grain Fe and Zn concentration ($r = 0.853$; $P < 0.01$). Earlier studies in sorghum also indicated that both Fe and Zn were quantitatively inherited and Zn is predominantly under the control of additive gene action and both additive and non-additive gene actions are important in controlling grain Fe concentration (Ashok Kumar et al., 2013). Therefore, both parents need to be improved for Fe and Zn for developing hybrids with high Fe and Zn. Further, it's possible to predict the hybrid performance based on the mean of parental lines (Ashok Kumar et al., 2013).

In the sorghum grain minerals are unevenly distributed. The highest concentration of Fe and Zn can be found in the aleurone layer and the embryo (in particular the scutellum) and a low concentration in starchy endosperm (Chavan and Patil., 2010). The concentration of Fe and Zn differ within a grain, due to accumulation of typically much higher concentrations in the embryo than in aleurone layer and endosperm of a grain (Singh et al., 2018). Micronutrients accumulate in grain by the uptake during seed development and requisitely pass through the plant to reach the grain. Thus, understanding the route of these micronutrients, and the role of genes responsible for their uptake, translocation and loading into the endosperm is crucial. The map positions of these genes and linked markers on a chromosome need to be determined for the effective genetic dissection and manipulation of complex traits in crop plants. Several QTLs and candidate genes governing grain Fe and Zn concentration have been reported in cereals such as, rice (Anuradha et al., 2012), wheat (Velu et al., 2018); barley (Sadeghzadeh et al., 2010) and in maize (Jin et al., 2013). However, no reports are available on the QTLs and genes controlling grain Fe and Zn in sorghum. Therefore, we attempted the identification of QTLs and underlying candidate genes in sorghum using recombinant inbred lines (RILs) population developed from a cross 296B × PVK 801.

2. Material and methods

2.1. Plant material and field evaluation

The RIL population consisting of 342 lines (F_6 generation) developed using two contrasting parents (Grain Fe/Zn, mold reaction, yield and other agronomic traits) 296B and PVK 801, was used in this study for phenotyping and genotyping. The phenotypic trials were conducted for two years (during postrainy seasons 2012–13 and 2013–14) at three locations viz., 1) International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) (17.53° N, 78.27° E) located at an altitude of 545 m above meansealevel, 2) ICAR - Indian Institute of Millets Research (IIMR) (17.19° N, 78.28° E) located at an altitude of 542.3 m above mean sea level and 3)Vasantrao Naik Marathwada KrishiVidyapeeth (VNMKV), Parbhani (18.45° N, 76.13° E) located at an altitude of 357 m above mean sea level, which are located in major grain sorghum growing areas in India. The three locations and two years form six environments, hereafter will be referred as ICRISAT 2012–13(E1), IIMR 2012–13(E2), VNMKV 2012–13(E3), ICRISAT 2013–14(E4), IIMR 2013–14(E5) and VNMKV 2013–14(E6). All the field experiments were conducted in post-rainy seasons to obtain the best quality grain for assessing grain Fe and Zn.

The soil Fe and Zn in the experimental field were analysed by DTPA extractable method at Charles Renard Analytical Laboratory, ICRISAT, Patancheru, and expressed as mg kg^{-1} (ppm). These Fe and Zn contents in the soil were in the sufficient range for normal plant requirements (2.6–4.5 mg kg^{-1} for Fe; 0.6–1.0 mg kg^{-1} for Zn) (Sahrawat and Wani, 2013). All the recommended agronomic practices were followed for raising a good crop. Observations were recorded on the following traits: Days to 50% flowering, plant height, 100-grain weight (test weight), grain yield and grain Fe and Zn concentration using standard procedures. The grains were harvested at physiological maturity stage. During harvest the main panicles of five random plants from each plot were harvested and stored separately in a cloth bag to produce clean grain samples for micronutrient analysis. The remaining panicles of the plot were harvested as a bulk. These panicles were sundried for 10–15 days. Five separately harvested panicles were manually threshed for the each replicate and approximately 20 g of grain was collected for Fe and Zn analysis and for 100 grain weight. Leftover grains from these panicles were added to the bulk grain produced by threshing in a multi head machine thresher. The grain yield including the 20 g sample taken for micronutrient analysis was recorded for each plot and extrapolated to tonnes per hectare for grain yield analysis. In all the experiments, self-pollinated (SP) grain samples were used to estimate grain Fe and Zn concentration expressed in mg kg^{-1}. The harvested panicles were put directly in a separate cloth bag to avoid soil contamination and dried in the sun to <12% post-harvest grain moisture content. Grains were cleaned from their glumes, panicle chaff, and debris and transferred to new, non-metal fold envelops and stored at cold temperatures. Due care was taken at each step to avoid contamination of the grains with dust or other extraneous matter.

The grain Fe and Zn concentration of the grains from all six environments were analysed at the Charles Renard Analytical Laboratory, ICRISAT, Patancheru, India following the standard method (Wheal et al., 2011). The digests were filtered and Fe and Zn in the digests were analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

3. Statistical analysis

3.1. Analysis of variance

Combined Analysis of Variance was carried out for six environments by modelling individual environment (a combination of location and year) error variances using mixed model procedure. Five variance

components (σ_g^2, σ_{gy}^2, σ_{gl}^2, σ_{gyl}^2, σ_e^2) were estimated for each of the six traits studied using restricted maximum likelihood estimation procedure (Paterson and Thompson, 1971) using GenStat Software, 17th edition (VSN International, Hemel Hempstead, UK). In these analyses, environment (location and year) was fitted as a fixed effect. Genotype, blocks, replicates and genotype interactions with environment, were fitted as random effects. The phenotypic observations z_{ijklm} on accession m in replicate k of block l of location j and year i was modelled as:

$$z_{ijklm} = \mu + y_i + e_j + ye_{ij} + r_{ijk} + b_{ijkl} + g_m + (yg)_{im} + (eg)_{jm} + (yeg)_{ijm} + \varepsilon_{ijklm}$$

Where μ is the grand mean; y_i is the fixed effect of year i; e_j is the fixed effect of location j; ye_{ij} is the fixed effect of interaction between year i and location j; g_m is the random effect of genotype m and is~NID(0, σ_g^2); r_{ijk} is the random effect of replication in location j and year $i$and is~NID (0, σ_r^2); b_{ijkl} is the random effect of block l nested with replication k in location j and year i and is~NID(0, σ_b^2); $(yg)_{im}$ is the random effect of the interaction between genotype m and year i and is~NID(0, σ_{yg}^2); $(eg)_{jm}$ is the random effect of the interaction between accession m in location j and~NID(0, σ_{eg}^2); $(yeg)_{ijm}$ is the random effect of the interaction effect of the genotype m in year i and location j and~NID(0, σ_{yg}^2); and ε_{ijklm} is the random residual effect and~NID(0, σ_ε^2).

Analyses of variance were also conducted using data from each environment for all six traits.

Heritability (H^2, broad sense) at individual environment was estimated from analysis of variance. The formula used as -

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_\varepsilon^2 / r}$$

whereas Heritability estimates across the environments were estimated by the formula as-

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{yg}^2 / y + \sigma_{eg}^2 / e + \sigma_{yeg}^2 / ye + \sigma_\varepsilon^2 / rye}$$

Where r, y, e denotes the number of replicates, years and environments respectively. The relationship between grain Fe and Zn concentration and agronomic traits like days to 50% flowering, plant height, 100-seed weight and grain yield were determined using Pearson correlation coefficient and genotypic correlation (Karl Pearson., 1895) using BLUPs (Best Liner Unbiased Predictors) of single environment as well as across the environments.

Genotyping, linkage map construction and QTL mapping:

DNA isolation (Mace et al., 2003) of RIL population and polymorphic screening with SSR markers were done at Center of Excellence in Genomics (CEG) Lab at ICRISAT, Patancheru, India. The DNA of 342 RIL population along with two parents was analysed for DArT analysis at Diversity Arrays Technology Pvt. Ltd. (DArT P/L), Yarralumla, ACT 2600, Australia (http://www.diversityarrays.com). The SSRs, DArT and DArTseq (SNPs) markers were used to genotype the parents and their derived RILs. A genetic linkage map was constructed using both the co-dominant microsatellite data and the dominant DArT and SNPs marker data using JoinMapv4.0 (van Ooijen et al., 2006). Markers within each group were mapped using the regression mapping algorithm with the LOD score 10 and map distance was estimated using the Kosambi mapping function. Quantitative trait locus (QTL) analysis was performed using the composite interval mapping (CIM) by the software Win Cartographer V2.5_011 with a one cM walk speed, threshold LOD of 2.5 and 1000 permutations. QTL analysis was performed using the BLUPs of across environmentsand individual environments. The original RIL population contained 342 lines. However, due to more than 10% non-parental data points (RIL from two parents couldn't be discriminated) few of RILs were removed in linkage mapping. Finally 309 lines were used for genetic linkage analysis and QTL detection.

3.2. Bioinformatics analyses

The stable QTLs across locations were analysed *insilico* to identify putative candidate genes involved in Fe/Zn metabolism within QTLs interval. The putative genes within the identified QTL region across the regions were retrieved using Plant GDB (http://www.plantgdb.org/SbGDB/cgi-bin/downloadGDB.pl). Further, literature search was performed to identify the putative candidate genes related to Zn, Fe uptake, homeostasis and accumulation in grains, that are present within the identified QTLs. Sorghum genes underlying Fe/Zn QTLs were used to analyze the gene synteny with *rice* and *maize* using Phytozome 10.3 (Sorghum bicolor v2.1_PhytoMine) software *Gene synteny - Phytozome 10.3 > JBrowse > bicolor v2.1 > Gene info > Homologues.*

4. Results

4.1. Mean performance, G × E interaction, heritability and trait association

The RIL population (296B × PVK 801) having 342 RILs of F_6 generation was phenotyped in six environments i.e. three locations over two years. The trials were conducted using Alpha Lattice Design with three replications for agronomic traits and grain Fe and Zn concentration along with parents to obtain means and variances (Tables 1a and 1b). The phenotypic data collected for the population during post-rainy seasons of 2012–13 and 2013–14 in six environments in total were analysed statistically to obtain variance components. The mean performance for grain Fe and Zn concentration in both the parents and RILs were higher in VNMKV 2012–13, whereas lowest in ICRISAT 2013–14, also both the parents exhibited wider range of variation for grain Fe and Zn studied in different environments.

The grain micronutrient (Fe and Zn) concentration showed highly significant genotypic variances in all the individual environments, werein, the grain Fe concentration showed the highest genotypic variance in VNMKV 2013–14 and the grain Zn concentrations showed highest variance in VNMKV 2012–13. The same trend continued across environments, both the traits showed highly significant genotypic variances, whereas genotype × year (σ^2gy) interaction for Zn was significant, but less in magnitude compared to genotypic variance. For grain Fe, the genotype × year (σ^2gy) interaction was non-significant. Genotype × location (σ^2gl) interactions were found to be non-significant for both the micronutrients but genotype × year × location (σ^2gyl) interactions were highly significant for both the traits, also the magnitude of variance was more than genotypic variances. The frequency distribution of Fe and Zn based on across environments BLUPs showed transgression beyond the parent's values for both the traits. Considerable transgressive segregation and significant differences in traits was obtained in present RIL population, indicating the occurrence of large variation among the RILs specifying that the RIL population is desirable for QTL mapping.

In the present study both grain Fe and Zn were highly heritable (>0.60) in individual environments except for grain zinc concentration in IIMR 2013–14 environment (Table 1b). Agronomic traits like days to 50% flowering, plant height, 100-seed weight and grain yield were found to be more heritable than grain micronutrients (data not shown). However, a partitioned genotype × environment interaction component reduced the heritability across the environments (pooled analysis). Broad sense heritability for all the traits was high (0.30–0.60) across the six environments. For grain Fe and Zn concentration, the heritability was high in first year (post-rainy 2012–13) compared to second year (post-rainy 2013–14), whereas environment-wise IIMR- 2013-14 showed lowest value for Fe and Zn heritability in tune with low genotypic variance for these traits in same environment.

Pearson's phenotypic and genotypic correlation among traits were

Table 1
Means (Table 1a) & variances (Table 1b) and heritability for grain Fe and Zn in sorghum (296B × PVK 801)-derived RIL population. a) The means and standard deviation of the mean for Fe/Zn measured for parents and RILs in individual environments. b) Variances and heritability for Fe and Zn in 296B × PVK 801 derived RIL population.

Trait	Environment	296B (P1)	PVK 801 (P2)	RILs	SD (±)
Fe (mg kg^{-1})	ICRISAT 12–13 (E1)	28.00	33.4	33.60	5.60
	IIMR 12–13 (E2)	28.50	33.00	33.00	6.34
	VNMKV 12–13 (E3)	46.33	49.4	49.26	6.93
	ICRISAT 13–14 (E4)	26.00	28.2	28.00	4.90
	IIMR 13–14 (E5)	30.80	35.9	35.85	5.08
	VNMKV 13–14 (E6)	27.24	33.6	34.00	7.89
Zn (mg kg^{-1})	ICRISAT12 -13 (E1)	21.32	24.33	24.63	4.78
	IIMR 12–13 (E2)	21.00	22.00	24.76	5.00
	VNMKV 12–13 (E3)	26.44	30.43	31.43	6.46
	ICRISAT 13–14 (E4)	14.63	16.46	17.33	3.50
	IIMR 13–14 (E5)	21.19	24.82	25.66	4.03
	VNMKV 13–14 (E6)	19.69	24.06	24.72	5.27

Pooled (across six environments)

Trait	σ^2g	SE (±)	σ^2gy	SE (±)	σ^2gl	SE (±)	σ^2gyl	SE	H^2
Fe	4.18**	0.69	−0.17	0.66	−0.7	0.80	14.32**	1.18	0.58
Zn	4.17**	0.51	0.71**	0.35	−0.14	0.37	5.22**	0.54	0.69

Individual environments

	ICRISAT 12–13 (E1)			**IIMR 12–13 (E2)**			**VNMKV 12–13 (E3)**		
	σ^2g	**SE (±)**	**H^2**	**σ^2g**	**SE (±)**	**H^2**	**σ^2g**	**SE (±)**	**H^2**
Fe	15.51**	1.68	0.78	20.79**	2.17	0.81	27.44**	2.85	0.8
Zn	10.00**	1.12	0.74	12.72**	1.33	0.8	23.41**	2.43	0.8
	ICRISAT 13–14 (E4)			**IIMR 13–14 (E5)**			**VNMKV 13–14 (E6)**		
Fe	8.72**	1.05	0.68	10.84**	1.3	0.68	31.36**	3.23	0.77
Zn	5.48**	0.6	0.73	4.60**	0.68	0.56	12.71**	1.4	0.74

Genotypic variance(σ^2g), Genotype × Year (σ^2gy) , Genotype × Location (σ^2gl), Genotype × Year × Location (σ^2gyl) interactions, standard error (SE), heritability's (H^2, broad-sense), SD= Standard Deviation, all variances ** Significant at 1% level.

computed for each individual environment and across the environments based on BLUPs of each individual environment and across the environments (Table 2). Significant positive and negative correlations were observed between the traits. Some of the traits were highly correlated while many of them had weak correlations. Based on BLUPs from the individual environments for phenotypic correlation, there was highly significant and high positive association between Fe and Zn concentrations in all the environments (E1 = 0.80, E2 = 0.69, E3 = 0.70, E4 = 0.72, E5 = 0.69 and E6 = 0.65; $p < 0.01$) and this trend was consistent in pooled analysis (AE = 0.79; $p < 0.01$). The correlation of grain Zn concentration and grain yield was negative, except in one environment (IIMR 2013–14(E5), but was smaller in magnitude compared to association between grain iron concentration and grain yield except in one environment (VNMKV 2013–14 (E6). For 100-seed

Table 2
Pearson phenotypic and genotypic correlation for agronomic traits and grain Fe and Zn concentration in sorghum RIL population.

Traits	ICRISAT 12–13 (E1)			IIMR 12–13 (E2)			VNMKV 12–13 (E3)		
	Fe	Zn	GY	Fe	Zn	GY	Fe	Zn	GY
Zn	0.88** (0.80**)	–	–	0.73** (0.69**)	–	–	0.76** (0.70**)	–	–
DTF	0.03 (0.04)	−0.08 (−0.06)	−0.23** (−0.22**)	−0.29** (−0.23**)	−0.17** (−0.1)	0.29** (0.23**)	0.04 (0.01)	−0.02 (−0.02)	−0.01 (−0.01)
PH	0.34** (0.29**)	0.30** (0.25**)	−0.02 (−0.01)	0.0008 (0.002)	0.14* (0.12*)	0.47** (0.46**)	0.34** (0.29**)	0.35** (0.30**)	0.17** (0.16**)
TW	0.34** (0.28**)	0.34** (0.30**)	−0.15** (−0.13*)	0.09* (0.06)	0.35** (0.30**)	0.29** (0.26**)	−0.01 (−0.01)	−0.04 (−0.023)	0.05 (0.045)
GY	- 0.41** (−0.32**)	−0.34** (−0.27**)	–	−0.33** (−0.28)	−0.16** (−0.16**)	–	−0.23** (−0.21**)	−0.33** (−0.29**)	–

	ICRISAT 13–14 (E4)			IIMR 13–14 (E5)			VNMKV 13–14 (E6)			Across Environment		
Traits	Fe	Zn	GY	Fe	Zn	GY	Fe	Zn	GY	Fe	Zn	GY
Zn	0.81** (0.72**)	–	–	0.83** (0.69**)	–	–	0.71** (0.65**)	–	–	0.88** (0.79**)	–	–
DTF	−0.16** (−0.14*)	−0.12** (−0.11*)	0.16** (0.13*)	−0.09 (−0.07)	−0.12* (−0.11*)	0.15** (0.12*)	0.1 (−0.08)	0.11* (0.08)	−0.04 (−0.03)	−0.18** (−0.12*)	−0.19** (−0.12*)	0.25** (0.19**)
PH	0.12** (0.09)	0.28** (0.23**)	0.30** (0.28**)	0.08 (0.06)	0.03 (0.04)	0.3** (0.35**)	0.26** (0.22**)	0.37** (0.31**)	0.17** (0.14**)	0.43** (0.31**)	0.39** (0.33**)	0.34** (0.31**)
TW	0.25** (0.20**)	0.47** (0.41**)	−0.17** (−0.12*)	0.12* (0.06)	0.03 (0.02)	−0.02 (−0.01)	0.17** (0.13*)	0.21** (0.18**)	−0.06 (−0.03)	0.56** (0.34**)	0.49** (0.36**)	−0.21** (−0.12**)
GY	−0.41** (−0.29**)	−0.31** (−0.24**)	–	0.06 (0.06)	0.02 (0.06)	–	−0.22** (−0.16**)	−0.22** (−0.19**)	–	−0.50** (−0.34**)	−0.45** (−0.34**)	–

DTF = Days to 50% flowering, PH= Plant Height (cm), TW = 100 seed weight (g), Fe= Iron (mg kg^{-1}), Zn = Zinc (mg kg^{-1}) and GY = Grain Yield (t/ha). Value in parenthesis is phenotypic correlation * Significant at 5% level; ** Significant at 1% level.

weight, out of six environments, grain Fe showed a significant positive association in only three environments (ICRISAT, 2012–13, ICRISAT, 2013-14 and VNMKV, 2013–14). Using across environment analysis, the Fe concentration showed significant positive (AE = 0.34; $p < 0.01$) association with 100-seed weight. In case of Zn concentration, four environments (ICRISAT, 2012–13, IIMR, 2012–13, ICRISAT-2013-14 and VNMKV, 2013–14) showed significant positive association and remaining two environments (VNMKV, 2012–13 and IIMR, 2013–14) did not show any significant association, whereas the across environment analysis of Zn concentration showed significant positive (AE = 0.36; $p < 0.01$) association with 100-seed weight.

The association between grain Fe concentration and days to 50% flowering ranged from (E2 = −0.23; $p < 0.01$ to E1 = 0.04; non-significant), while across the environmentsshowed significant negative (AE = −0.12; $p < 0.05$) association between Fe concentration and days to 50% flowering. The association of Zn concentration with days to 50% flowering ranged from (E4 = E5 = −0.11; $p < 0.05$ to E6 = 0.08; non-significant), while across the environments it showed significant negative (AE = −0.12; $p < 0.05$) association between Zn concentration and days to 50% flowering. The association between plant height and grain Fe concentration ranged from (E2 = 0.002; non-significant to E1 = E3 = 0.29; $p < 0.01$), while across environments it showed significant positive (AE = 0.31; $p < 0.01$) association. The plant height association with grain Zn concentration, ranged from (E5 = 0.04; $p <$ non-significant to E6 = 0.31; $p < 0.01$), while across environments it showed significant positive association (AE = 0.33; $p < 0.01$). The association between 100-seed weight (seed size) and grain yield ranged from (E1 = −0.13, $p < 0.05$ to E2 = 0.26, $p < 0.01$), while across environments it showed significant negative association (AE = −0.12, $p < 0.05$).

The association analysis using genotypic correlation also showed similar results indicating significant positive correlation between grain Fe and Zn (E1 = 0.88, E2 = 0.73, E3 = 0.76, E4 = 0.81, E5 = 0.83 and E6 = 0.71; $p < 0.01$) and this trend was consistent in pooled analysis (AE = 0.88; $p < 0.01$). The correlation of other agronomic traits with grain Fe and Zn showed same trend as Pearson phenotypic correlation (Table 2).

4.2. Construction of high density genetic map

A total of 271 SSR markers were used for a polymorphism survey of two parental lines. Forty-five out of 271 (16.60%) SSR markers detected polymorphism between 296B and PVK 801. From the Diversity Arrays Technology (DArT) analysis, a set of 6126 (70.15%) polymorphic DArT clones were identified in a total of 8732 clones. In addition, 3331 polymorphic DArtseq (SNPs) were identified in a total of 3640 clones (91.51%) on the array of 296B and PVK 801. After removal of the markers with distorted segregation and loci that contained >10% missing data, a total of 2088 markers were retained. A linkage map was constructed using 2088 polymorphic DArT (1,148), DArTseq (SNPs) (927) and SSRs (13) markers of sorghum RIL population. A length of 1355.52 cM was covered across the 10 chromosomes, with an average marker interval of 0.6 cM. The largest linkage group contained 257 markers and spanned 180.6 cM (SBI-03), while the smallest linkage group contained 79 markers and spanned only 102.6 cM (SBI-10). The average linkage group length was 135.5 cM with an average of 208.7 loci. The average adjacent marker interval length ranged from 0.51 cM (SBI-01 and SBI-05) to 1.29 cM (SBI-10) followed by 0.89 cM (SBI-08) and 0.0023% of the intervals (5 out of 2088) were more than 5 cM.

4.3. QTL analysis ofgrain Fe and Zn concentration

The QTL analysis for individual environments (Table 3) and across the environments (Table 4) was carried out to better understand the QTLs controlling grain Fe and Zn (Fig. 1).

4.4. Individual environment (E) analysis

4.4.1. ICRISAT

A total of 6 QTLs were identified for grain Fe and Zn concentration in ICRISAT 2012–13 (E1) environment. The 3 QTLs for Fe concentration

Table 3
Description of identified QTLs for grain Fe and Zn concentration in the (296B × PVK 801) - derived RIL population from six environments (E1 to E6) and across the environments (pooled data of six environments).

QTL name	Chr. No	QTL Position (cM)	Flanking Markers	Genetic position of marker (cM)	Physical position marker (bp)	LOD	Additive effect	PVE (%)
ICRISAT 12–13 (E1)								
qfe6.1(E1)	SBI-06	90.4	Sn2647940 – Sn2657501	90.3–91.5	54954413–54555447	4.6	0.84	5.44
qfe7.1(E1)	SBI-07	42.9	Dt3625344 - Sn2644846	36.8–49.6	57701276–60259798	3.6	0.87	5.82
qfe7.2(E1)	SBI-07	123.5	Sn2653248 - Xtxp525	123.4–126.7	4593373–2340000	4.3	0.81	5.09
qzn7.1 (E1)	SBI-07	42.9	Dt3625344 - Sn2644846	36.8–49.4	57701276–60259798	5.6	0.88	9.42
qzn7.2 (E1)	SBI-07	54.0	Dt2646020 - Sn1925068	53.0–54.7	56910000–57064983	6.8	0.86	8.80
qzn7.3 (E1)	SBI-07	57.0	Sn1895281 – Sn2650637	57.0–57.9	56502177–56741914	5.9	0.77	6.96
VNMKV 12–13 (E3)								
qfe7.1(E3)	SBI-07	65.5	Dt3627294 - Dt2648007	65.4–65.6	55123911–55156270	3.9	0.88	5.19
qzn7.1 (E3)	SBI-07	64.2	Sn1875097 - Xiabtp360	63.5–64.2	55324468–55900000	4.9	0.78	6.29
qzn7.2 (E3)	SBI-07	67.2	Dt3628977 - Dt2649259	67.1–67.6	54659732–54837270	4.3	0.75	5.83
ICRISAT 13–14 (E4)								
qfe1.1(E4)	SBI-01	107.4	Sn1933402 – Sn2650907	107.3–107.6	20084094–20071525	5.5	0.79	6.80
VNMKV 13–14 (E6)								
qfe7.1(E6)	SBI-07	61.0	Sn2645682 – Sn2653275	60.9–61.4	55409292–55538220	4.4	0.99	5.66
Across the environments (pooled data of six environments)								
Fe								
qfe7.2	SBI-07	123.5	Xtxp525 -Sn2653248	123.4–126.5	2340000–4593373	5.6	0.6	6.7
Zn								
qzn7.4	SBI-07	67.2	Dt2649259 -Dt3628977	67.1–67.6	54837270–54659732	4.5	0.53	5.7
qzn7.1	SBI-07	55.5	Dt2645576 -Sn2033434	55.4–56.2	56915197–56741980	4.5	0.54	5.7

Table 4
Details of putative candidate genes identified for Fe and Zn concentrations (Pooled data across all locations) in identified QTL region in sorghum.

QTL Name	Locus ID	Chr	From	To	Transcripts	Locus Description	Reference
qfe7.2	Sb07g003000	7	3191492	3193591	1	PF00067 PTHR19383 KOG0156 AT3G26300.1 CYP71B34 CYP71B34; electron carrier/heme binding/iron ion binding/monooxygenase/oxygen binding	Chen and Jia (2006) (Wheat), Xu et al. (2010) (Maize),
qzn7.4	Sb07g021130	7	54829074	54830555	1	PF10551 PF04434 PF00098 AT4G38180.1 FRS5 FRS5 (FAR1-related sequence 5); zinc ion binding	Nozoye et al. (2007) (Rice), Mills et al. (2012) (Barley), Ricachenevsky et al. (2018) (Rice), Yang et al., 2011 (Wheat)
qzn7.1	Sb07g021910	7	56151914	56153758	1	PF00097 PTHR22764 AT4G30400.1 zinc finger (C3HC4-type RING finger) family protein	Nozoye et al. (2007) (Rice), Mills et al. (2012) (Barley), Ricachenevsky et al. (2018) (Rice), Yang et al., 2011 (Wheat)
	Sb07g022160	7	56501258	56504350	1	PF01485 PTHR11685 AT3G14250.1 protein binding/zinc ion binding	Nozoye et al. (2007) (Rice), Mills et al. (2012) (Barley)
	Sb07g022165	7	56514545	56515453	1	PF01485 PTHR11685 AT3G14250.1 protein binding/zinc ion binding	Nozoye et al. (2007) (Rice), Mills et al. (2012) (Barley)
	Sb07g022570	7	57198318	57199295	1	AT2G41940.1 ZFP8 ZFP8 (ZINC FINGER PROTEIN 8); nucleic acid binding/transcription factor/zinc ion binding	Hindu et al., 2018 (Maize)

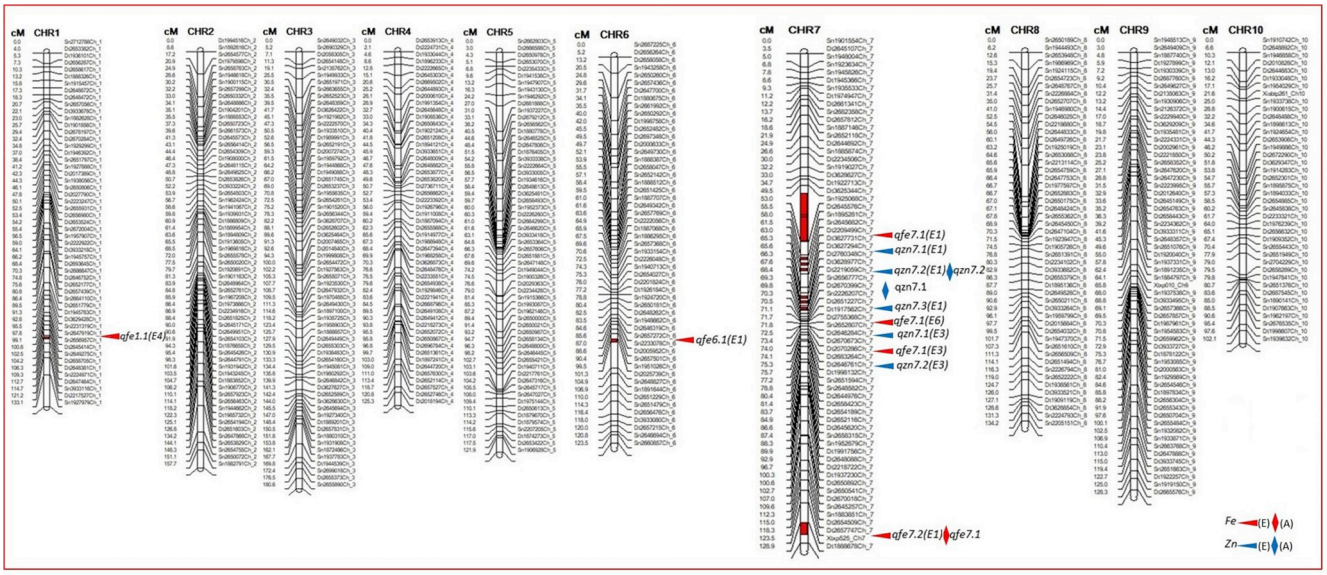

Fig. 1. Linkage map of sorghum RIL population derived from (296B × PVK 801) and chromosomal location of QTLs (individual and across environments) for gain Fe and Zn concentrations. All the QTLs were derived from higher parent PVK 801. In figure (A) indicates QTLs from across environments.

out of 6, were located on chromosomes SBI-06 and SBI-07 and 3 QTLs for Zn were on chromosome SBI-07. The phenotypic variation (PVE) for Fe explained by these QTLs ranged from 5.09% to 5.82%, and for Zn from 6.96% to 9.42%. Only one Fe QTL with 6.80% PVE was identified in ICRISAT 2013–14 (E4) environment on chromosome SBI-01. Two significant QTLs *qfe7.1* (E1) and *qzn7.1* (E1) associated with high PVE (Zn 9.4% and Fe 5.8%) co-located on SBI-07 (Flanking markers Sn2644846 - Dt3625344) for Fe and Zn concentration were found within the region of 57–60.3 Mb and for both of the alleles increasingly derived from PVK 801 parent.

4.4.2. IIMR

IIMR- 2013 (E2) and 2014 (E5) in both of the environments did not find any significant QTLs.

4.4.3. VNMKV

One Fe and 2 Zn QTLs were identified in VNMKV 2012–13 (E3) environment on chromosome SBI-07. The PVE by these QTLs for Fe was 5.19% and for Zn 5.83%–6.29%. One Fe QTL with 5.66% PVE was identified in VNMKV2013-14 (E6) environment on chromosome SBI-07.

4.4.4. Across environment analysis

One Fe and 2 Zn QTLs were identified across environments (pooled data of six environments) QTL analysis on chromosome SBI-07 (Table 4). The PVE explained by these were for both Fe and Zn QTLs ranged from 6.66% to 5.66–5.74% respectively. Two (*qfe7.1 and qzn7.1)* QTLs out of 3 were repeated from individual environment analysis.

4.4.5. Candidate gene prediction

Three QTLs were identified in this study (pooled data of six environments) which were analysed *in silico* for identifying putative candidate genes within the QTL marker intervals. In all, six genes governing Fe and Zn concentrations were found within three QTLs (Table 3). One gene belonging to Cytochrome P450 family and 5 genes related to zinc finger protein family were identified on chromosome SBI-07 (Table 4). In addition, we have also identified the genes on QTLs from individual locations and studied the synteny with other crops such as rice and maize (Fig. 2). In this, we identified 42 genes governing Fe and Zn concentrations within the identified QTLs.

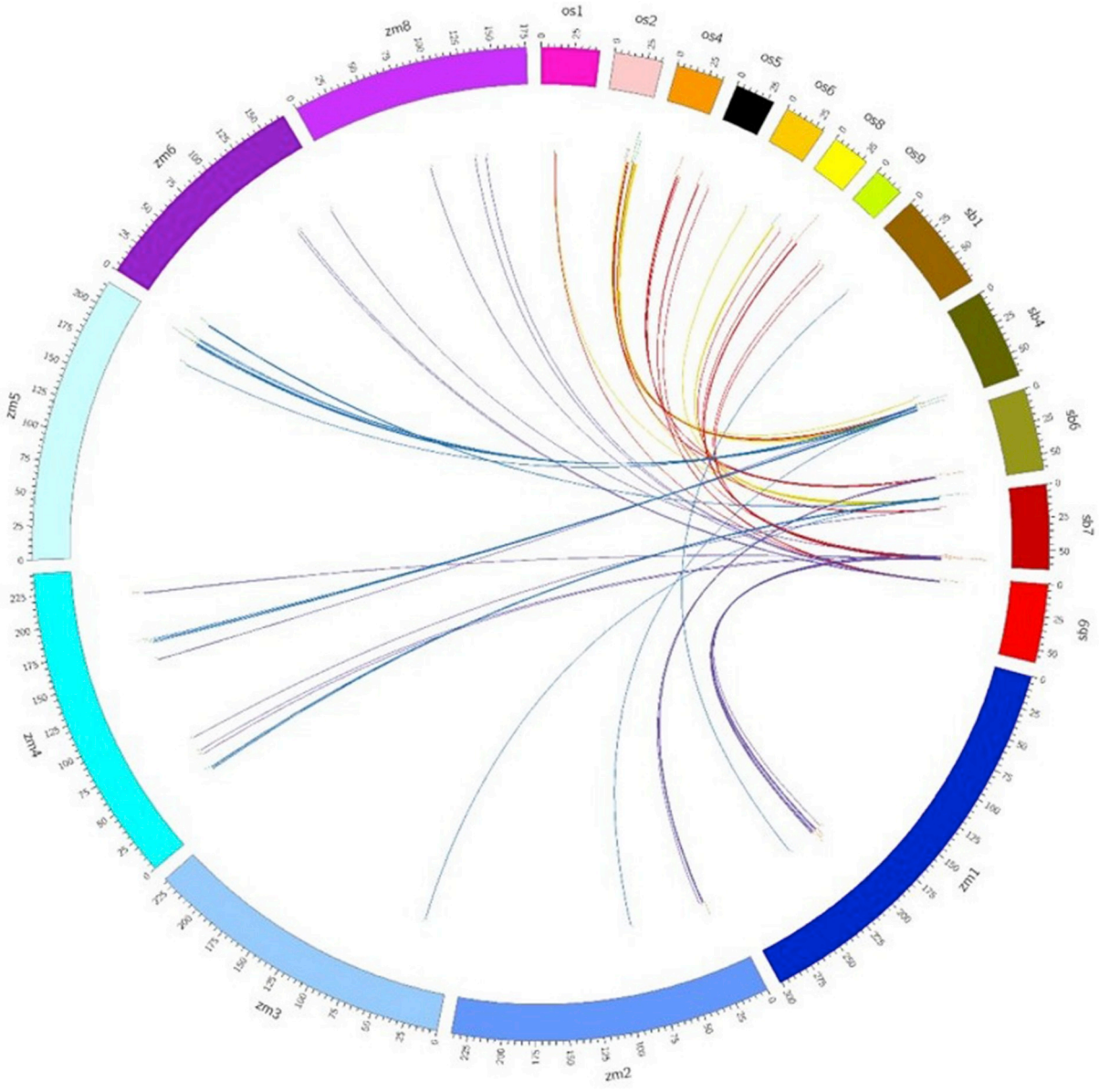

Fig. 2. Putative candidate genes underlying the Fe/Zn QTLs on sorghum chromosomes SBI-1, SBI-06 and SBI-07 showing gene syntenic relationship with Zea mays on chromosomes 1, 2, 3, 4, 6, 8 and 10 and Oryza sativa on chromosomes 1, 2, 4, 5, 6, 8, 9, 10, and 11.
Note: Homologues genes are connected by lines, Sb-*Sorghum bicolor*, Zm-*Zea mays* and Os-*Oryza sativa*.

4.4.6. Gene synteny analysis

The Synteny analysis was carried out using sequence information of the genes underlying QTLs located on the present map of sorghum with published information from other well-studied cereal crop species. Identified sorghum genes within QTL intervals were used to evaluate gene synteny with (*Zea mays*) and rice (*Oryza sativa*). The *S*ynteny sequence level between sorghum-maize ranged from 49% to 99%, while sorghum-rice ranged from 44% to 97%. Putative candidate genes underlying the regions associated with grain Fe/Zn QTLs in sorghum shown gene syntenic relationship with *Zea mays* on chromosomes 1, 2, 3, 4, 6, 8 and 10 and *Oryza sativa* on chromosomes 1, 2, 4, 5, 6, 8, 9, 10 and 11.

5. Discussion

The grain Fe and Zn are quantitatively inherited traits and the correlation between grain Fe and Zn concentration and agronomic traits is complex. Therefore, it is essential to understand the genetic architecture of these traits. Measuring the constancy of grain micronutrients for biofortification is critical, as sorghum is grown in diverse soil types with varying levels of fertility and nutrient management in different agro-eco zones. The current study showed that the parents have substantial differences for both Fe and Zn in all the environments. The average Fe and Zn concentrations were highest at VNMKV followed by IIMR and ICRISAT in first year analysis (2012–13), whereas in second year (2013–14) the average Fe and Zn concentrations were highest at IIMR followed by VNMKV and ICRISAT. The VNMKV soils are deep black with high moisture holding capacity compared to the soils of IIMR and ICRISAT. This indicates Genotype × Environment interaction possibly effecting mineral uptake, translocation and distribution calling for extensive testing of genotypes for commercialization. The association between grain Fe and Zn showed significant and high positive values and the trend was similar in across environment analysis. A significant positive association between Fe and Zn gives opportunity for simultaneous selection for both traits. No or weak association of grain Fe and Zn with other agronomic traits including yield enables breeders to develop high

yielding genotypes possessing high grain Fe and Zn in different maturity backgrounds (Ashok Kumar et al., 2015). The recent (2018) release of biofortified sorghum variety 'Parbhani Shakti' by VNMKV-Parbhani, Maharasthra, India in partnership with ICRISAT vindicated this. It showed 45 ppm Fe and 32 ppm Zn compared to the base level of 30 ppm Fe and 20 ppm Zn in sorghum. The grain yield (3.4 tons ha^{-1}) of Parbhani Shakti is higher than the best varieties currently grown in Maharashtra, India. https://www.scienceforum2018.org/sites/default/files/2018-09/SF18_case_study_bioavailable_micronutrients.pdf verified on 29 March 2019.

Multi-location and multi-season evaluation is a prerequisite for identifying stable donors for micronutrient enhancement and for identifying the most promising genotypes for commercialization. In the present study, the analysis of variances for all the traits revealed highly significant genotypic variances in individual environments as well as across environments indicating high degree of genotypic variance for the traits studied. For agronomic traits, the genotype × year × location (σ^2gyl) interaction values were significant, but lower than genotypic variance, suggesting that the traits are predominantly under genetic control and influenced by environments to a limited extent, which implies that there is no need for G × E portioning. Whereas, for both micronutrients, the genotype × year × location (σ^2gyl) interactions were significant and also higher than genetic variances indicating environment in different years played significant role in expression of grain Fe and Zn concentrations. Therefore, Fe and Zn concentration in grains show variation according to micronutrients concentration in soil and their availability to plants besides genotypic variability in extraction, translocation and loading. Another reason for greater G × E interaction for Fe and Zn concentrations could be their quantitative inheritance as reported in maize, rice and sorghum (Gregorio, 2002; Long et al., 2004; Ashok Kumar et al., 2013; Hariprasanna et al., 2012), therefore the progress in the genetic selection and improvement of these traits is expected to be slower than many other traits. However, in spite of these challenges, there is evidence that breeding for increased levels of micronutrients is feasible (Ashok Kumar et al., 2015). While stronger G × E effects call for testing the genotypes in large number of locations for many years for assessing the stability of genotypic performance, large variability, high heritability, stronger positive association between Fe and Zn traits offers great breeding opportunity for improving Fe and Zn in sorghum.

Identification of QTLs helps to have better grip over the traits by identifying genomic regions responsible for phenotypic variation. In that direction, to identify QTLs for Fe and Zn in sorghum, a RIL population was phenotyped in multiple environments over two years and genotyped using the SSR, DArT and DArTSeq markers. A linkage map length covering 1355.52 cM with an average marker interval of 0.6 cM indicates that good genome coverage was developed in the present study. In the first linkage map of sorghum using DArT and other markers developed by Mace et al. (2009), the reported map length was 1603.5 cM with average marker density of 0.79 cM. Compared to previous maps in sorghum, the present linkage map average adjacent-marker distance is smaller with high density of markers. Genome coverage reported in this map will be good source to select markers for use in whole-genome breeding strategies. Compared to SSR based genetic linkage map, a well saturated genetic linkage map developed using DArt/DArtseq markers for the RIL population is more suitable for precise QTL mapping.

The QTL analysis for grain Fe and Zn in individual environments identified different QTLs in different environments, which shows the effect of the environment on expression of traits beside the soil type and soil Fe and Zn concentration. It is noteworthy to highlight that several genes controlling Fe and Zn homeostasis in cereal grains and also grain Fe and Zn are highly quantitative in nature. The Occurrence of multiple QTLs nearly in all environments with small phenotypic variance, explains the cumulative effect of different genomic regions contributing to a small percentage of phenotypic variation on expression of QTLs. This suggests that the transport and accumulation of minerals in seed is a complex process and highly influenced by environment. However, the present QTL mapping results for grain Fe and Zn showing several genes with small effects cannot be investigated individually. The detection of a few QTLs with large phenotypic effect is often the result of a small population size or can be the artifact of the strong directional selection often used to create phenotypically divergent lines used for mapping. However, in the present study the mapping population used was oflarge size and the variation in parents of RIL mapping population is nearly 4 $mgkg^{-1}$ for both the traits Fe and Zn, which is lesser than variation used to select phenotypically divergent parental lines resulting in strong directional selection.

In this study, Fe and Zn QTLs were detected using a high-density genetic map, which serves as a reliable reference for mapping important micronutrient traits in sorghum. A total of 11 genomic regions identified for both micronutrients. A common genomic region identified with high phenotypic variance Zn 9.42% *(qzn7.1 (E1))* and Fe 5.82% *(qfe7.2 (E1))* on SBI-0 7 in ICRISAT environment. The results suggest that identification of 9 out of 11 genomic regions and QTLs with high phenotypic variance on chromosome SBI-07 might be the useful target regions for further improvement of both the micronutrients in sorghum. Two QTLs *qfe7.2* (E1) and *qzn7.2* (E3) on SBI-07 also were observed in across the environment analysis. The co-localized and stable (across) QTLs on chromosomes SBI-07 for Fe and Zn providing evidence for the significant positive correlation between for Fe and Zn in sorghum. The co-localization of QTLs for Fe and Zn was previously observed in rice on chromosome 7 and 12 (Anuradha et al., 2012) and in maize on chromosomes 2, 5, 7 and 9 (Jin et al., 2013). This may be due to some QTLs having common molecular mechanisms controlling the uptake and translocation of the both traits. The direction and intensity of association suggests a good possibility of simultaneous genetic improvement of both micronutrients by co-transferring these traits into the genetic background of elite lines. The stability of grain micronutrient constituents across environments as well as co-localization of QTLs plays an essential role in marker-assisted breeding.

The availability of the complete genome sequence enabled detailed study of the identified QTLs and the genes involved in grain Fe and Zn concentration in sorghum. In all, six genes related to Fe/Zn concentrations were found to be present within the selected QTLs on chromosome SBI-07 (pooled data of six environments). Annotations of these genes were observed as, Heme binding/iron ion binding, Zinc ion binding/metal ion binding, *bZIP* transcription factor, one Fe gene governing Fe metabolism out of the 6, and were found in QTL with phenotypic effect (6.7%) present on chromosome SBI-07. Five genes for Zn (*Zinc ion binding and zinc finger protein)* were found within two QTLs *qzn7.4* and *qzn7.1*. The identified gene CYP71B34 is important for membrane integrity and most importantly phytochrome activities (Table 4). Zinc ion binding and zinc finger protein ZFP motifs are required for their transcriptional activation activity. Upon looking into the QTLs from individual locations we found 42 genes related to Fe/Zn metabolism on chromosome SBI-07. With the progress made in identification of Fe and Zn QTLs we performed gene synteny analyses with maize and rice genomes using sorghum Fe and Zn genes found within QTLs interval. Significant gene sequence syntenic regions were detected, the gene synteny relations could be due to close evolutionary relationship of major cereal crops. These genes play a crucial role in protein metabolism wherein the stability of zinc finger protein is regulated by appropriate supply of Zn to the grain. Zinc finger proteins, are required for the expression and regulation of gene expression (Marschner, 1995). These results strongly suggest that simultaneous selection for grain Fe and Zn will be effective in sorghum. Many of these genes have been previously reported for metal homeostasis, metal chelation, biosynthesis of phytosiderophore, uptake, transport, loading and storage in cereals, such as rice, maize, barley and wheat. Availability of the complete genome sequence of sorghum, maize and rice enabling to study comparative genomics studies for better understanding of grain Fe/Zn QTLs and

genes. These results will contribute to better understanding of the genetic control of grain Fe and Zn concentration in sorghum. The genomic resources generated from this study through QTL mapping, identification of putative candidate genes underlying these QTLs, gene synteny, would provide a basis for functional analysis, which can be potentially used after validation in marker-assisted selection for grain Fe and Zn in sorghum.

6. Conclusions

The present study on sorghum RIL population showed wide variability for both grain micronutrients (Fe and Zn), also the presence of G × E interaction for both the micronutrients indicating strong influence of environment on the expression of these traits. It is important to test the genotypes in multiple environments over years to ascertain their stability. Constant positive and highly significant correlation between grain Fe and Zn concentration showed that simultaneous selection for both the micronutrients will be highly effective. The recent release of 'Parbhani Shakti' the first biofortified sorghum variety in India by the same authors vindicated this. This work is the first report on QTL mapping utilizing high-density of DArT and DArTseq markers for identifying QTLs for grain Fe and Zn in sorghum. Overall, this study identified a total of 11QTLs (individual) and 3QTLs (across) environments controlling both the traits. QTLs that are co-located and stable across environments, with common markers provides opportunity for early generation testing for enhancing grain Fe and Zn in sorghum. This study may also serves as a model for further genetic investigations of the sorghum grain Fe and Zn concentration, as well as in other C4 crops.

Compliance with ethical standards

No animal or humans have been used in this study. The work was done and manuscript was developed with high ethical standards.

Author contributions

Experiments were designed by AAK, SPD, KA; trials were carried out by RP, KA, AAK, BVSR, SG, HK, SPM, AG and VS; the data were analysed and interpreted by AAK, RKS, AR, RP,RD, RK, FJ; genotyping done by SPD, KA, CTH; genetic map construction and QTL analysis by KA, SPD, RKS; Candidate gene search made by KA, SG; gene synteny analysis by KA, BP. The manuscript was developed by KA, RP, AAK, RKS, SG, SPD.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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