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Research Article

QTL underlying iron toxicity tolerance at seedling stage in backcross recombinant inbred lines (BRILs) population of rice using high density genetic map

Adnan RASHEED[1a], Ghulam M. WASSAN[1c], Hira KHANZADA[1b], Abdul M. SOLANGI[1], Muhammad AAMER[2], Ruicai HAN[1], Jianmin BIAN[1*], Ziming WU[1*]

[1]*Key Laboratory of Crops Physiology, Ecology and Genetic Breeding, Ministry of Education/College of Agronomy, Jiangxi Agricultural University, Nanchang, 330045, China; adnanrasheed@jxau.edu.cn; wassanmk@outlook.com; hira.khanzada@outlook.com; solangi.abdulmalik@outlook.com; hrc1988113@163.com; jmbian81@126.com; wuzmjxau@163.com (*corresponding author)*
[2]*Research Center on Ecological Sciences, Jiangxi Agricultural University, Nanchang 330045, China; aamirwattoo2009@gmail.com;*
[a,b,c]*These authors contributed equally to this work*

Abstract

Fe is a trace element considered to be essential for rice, and it drives several metabolic processes. Fe toxicity occurs due to excessive Fe ions (Fe^{2+}) and which, disturb cellular homeostasis and dramatically reduces the rice yield. A set of 118 BRILs made from a cross of *japonica* cv.'02428' and *indica* 'Changhui 891' was used with high density bin map constructed by using high quality SNP to identify the QTL for Fe toxicity tolerance. As a whole total of 23 QTL were identified for various seedling traits, 3 under control with phenotypic difference ranging from 14.21% to 62.46%, 11 QTL under stress with phenotypic difference ranging from 7.89% to 47.39% and 9 under stressed/control ratio with phenotypic variance ranging from 9.17% to 183.50%. LOD values of QTL ranging from 4.05 to 17.04 in control, 3.41 to 8.09 in stress and 2.84 to131.63 in stress/control ratio. Shoot length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW), were used to estimate the degree of Fe tolerance. Many stable QTL, *qSSDW-4*, *qSSDW-6*, *qRSDW-4* and *qRSDW-6* affecting SDW were detected and beside this some new QTL, *qRSFW-1*, q*RRFW-10* and *qRRDW-1* were successfully identified significantly contributing to Fe toxicity tolerance in rice. The results of current study indicated that these novel regions could be transferred via markers assisted section and QTL pyramiding to develop Fe resistant lines in rice.

Keywords: bin map; Fe toxicity; genetic factors; rice; tolerance

Abbreviations: Fe: Iron; BRILs: backcross recombinant inbred lines; QTL: quantitative traits loci; SL: shoot length (cm); RL: root length (cm); SFW: shoot fresh weight (mg); RFW: root fresh weight (mg); SDW: shoot dry weight (mg); RDW: shoot dry weight (mg); RSL: relative shoot length (mg); RRL: relative root length; RSFW: relative shoot fresh weight; RRFW: relative root fresh weight (mg); RSDW: relative shoot dry weight; RRDW, relative root dry weight; MAS: marker-assisted selection; RILs: recombinant inbred lines;

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NILs: near isogenic lines; CSSL: chromosomal segment substitution lines; SNP: single nucleotide polymorphism.

Introduction

Rice (*Oryza sativa* L.) is the most important and staple food crop of the world, particularly in Southeast Asia and China (Mishra and Panda, 2017; Vivitha *et al.*, 2017) and it provides about 40 % of the daily calories to world's population (Parengam *et al.*, 2010). It has been estimated that rice production need to be increased by an additional 100 million tons to satisfy the need of 9.1 billion people by the year 2050 (Mahender *et al.*, 2019). Heavy metals are usually defined as metals with comparatively high atomic weights, as well as atomic numbers. Fe has atomic number 26 that's why it is called heavy metal (Tchounwou *et al.*, 2012). Many heavy metals such as cobalt, nickel, copper and Fe. Fe which is a trace element, essential to humans, plants and animals, but it is toxic to many plants at higher concentration (He *et al.*, 2005; White and Brown, 2010). One of the serious constraint to production of lowland rice grown in acid flooded soil is Fe toxicity (De Dorlodot *et al.*, 2005). About 18% of the global soils are Fe toxic (Mahender *et al.*, 2019) and toxic effects of Fe have been noted by many scientists (De Dorlodot *et al.*, 2005; Dufey *et al.*, 2012; Dufey *et al.*, 2015a). Rice production in Southeast Asia, Brazil and Africa is severely affected by Fe toxicity (Audebert and Fofana, 2009).

Fe has a key role in plants functions such as, cell division, respiration, and chlorophyll synthesis etc.(Müller *et al.*, 2015), however, Fe produces reactive oxygen species (ROS) by its function as catalyst in Fenton reaction which are harmful to the cell and cause oxidative damage (Onaga *et al.*, 2016). Fe in excessive from cause alterations in morphological, biochemical and physiological features of plant by shifting redox potential towards a pro-oxidant state (Hell and Stephan, 2003). Fe toxicity can also induce nutrients disorders such as potassium, phosphorous, calcium, magnesium, and manganese etc. Rice plant absorbs Fe both in divalent Fe^{2+} and trivalent Fe^{3+} form, but Fe^{3+} is less available to plants. Due to depletion of oxygen by plant roots and microbes in soil result in anaerobic conditions. This led to conversion of Fe^{3+} to Fe^{2+} which cause Fe toxicity. This toxic form of Fe is very lethal to rice plant as it cause severe lose in yield. (Boruah and Bharali, 2015). ROS are produced by Fe toxicity damage protein, nucleic acids and membrane lipids (Becana *et al.*, 1998), oxidize chlorophyll and reduce photosynthesis (Pereira *et al.*, 2013; Onaga *et al.*, 2016).

Fe toxicity include, lead bronzing score (0-9) and decrease in root and shoot length and as well as fresh weight at seedling stage (Dufey *et al.*, 2009). In case of severe Fe toxicity, rice plants showed reduction of the water content, and chlorophyll content, water content, increase in the stomatal resistance as well as iron concentration of roots and shoots as studied by (Dufey *et al.*, 2009). Damaged root system can also be noticed under severe Fe stress. Fe toxicity lead to the reduction in uptake of many essential nutrients like nitrogen, phosphorous, potassium, etc. The reduction in nutrients uptake is due to barrier made by Fe coating, or due to chemical action in soils. It shows antagonistic effects and ultimately reduce yield (Rasheed *et al.*, 2020c). Fe toxicity effects the competence of the water flow by depressing transpiration rate through fluctuations in stomatal resistance in leaves. Due to Fe stress the soil solution restrict the water uptake and cause osmotic stress and plant shows the symptoms of lethal Fe toxicity (Rucińska-Sobkowiak, 2016). Plants use several resistance mechanisms to overcome these toxic effects such as, extrusion, restricted nutrients uptake, chelation and alleviation of toxic heavy metals (Dufey *et al.*, 2009). Other strategies include, avoidance and tolerance strongly depends upon Fe toxicity type in growth condition, its duration and intensity (Frei *et al.*, 2016). Rice plant use three type of tolerance mechanisms, chelation of Fe^{2+} at root level, secondly, Fe^{+2} avoidance through its distribution and storage in less reactive form (Briat *et al.*, 2009) and type three included use of ascorbic acids to scavenge ROS (Gallie, 2012). Some of the cultural practices could be used to counteract harmful effects of Fe in soil and water (Herviyanti *et al.*, 2019).

Generally, rice is categorized in two groups (*indica* and *japonica*) based on large genetic variability. Many researchers have studied large phenotypic diversity among *japonica* and *indica* rice varieties (Liu *et al.*, 2005), as *japonica* is more resistant to Fe toxicity than *indica*. Use of resistant genotypes is one of the best strategy to improve rice yield on soils effected by Fe toxicity (Audebert, 2006). Fe toxicity tolerance is appear to be polygenic inherited traits and therefore QTL mapping along with marker assisted selection is most reliable technique to improve genotypes resistance (Mackill *et al.*, 1999). Earlier screening studies demonstrated that , environmental conditions, Fe stress level, screening systems played a key role to determine the response of rice cultivars to Fe stress (Wu *et al.*, 2014).Therefore it is important to breed the special varieties adopted to specific environment and those adopted to wide range of environments. Several genes controlling Fe uptake, transport and accumulation belong to five protein groups have been identified, *OsNRAMPs*, *OsFROs*, *OsZIPs*, *OsFERs* and *OsYSLs* (Chandel *et al.*, 2010). Identification of specific traits involved in contributing Fe toxicity tolerance would be the most promising step for reliable mapping studies and successful identification of Fe tolerant QTL in the environments diverse in Fe toxicity patterns. The cultivars 'EPAGRI 108', 'BR-IRGA 409' and japonica cultivar '02428' are Fe tolerant cultivars (Stein *et al.*, 2019; Rasheed *et al.*, 2020c).

Various QTL against Fe toxicity tolerance have been identified in rice using population derived from two parents (Dong *et a*l., 2006; Dufey *et al.*, 2009) using easily measurable parameters, such as SL, RL, SDW and RDW, because it is difficult to measure physiological traits at whole population level (Dufey *et al.*, 2015b). Numerous mapping and screening experiments have been conducted to study Fe toxicity tolerance with different tolerant response in rice. A *japonica* variety, 'Azucena' was studied for its tolerance by using (250 mg L^{-1} $Fe^{2+)}$ for duration of 4 weeks using hydroponics conditions and marked as tolerant (Dufey *et al.*, 2009) but it showed susceptibility when exposed to (1,500 mg L^{-1} Fe^{2+}) (Engel *et al.*, 2012). Contradictory performance of same genotype under different Fe toxic levels provided an opportunity to understand the genetics basis of iron toxicity tolerance. Lot of studied have been conducted in past to identify the QTL behind Fe toxicity tolerance (Dufey *et al.*, 2009; Dufey *et al.*, 2012; Fukuda *et al.*, 2012). Due to strong interaction of genotype and environment in field, the testing of genotype tolerance against iron toxicity in hydroponic condition is more rapid and reliable way. Few studies were conducted using genome wise association analysis for complex traits related to iron tolerance (Matthus *et al.*, 2015). Comprehensive understanding of genetic mechanism of Fe toxicity tolerance is important to speed up the development of Fe tolerant varieties.

Many QTL for rice tolerance to Fe toxicity have been identified by earlier researchers. A total of 29 QTL in rice for Fe toxicity tolerance using GWAS technique have been identified for SL, RL, SFW, RFW, SDW and RDW (Zhang *et al.*, 2017). Dufey *et al.* (2015a) conducted QTL analysis using 220 BC3DH lines tested in hydroponics condition in presence or absence of Fe toxic levels and reported 28 QTL for various morphological traits. Similarly Liu *et al.* (2016*)* used two reciprocal introgression lines with same parents to identify QTL against Fe toxicity tolerance. They have successfully identified 9 QTL for Fe toxicity tolerance using several morphological indicators such as, RDW, SDW, and total dry weight (TDW). Fe toxicity is still a challenge for rice breeders to sustain its production on Fe toxic soils by identifying putative QTL, because until now this mechanism is not fully understood (Nughara *et al.*, 2016; Zhang *et al.*, 2017). Exploitation of appropriate mapping population is a reliable and effective strategy in QTL analysis, because of the fact that standard segregating populations cannot give accurate knowledge about size and position of individual QTL, mainly QTL with small effect, due of genetic background noise (Jiang *et al.*, 2017). In such circumstances, use of near isogenic lines (NIL) and chromosomal segment substitution lines (CSSL) can be ideal to overcome this problem and therefore considered as ideal for identification of QTL (Kubo, 1999; Bian *et al.*, 2010; Jiang *et al.*, 2017), but using these population are time consuming and increase labor cost, which limited the rapidity of gene cloning. BRILs population holds higher percentage of genome from recurrent parent and easily crossed with recurrent parent to develop NIL population for achieving the cloning of targeted QTL. Hence BRILs is considered a permeant and stable population and easier to develop than that of CSSL and NILs population. With the passage of time idea of developing BRILs population gained more attention for genetic analysis of polygenic traits in rice against various abiotic stresses (Wang *et al.*, 2011; Hosseini *et al.*, 2012).

QTL mapping is one of the most reliable and powerful technique to locate gene of interest on chromosome (Nughara *et al.*, 2016) and mapping of QTL on chromosome depend on marker density in linkage group as well as size of QTL interval (Da *et al.*, 2000). Idea of developing reliable markers gained more and more attention with passage of time by researchers to construct high density linkage map. Use of restriction fragment length polymorphism (RFLP) and single sequence repeat (SSR) markers do not meet our needs of QTL identification when linkage distance is zero, therefore use of additional markers would be better to enhance QTL mapping resolution (Liu *et al.*, 2008). Use of SNP would facilitate the probability of increase in high resolution QTL mapping. Use of SNP is more beneficial as comparted to other markers because old markers like, SSR, RFLP are not appropriate when their linkage distances are zero and cannot meet the necessities of the high QTL mapping resolution (Rasheed *et al.*, 2021). Use of SNP markers by next generation sequencing tools will rise the possibility to rise resolution of QTL mapping (Jiang *et al.*, 2017). In this study high resolution bin-map was constructed using re-sequence strategy for recognition of QTL for Fe^{2+} tolerance. Aim of currently study was to evaluate a set of 118 BRIL lines population under hydroponic conditions to determine the degree of Fe toxicity tolerance in BRILs and to recognize the putative QTL controlling Fe tolerance.

Materials and Methods

Population development

A BRILs population consists of 118 lines and its parents were used to estimate the degree of Fe toxicity tolerance at seedling traits and to identify the putative QTL linked with Fe toxicity tolerance. Population was made from a cross of '02428' (*japonica*) and 'Chunghui/891' (*indica*) parents at experimental station of Jiangxi Agricultural University and Linwang of Hainan Province. Parents were crossed and population was allowed to self-fertilization for six generations to produce BC_1F_6.

Screening for Fe tolerance using hydroponic experiment

Experiment was conducted at experimental station of Jiangxi Agricultural University, Nanchang PR. China during 2019. A total of 118 rice BRILs along with their parents were evaluated in culture solution experiment to investigate the consequence of Fe toxicity on each line and parent seedlings. Experiment was laid out as RCBD with three replications per block, 10 seedlings from (3-4) lines per replication. Firstly seeds of the parents and BRILs population were disinfected with 1% H_2O_2 for 30 minutes and rinsed several times with distilled water (Satoh *et al.*, 2016) and soaked in de-ionized water for oven night than grown on filter papers placed in petri plates. One BRILs line was grown in three dissimilar plates alongside with its parents; hence float in 0.5 mmol l^{-1} $CaCl_2$ (pH 5.0) solution. Simple $CaCl_2$ solution could be used to screen at young seedling stage when seed can provide vital nutrients and to escape precipitation in Yoshida solution.

The whole experiment was conducted in culture room with temperature 27±2 °C, 16 hours of light and relative humidity of 70%. Seeds were allowed to germinate at this temperature for 4 days, and on 4^{th} day 10 seedlings with identical size were selected and transferred in plastic trays protected PVC sheet containing nylon screen attach holes. On 5^{th} day $CaCl_2$ at concentration of 0.5 $mmol^{-1}$ (as control) and Fe^{2+} in form of $FeCl_2$ at concentration of 100 µM (as stress) were applied to the seedlings in nutrients solution following method of (Yoshida *et al.*, 1976). We have conducted a preliminary experiment and treated rice lines with different doses of Fe toxicity and we have concluded that 100 µM is more effective dose as plant showed large variation towards Fe toxicity on this concentration. The pH of the solution during the experiment was adjusted to 5.0 using I N NAOH/HCL (Zhang *et al.*, 2017). The nutrients solution of the experiment was replaced after one weak and seedlings were exposed to stress for a period of 2 weeks to determine the effect of Fe toxicity on seedling traits. We have used the $CaCl_2$ to prepare the solution for control and we used the $FeCl_2$ to prepare the Fe stress solution. $FeCl_2$ was source of Fe stress and was applied at concentration of100 µM per liter.

Phenotypic data collection

After 15th day of treatment, when significant differences among the control and stress treatments were clearly noticed than, 10 plant samples from each parent and line were harvested to determine the SL and RL. SL was measured from the longest leaf to the base of shoot with the help of ruler and longest root was measured from longest root to the base of root (Nughara *et al.*, 2016). After this root and shoot were detached to measure the SFW and RFW with the help of an electronic balance. The samples of shoot and root were than oven dried at 60 °C for 72 hours to determine the SDW and RDW. The relative values like, relative shoot length (RSL), relative root length (RRL), relative shoot fresh weight (RSFW), relative root fresh weight (RRFW), relative shoot dry weight (RSDW) and relative root dry weight (RRDW) were taken by using formula (trait value in treatment/trait value in control) x 100 (Nughara *et al.*, 2016).

Data analysis

The phenotypic data was subjected to the analysis of variance using Minitab v.18 software (Pennsylavania state University, PA, USA) based on general linear model at $p < 0.05$ level and Tukey honest significant difference (HSD) test was used for comparison of the mean values. SPSS software version 20 (SPSS, Chicago, IL, USA) was used to carry out further analysis, descriptive statistics, Pearson correlation, frequency distribution, histogram and box plot. The broad sense heritability was calculated using the formula: h^2=Vg (Vg+Ve/r), where, (Vg) genotypic variance, (Ve) error variance and r is replication.

QTL analysis in BRILs population

A high-density linkage map was developed by using SNP with high quality (Jiang *et al.*, 2017). SNP used in this study are more stable and consistent markers. QTL analysis was carried out using the software IciMapping v 4.1 software (*http://www.isbreeding.net*). Significance of the QTL for evaluated traits was claimed using LOD value of >2.50 (Bian *et al.*, 2015; Jiang *et al.*, 2017). Technique of McCouch (McCouch, 2008) was applied for QTL nomenclature.

Results

Phenotypic variation and traits correlation

Analysis of variance was performed for all investigated traits of 118 BRILs across different experimental conditions. Different range of variation of all investigated traits was observed across both control and stress conditions (Table 1). Higher estimates of heritability were recorded for all traits, indicating the large scope of selection present for all traits. Regarding heritability estimates all traits showed varying pattern, SL (93.81%), RL (96.88%), SFW, (96.31%), RFW, (93.44%), SDW (92.97%) and RDW (69.06%) (Table 3). Higher estimates of broad sense heritability revealed that all of these traits are highly heritable and selection of genotypes based on these traits would be highly appreciable. Mean values of SL, SFW and SDW under control conditions were considerably decreased as compared to stress indicated that these are least affected by the Fe toxicity under hydroponic conditions. Mean values of RL and RFW under stress conditions were considerably reduced compared to control (Table 2). Box plot analysis for all traits across both experimental showed that all traits are normally distributed in the population (Supplementary Figure 1). Mean values for six seedling traits are given below. The minimum and maximum mean values of SL under control and stress are 6.61 to 13.92 and 6.76 and 12.28. The Skewness and Kurtosis for SL under control and stress are 1.00 to 2.75 and 0.07 to -0.55. Likewise, for RL the range of mean values under control and stress is 2.61 to 9.72 and 2.37 to 7.65. The Skewness and Kurtosis values are 0.56 to 0.76 and 0.16 to -0.34. For SFW the minimum and maximum values under control and Fe stress are 0.17 to 0.53 and 0.19 to 0.47. Skewness and Kurtosis values are 0.55 to 1.22 and 0.19 to -0.30. The minimum and maximum values for RFW under control and stress are 0.03 to 0.28 and 0.04 to 0.27. In the same way Skewness and Kurtosis values are 0.47 to -0.08 and 0.82 to 0.67. The minimum and

maximum mean values of SDW under control and stress are 0.05 to 0.13 and 0.05 to 0.13. Skewness and Kurtosis values are 0.84 to 0.73 and 0.27 to -0.16. For RDW the range of mean values under control and stress is 0.01 to 0.06 and 0.01 to 0.12. Skewness and Kurtosis values are 0.73 to 1.14 and 3.27 to 19.23.

Table 1. Analysis of variance for various seedling traits of 118 BRILs population derived from a cross of *japonica* cv. '02428' × *indica* 'Chunghui 891'

Trait	SOV	DF	SS	MS	F-Value	P -Value	H^2
SL	Trait	1	56.01	56.0142	116.10	0.000	93.81
	Line	117	912.08	7.7956	16.16	0.000	
	Rep	2	1.06	0.5305	1.10	0.334	
	Treat*Line	117	178.79	1.5281	3.17	0.000	
	Error	470	226.75	0.4824			
RL	Trait	1	0.03	0.03347	0.17	0.677	96.88
	Line	117	722.50	6.17525	32.07	0.000	
	Rep	2	1.43	0.71286	3.70	0.025	
	Treat*Line	117	235.87	2.01597	10.47	0.000	
	Error	470	90.49	0.19253			
SFW	Trait	1	0.07020	0.070201	113.51	0.000	96.31
	Line	117	1.96246	0.016773	27.12	0.000	
	Rep	2	0.00118	0.000589	0.95	0.387	
	Treat*Line	117	0.44158	0.003774	6.10	0.000	
	Error	470	0.29069	0.000618			
RFW	Trait	1	0.00342	0.003420	4.54	0.034	93.44
	Line	117	1.34646	0.011508	15.26	0.000	
	Rep	2	0.00326	0.001630	2.16	0.116	
	Treat*Line	117	0.30643	0.002619	3.47	0.000	
	Error	470	0.35439	0.000754			
SDW	Trait	1	0.014193	0.014193	131.65	0.000	92.97
	Line	117	0.179778	0.001537	14.25	0.000	
	Rep	2	0.000601	0.000300	2.79	0.063	
	Treat*Line	117	0.027245	0.000233	2.16	0.000	
	Error	470	0.050671	0.000108			
RDW	Trait	1	0.000145	0.000145	0.77	0.382	69.06
	Line	117	0.071517	0.000611	3.23	0.000	
	Rep	2	0.000224	0.000112	0.59	0.553	
	Treat*Line	117	0.026922	0.000230	1.22	0.081	
	Error	470	0.088843	0.000189			

SL, shoot length; RL, root length; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight; RDW, root dry weight; SOV, source of variation; DF, degree of freedom; SS, sum square; MS, mean square; H2, heritability; * shows significant at P ≤ 0.05 and ** shows highly significant at P ≤ 0.01.

Under control condition positive non-significant correlation was observed between RL and SL (0.19), while SFW also exhibited non-significant positive correlation with SL (0.31) and RL (0.15) (Table 3). RFW showed negative non-significant correlation with SL (-0.01), positive non-significant correlation with RL (0.14) and highly significant positive correlation with SFW (0.70**). SDW exhibited non-significant positive correlation with SL (0.32), RL (0.35), RFW (0.35) and highly significant positive correlation with SFW (0.68**). RDW demonstrated non-significant positive correlation with SL (0.01), RL (0.35), SDW (0.43), significant positive correlation with SFW (0.50*), and highly significant positive correlation with RFW (0.78**) and under control. Significant correlation demonstrated that these traits could be improved via direct selection. Under $FeCl_2$ RL exhibited non-significant positive correlation with SL (0.33) and, SFW showed significant positive correlation with SL (0.48*) and positive non-significant correlation with RL (0.30). RFW had negative non-significant correlation with SL (-0.05), positive non-significant correlation with RL (0.11)

and positive and significant correlation SFW (0.52*). SDW revealed positive non-significant correlation with SL (0.39), RL (0.30), RFW (0.37) while highly significant positive correlation with SFW (0.79**). RDW exhibited non-significant positive correlation with SL (0.08), RL (0.20), SFW (0.29), RFW (0.42) and SDW (0.31). Over all, highly significant positive correlation was noticed among RDW and RFW in control, while among SDW and SFW in stress. According to results presented in (Table 3), all the variables which showed significant and highly significant positive correlation could be selected to improve direct selection.

Table 2. Descriptive statistics of the traits measured in BRILs population under control and stress environment

Traits	Treatments	Mean	SD	CV	Minimum	Maximum	Skewness	Kurtosis
SL	$CaCl_2$	8.80	1.14	12.98	6.61	13.92	1.00	2.75
	$FeCl_2$	9.36	1.34	14.33	6.76	12.28	0.07	-0.55
RL	$CaCl_2$	4.94	1.23	24.94	2.61	9.72	0.56	0.74
	$FeCl_2$	4.93	1.10	22.29	2.37	7.65	0.16	-0.34
SFW	$CaCl_2$	0.30	0.05	19.27	0.17	0.53	0.55	1.22
	$FeCl_2$	0.32	0.05	17.68	0.19	0.47	0.19	-0.30
RFW	$CaCl_2$	0.12	0.05	42.42	0.03	0.28	0.47	-0.08
	$FeCl_2$	0.11	0.04	38.89	0.04	0.27	0.82	0.67
SDW	$CaCl_2$	0.08	0.01	20.15	0.05	0.13	0.84	0.73
	$FeCl_2$	0.09	0.01	19.53	0.05	0.13	0.27	-0.16
RDW	$CaCl_2$	0.03	0.009	31.37	0.01	0.06	0.73	1.14
	$FeCl_2$	0.03	0.01	43.95	0.01	0.12	3.27	19.23

SL, shoot length; RL, root length; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight; RDW, root dry weight; $CaCl_2$, calcium chloride; $FeCl_2$, iron chloride; SD, standard deviation; CV, coefficient of variability; * indicates significant at $P \leq 0.05$ and ** indicates highly significant $P \leq 0.01$.

Table 3. Pearson correlation coefficient among seedling traits of 118 BRILs population under $CaCl_2$ and $FeCl_2$

	$CaCl_2$					$FeCl_2$				
Traits	SL	RL	SFW	RFW	SDW	SL	RL	SFW	RFW	SDW
RL	0.19					0.33				
SFW	0.31	0.15				0.48*	0.30			
RFW	-0.01	0.14	0.70**			-0.05	0.11	0.52*		
SDW	0.32	0.35	0.68**	0.35		0.39	0.30	0.79**	0.37	
RDW	0.01	0.35	0.50*	0.78**	0.43	0.08	0.20	0.29	0.42	0.31

SL, shoot length; RL, root length; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight; RDW, root dry weight; $CaCl_2$, calcium chloride; $FeCl_2$, iron chloride; * indicates significant at $P \leq 0.05$ and ** indicates highly significant $P \leq 0.01$.

Identification of the QTL for seedlings traits of BRILs in response to control and Fe stress

A total of 14 QTL in control and Fe stress conditions were identified for seedling traits in BRILs population (Tables 4 and 5, Figures 1 and 2), including 3 QTL detected only under control environment and 11 detected only in Fe stress condition. Three QTL underlying three traits, CRL, CRFW and CRDW, specifically one QTL, *qCRL-1* for CRL, one QTL, *qCRFW-3* governing CRFW and one QTL, *qCRDW-3* underlying CRDW were identified under control experiment on chromosome 1 and 3 respectively. The QTL, *qCRDW-3* was identified in both control and stress environment, which revealed its background independence. Range of LOD values and phenotypic variances shown by these QTL were 4.05, 3.31, and 17.04 and 14.36%, 14.21% and 62.46% respectively. For three traits, CSL, CSFW and CSDW no QTL were mapped on any chromosome in control background. No QTL was detected on chromosome, 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 under control experiment. No QTL was detected for CSL, CSFW, and CSDW in control condition.

Table 4. Identification of QTL for Fe tolerance in BRILs population under control environment

Traits	QTL	Chr	Position	Left Marker	Right Marker	LOD	PVE%	Add
CRL	*qCRL-1*	1	373.5	bin1-114	bin1-115	4.05	14.36	-0.54
CRFW	*qCRFW-3*	3	776	bin3-277	bin3-278	3.31	14.21	-0.43
CRDW	*qCRDW-3*	3	729	bin3-253	bin3-254	17.04	62.46	-1.04

CRL, control root length; CRFW, control root fresh weigh; CRDW, control root dry weight; QTL, quantitative trait loci; Chr, chromosomes; LOD, lod of algorithm; PVE (%), percentage of phenotypic variance explained; Add, additive effect; * indicates significant at P ≤ 0.05 and ** indicates highly significant P ≤ 0.01.

Table 5. Identification of QTL for Fe tolerance in BRILs population under stress environment

Trait	QTL	Chr	Position	Left Marker	Right Marker	LOD	PVE (%)	Add
SSFW	*qSSFW-6*	6	609	bin6-159	bin6-160	3.41	12.26	0.01
SRFW	*qSRFW-10*	10	578	bin10-161	bin10-162	3.94	13.52	-0.02
SSDW	*qSSDW-4*	4	792.5	bin4-253	bin4-254	6.66	21.42	-0.01
	qSSDW-6	6	209	bin6-70	bin6-71	2.64	7.89	0.004
SRDW	*qSRDW-1-1*	1	218.5	bin1-72	bin1-73	9.39	47.39	-0.03
	qSRDW-1-2	1	224.5	bin1-73	bin1-74	9.62	47.39	-0.03
	qSRDW-1-3	1	348.5	bin1-96	bin1-97	9.70	47.12	-0.03
	qSRDW-1-4	1	391.5	bin1-121	bin1-122	6.79	47.39	-0.03
	qSRDW-1-5	1	486.5	bin1-142	bin1-143	7.08	47.38	-0.03
	qSRDW-3-1	3	729	bin3-253	bin3-254	11.46	47.39	-0.03
	qSRDW-3-2	3	735	bin3-257	bin3-258	8.09	47.26	-0.03

SSFW, stress shoot fresh weight; SRFW, relative root fresh weight; SSDW, stress shoot dry weight; SRDW, stress root dry weight; QTL, quantitative trait loci; Chr, chromosome; LOD, lod of algorithm; PVE (%), percentage of phenotypic variance explained; Add, additive effect; * indicates significant at P ≤ 0.05 and ** indicates highly significant at P ≤ 0.01.

Under Fe stress 11 QTL affecting four seedling traits, SSFW, SRFW, SSDW, and SRDW were detected on chromosomes 1, 3, 4, 6, 10. Two QTL, *qSSFW-6*, *qSRFW-10* governing SSFW and SRFW were identified on chromosomes 6 and 10 among the flanking markers (bin6-159, bin6-160 and bin10-161, bin10-162), with LOD values 3.41 and 3.94 and accounting for 12.26% and 13.52% of phenotypic variances respectively. Allele for *qSSFW-6* was shared by male parent as shown by positive additive effect and increased trait value. Two QTL, *qSSDW-4* and *qSSDW-6*, affecting SSDW were detected in Fe stress experiment on chromosome 4 and 6, with LOD value of 6.66 and 2.64, accounting 21.42% and 7.89% of phenotypic variance, respectively. QTL, *qSSDW-6* had positive additive effect, showed that allele at this locus was shared by donor parent and increases index value. Negative additive effect showed that female parent is involved in increasing SSDW of BRILs population under stress environment. Seven QTL for SRDW were identified in Fe stress on chromosome 1 and 3, with LOD values ranging from 6.79 to11.46 and phenotypic variances 47.12% to 47.39%, respectively. Among the above QTL for SRDW, five QTL, *qSRDW-1-1*, *qSRDW-1-2*, *qSRDW-1-3*, *qSRDW-1-4*, and *qSRDW-1-5* were identified on chromosome 1, explained 47.39%, 47.39%, 47.12%, 47.39% and 47.38% of phenotypic variance, which indicating that all of these were major QTL for tolerance to Fe toxicity in BRILs population. Other two QTL *qSRDW-3-1* and *qSRDW-3-2* affecting SRDW were mapped on chromosome 3 and average of phenotypic variances explained by them were 47.39% and 47.26%, respectively. Among the above QTL one QTL, *qSRDW-3* affecting SRDW was simultaneously detected in both environments (control and Fe stress) indicating that these QTL expressions are independent of genetic background. No QTL were detected on chromosomes 2, 5, 7, 8, 9, 11 and 12 in Fe stress. No QTL was identified for SSL and SRL under Fe stress condition.

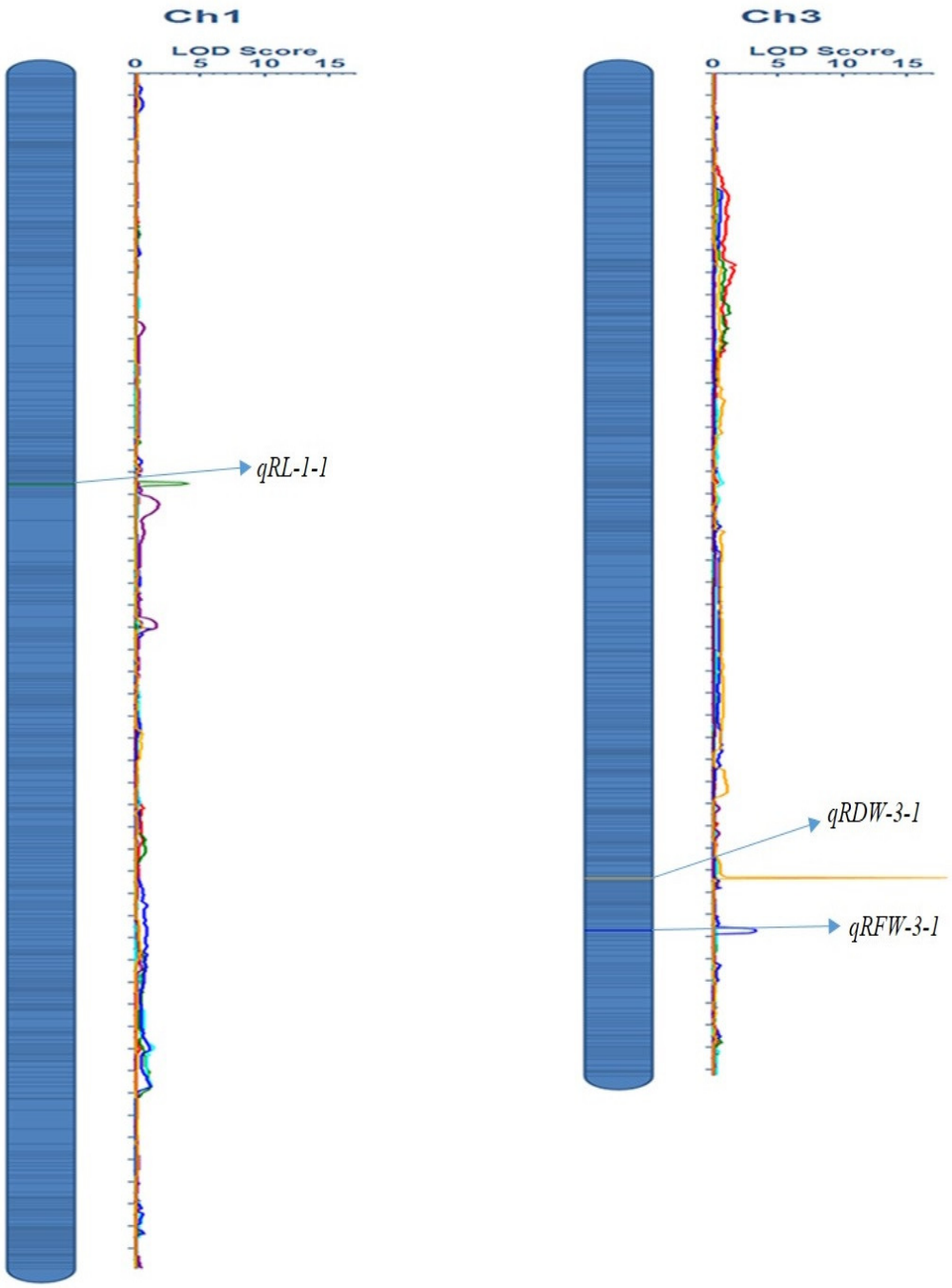

Figure 1. Location of putative QTL detected by QTL mapping under $CaCl_2$ condition for the traits linked with Fe toxicity tolerance, i. e. RL, RFW and RDW

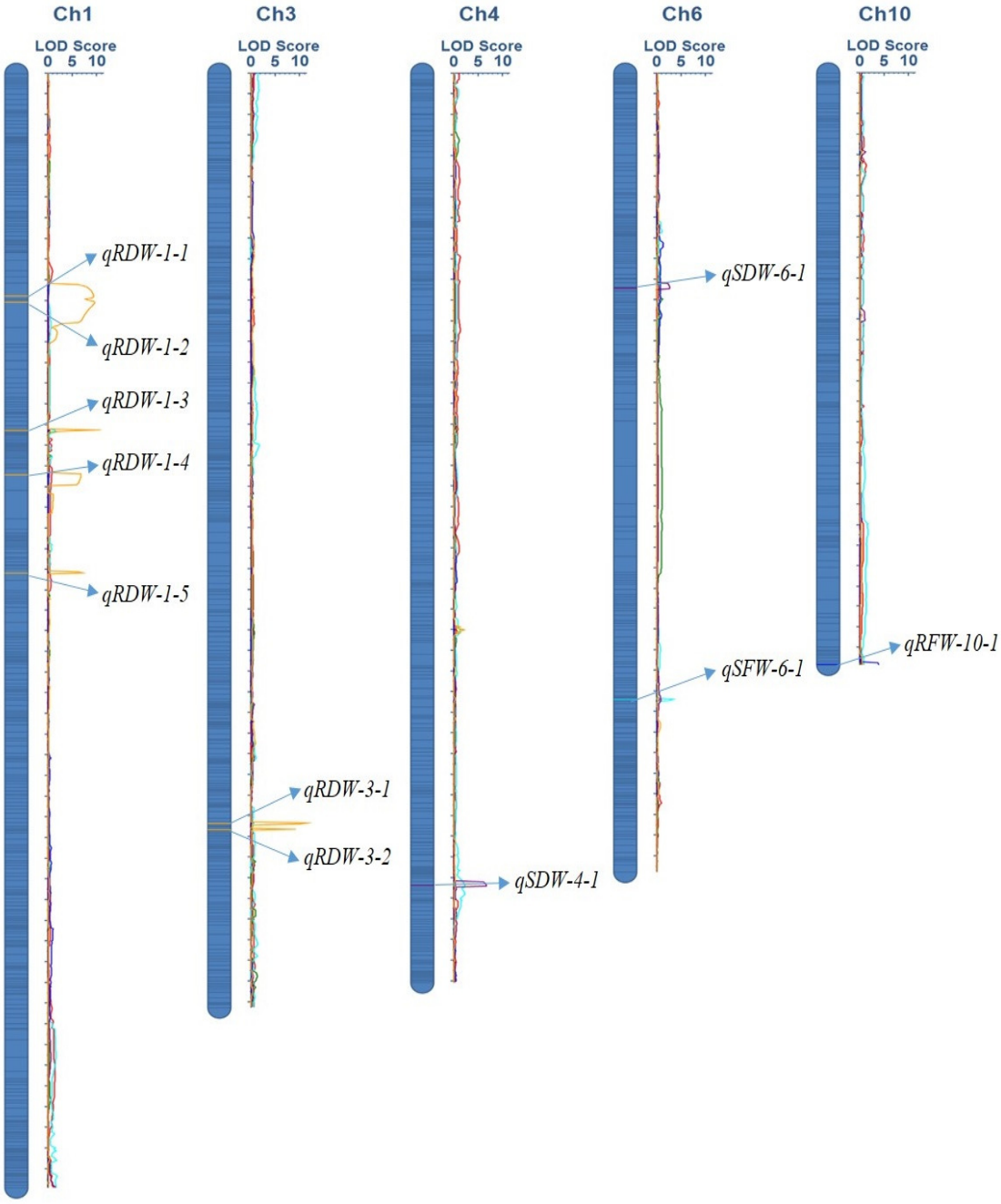

Figure 2. Location of putative QTL detected by QTL mapping under $FeCl_2$ condition for the traits liked with Fe toxicity tolerance, i. e. SFW, RFW, SDW and RDW

Detection of QTL for seedlings traits of BRILs in stressed/control experiment

Various QTL affecting the indexes in stress/control environment were identified and mapped on different chromosomes shown in (Table 6, Figure 3). In current study a total of 9 QTL were detected in stressed/control environment on chromosomes 1, 4, 6, 10, and 12 in *japonica/indica* BRILs. Three QTL, *RSL-12*, *RSFW-1* and *RRFW-10*, underlying RSL, RSFW and RRFW were detected in stressed/control experiment on chromosome 12, 1 and 10, respectively. QTL *qRSL-12*, affecting RSL was detected on chromosome 12 among the markers (bin12-108, bin12-109), with LOD value 2.84 and accounting for 9.92% of phenotypic

variance, while QTL, *qRSFW-1* controlling RSFW was detected on chromosome 1 with LOD value of 2.72 and 10.78% of phenotypic variance. One QTL, *qRRFW-10* afflicted RRFW was identified in stress/control condition on chromosome 10, with LOD value of 2.61 and explained variance of 9.17%, respectively.

Two QTL, for RSDW were detected on chromosome 4 and 6. The *qRSDW-4* was mapped on chromosome 4 between the markers (bin4-252, bin4-253), secured LOD value 5.65 and accounted for 19.23% of phenotypic variance, *qRSDW-6* was detected on chromosome 6 among the flanking markers (bin6-65, bin6-66), secured LOD value 3.70 and with 11.94% of total explained variance. Donor parent japonica contributed allele at locus of this QTL. Regarding RRDW four QTL, *qRRDW-1-1*, *qRRDW-1-2*, *qRRDW-1-3*, and *qRRDW-1-4* were mapped on chromosome 1 in stressed/control experiment, with LOD values ranging from 106.48, 106.60, 115.37 and 131.63. Ratio of phenotypic variances explained by these QTL was 54.49%, 54.49%, 98.12% and 183.50%. QTL, *qRRDW-1* was simultaneously detected under both stress and stressed/control environment with allele for increasing effect shared by 'Chunghui/891' parent. No QTL were detected on chromosomes 2, 3, 5, 7, 8, 9, and 11. No QTL was detected for RRL.

Table 6. Identification of QTL for Fe tolerance in BRILs population under stress/control environment

Trait	QTL	Chr	Position	Left Marker	Right Marker	LOD	PVE (%)	Add
RSL	*qRSL-12*	12	341	bin12-108	bin12-109	2.84	9.92	-0.40
RSFW	*qRSFW-1*	1	964.5	bin1-295	bin1-296	2.72	10.78	-0.02
RRFW	*qRRFW-10*	10	578	bin10-161	bin10-162	2.61	9.17	-0.01
RSDW	*qRSDW-4*	4	789.5	bin4-252	bin4-253	5.65	19.23	-0.01
	qRSDW-6	6	190	bin6-65	bin6-66	3.70	11.94	0.005
RRDW	*qRRDW-1-1*	1	219.5	bin1-72	bin1-73	106.48	54.49	-0.44
	qRRDW-1-2	1	224.5	bin1-73	bin1-74	106.60	54.49	-0.44
	qRRDW-1-3	1	499.5	bin1-143	bin1-144	115.37	98.12	-0.42
	qRRDW-1-4	1	504.5	bin1-144	bin1-145	131.63	183.50	-0.41

RSL, relative shoot length; RSFW, relative shoot fresh weight; RRFW, relative root fresh weight; RSDW, relative shoot dry weight; RRDW, relative root dry weight; QTL, quantitative trait loci; Chr, chromosome; LOD, lod of algorithm; PVE (%), percentage of phenotypic variance explained; Add, additive effect; * indicates significant at $P \leq 0.05$ and ** indicates highly significant at $P \leq 0.01$.

Discussion

Use of BRILs population enables the genetic analysis of QTL

Use of suitable mapping population is a reliable and effective strategy in QTL analysis, because of the fact that standard segregating populations cannot give accurate knowledge about size and position of individual QTL, mainly QTL with small effect, due of genetic background noise (Jiang *et al.*, 2017). In such circumstances, use of NILs and CSSL can be ideal to overcome this problem and therefore considered as ideal for identification of QTL (Kubo, 1999; Bian *et al.*, 2010; Jiang *et al.*, 2017), but using this populations is time consuming and increase labor cost, which limited the rapidity of gene cloning. BRILs population holds higher percentage of genome from recurrent parent and easily crossed with recurrent parent to develop NIL population for achieving the cloning of targeted QTL. Hence BRILs is considered a permeant and stable population and easier to develop than that of CSSL and NIL population. With the passage of time idea of developing BRILs population gained more attention for genetic analysis of polygenic traits in rice against various abiotic stresses (Hosseini *et al.*, 2012; Wang *et al.*, 2011). Currently a population of 118 BRILs was developed by crossing *japonica* ('02428')/ 'Changhui/891', hence; we propose that, use of this population would be a better choice to understand the complicacy of important quantitative traits in rice.

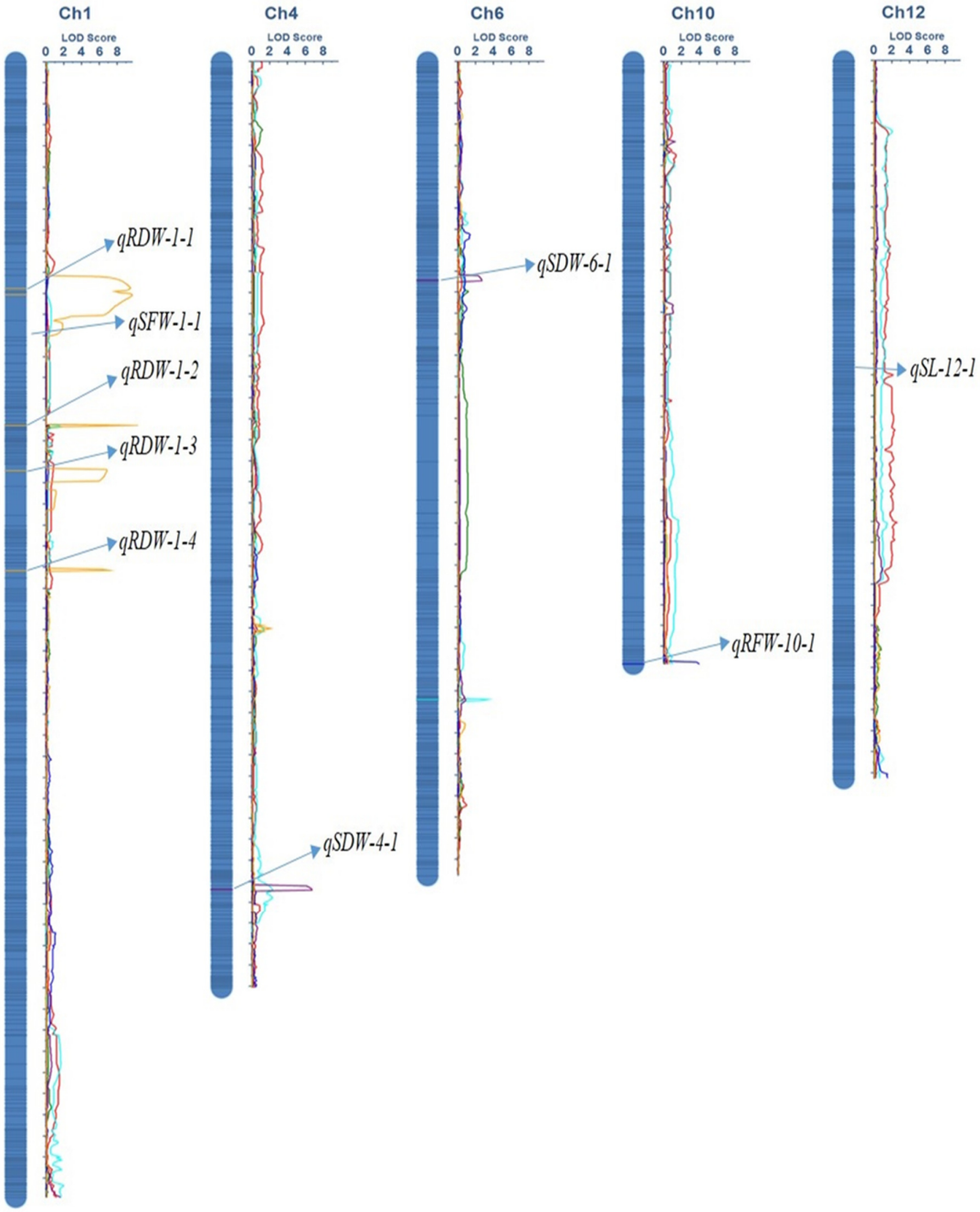

Figure 3. Location of putative QTL detected by QTL mapping under $FeCl_2/CaCl_2$ ratio condition for the traits liked with Fe toxicity tolerance, i. e. SL, SFW, RFW, SDW and RDW

Relationship of currently identified and previously reported QTL

For successful identification of QTL an effective nutrient solution with toxic levels of Fe is required (Liu *et al.*, 2016). Fe stress tolerance in rice can be characterized at morphological, biochemical and physiological levels (Blum, 2011). Fe tolerance in rice is mainly contributed by morphological attributes and these traits are polygenic in nature (Tiwari and Mamrutha, 2013). Crop tolerance to any stress is influenced by the environment to a great extent, hence previously it was proposed to improve crop tolerance to Fe stress for morphological traits using MAS selection (Zhang *et al.*, 2017). All of the secondary traits are linked with stress

tolerance and should have following criteria (1) genetically associated with Fe stress tolerance in Fe stress condition; (2) their heritability should be high in screening system; (3) lines should possess large diversity for given traits (4) Traits should be measured rapidly and they should be economic (Lafitte and Courtois, 2000). Currently we have measured Fe stress tolerance related to morphological attributes i.e. SL, RL, SFW, RFW, SDW and RDW under control, stress and stress/control ratio. QTL detected for the ratio traits measured in stress/control experiment were the major QTL that directly contributed to Fe toxicity tolerance in rice (Yue *et al.*, 2006), but traits variation can be reduced as a result some of the QTL may be un-identified (Zhang *et al.*, 2017). Hence, comparison of QTL detected under control and stress should also be done. All of the detected QTL in our study were associated with seedling traits, RL, SL, SFW, RFW, SDW and RDW. Of the 23 QTL identified, 3 were detected under control, 11 under stress and 9 under stress/ratio.

Only one QTL, *qRSL-12* affecting RSL was identified in current study under stress/control condition accounting for 9.92% of phenotypic variance. Meng *et al.* (2017) used five MAGIC populations against Fe toxicity and identified four QTL influencing RSL out of them one QTL *qRSL-12* with 8.1% variance was common to our reported QTL contributing to Fe toxicity tolerance in rice. However, Zhang *et al.* (2017) previously presented contradictory results and identified two QTL, *qCSL-12*, *qSSL-12* affecting CSL and SSL on chromosome 12 in both control and Fe stress condition with 4.4% and 6.9% of phenotypic variance. Similarly, Zhang *et al.* (2013) used reciprocal advanced backcross introgression lines and identified 42 QTL, out of them 17 QTL were reported for SL on chromosomes 1, 2, 3, 5, 6, 7, 8, 9, 11 not common to our detected QTL. This difference is mainly due to use of different populations and markers. RL is important seedling trait that is easily to measure for Fe toxicity tolerance. Currently, only one QTL, *qCRL-1* is mapped on chromosome 1 under control experiment. Three QTL, *qRL-6*, *qRL-7* and *qRL-8* were identified by Meng *et al.* (2017) for RL differed from our reported QTL due to use of different populations and nutrient solution. Zhang *et al.* (2017) detected, *qCRL-2* for CRL in control, and *qSRL-3* for SRL in Fe stress condition, which were in contrast with our identified QTL. By comparing with previous results, currently identified QTL, is not reported before.

SFW is an important secondly Fe tolerance index used to estimate the degree of Fe tolerance in many studies (Zhang *et al.*, 2017; Tao *et al.*, 2018). Two QTL were identified for SFW in current study, one *qSSFW-6* between the bins (bin6-159, bin6-160) under Fe stress experiment and second *qRSFW-1* between the bins (bin1-295, bin1-296) in stressed/control experiment with 12.26% and 10.78% of phenotypic variance. QTL, *qSSFW-6* had positive additive effect which showed that allele at this locus was shared by donor parent. A total of five genomic regions, *qSSFW*-2, *qSSFW-3*, *qSSFW-5*, *qSSFW-6*, *qSSFW-11* affecting SFW were mapped on chromosome 2, 3, 5, 6, 11 by Zhang *et al.* (2017) under Fe stress condition. QTL, *qSSFW-6* in their study mapped on chromosome 6 only was in line with our detected QTL under Fe stress condition.

So, what QTL *RSFW-1* detected in current experiment is newly reported QTL with the allele from recurrent parent. This region is strongly affects the stress tolerance index of SFW. Zhang *et al.* (2013) detected 42 QTLs for Fe and zinc toxicity tolerance across all environments but, they have not reported any QTL for SFW. Regarding RFW we have detected three QTLs, *qCRFW-3*, *qSRFW-10*, *qRRFW-10*, one under control, one under stress and one under stressed/control ratio experiment with 14.21%, 13.52% 9.17% of phenotypic variances. Previously no QTL was reported for RFW in any study. However, RFW is an important parameter in studying Fe toxicity tolerance. These novel regions may contribute to Fe toxicity tolerance in BRILs and could be cloned and transfer via MAS strategy.

Fe toxicity strongly influences the SDW in hydroponic condition, treated with toxic level of Fe. Most of the QTL, reported in previous studies were mainly related to SDW and RDW. It is of great interest that four QTL, two *qSSDW-4*, *qSSDW-6* in Fe stress and two *qRSDW-4*, *qRSDW-6* in stressed/control environment were identified in this study on chromosome 4 and 6 with 21.42%, 7.89%, 19.23%, and 11.94% of variance. These regions were identified on same chromosomes but among different markers. QTL on chromosome 6 in both the environments had positive additive effect which indicated that at this locus the allele was shared by donor parent. In many studies that QTL for SDW have been detected this revealed that this

morphological trait is largely contributing to Fe toxicity tolerance. Dufey *et al.* (2012) identified a QTL, *qSDW-4* for Fe toxicity stress on chromosome 4 accounted for 10.9% of phenotypic variance.

This genetic factor is on the same chromosome as reported in current experiment. These finding revealed that chromosome 4 may provide important regions that contributed to Fe toxicity tolerance. Zhang *et al.* (2017) identified five QTL influencing SDW on chromosome 3, 6, 11, 12. QTL, *qSSDW-6* detected under Fe stress and *RSDW-6* detected under stressed/control condition accounted for 9.2% and 7.9% of variances were common to the QTL in identified in current study. Zhang *et al.* (2013) also mapped QTL, *qRSDW-6* under Fe stress/control ratio and *qCSDW-4* under control condition on chromosome 6 and 4. *qRSDW-6* is identical to our detected QTL for RSDW on chromosome 6. Comparison of these regions provided a strong base to isolate and transfer these regions for improving growth under Fe toxic condition.

Similarly, Liu *et al.* (2016) used two reciprocal IL and identified two QTL for RSDW in Fe stressed/control environment. QTL *qRSDW-11-1* and *qRSDW-11-2* were mapped on different chromosome 11 in this study. In another experiment conducted by Meng *et al.* (2017) two QTL, *qSDW-2*, *qSDW-5* were mapped on chromosome 2 and 5 for Fe toxicity tolerance with positive additive effect. These mapped QTL are on different chromosomes and at different positions. In another study conducted by. Dufey *et al.* (2015a) three QTL for SDW under Fe toxicity condition were mapped on chromosome 3, 5 and 12 with positive additive effect.

Most of the QTL identified in this study are related to RDW, one under control, seven under Fe stress and four under stressed/control ratio. Previously lot of studies has identified QTL related to RDW. QTL, *qCRDW-3*, *qSRDW-3* identified in this study with highest phenotypic variances 62.46%, 47.39% were consistent to previously identified QTL, *qCRDW-3*, qSRDW-3 reported by Zhang *et al.* (2017) explained 8.5% and 7.8% variances. However, Wan *et al.* (2003) identified a QTL, *qRDW-3* for RDW at chromosome 3 at the region of C25-C515 with explained variance of 35% and positive additive effect. These results are strongly supported by earlier findings. Five QTL for SRDW were mapped on chromosome 1 under Fe stress and four QTL were mapped for RRDW on chromosome 1 in stressed/control ratio condition in current experiment. In comparison with earlier reports, Dufey *et al.* (2015a) identified *qRDW-1* affecting RDW, Wan *et al.* (2003) also detected two QTL for RDW on chromosome 1. Results of Liu *et al.* (2016) are in line with our findings, they identified two QTL, *qRRDW-1-1* and *qRRDW-1-2* on chromosome 1 in stressed/control ratio, but we identified four QTL on same chromosome due to use of different breeding material and markers. We have identified many new QTL for RDW on chromosome 1 in Fe stress and stressed/control ratio and detection of large QTL on same chromosome is of great interest for Fe tolerance. Hence, RDW is strongly associated with Fe toxicity tolerance in rice. These QTL could be cloned and transfer to develop Fe resistant lines in future studies (Rasheed *et al.*, 2020a, 2020b).

Implications in plant breeding

It is noteworthy, that chromosome 1 holds many QTL, *qCRL-1* for CRL, *qSRDW-1-1*, *qSRDW-1-2*, *qSRDW-1-3*, *qSRDW-1-4*, *qSRDW-1-5* for SRDW, *qRSFW-1* for RSFW and *qRRDW-1-1*, *qRRDW-1-2*, *qRRDW-1-3*, and *qRRDW-1-4*, for RRDW. Chromosome 1 harbored 5 QTL, for RDW in Fe stress and four QTL in Fe stressed/control experiment. Detection of large number of QTL on same chromosome across all environments could be a significant point for Fe toxicity tolerance in BRILs and these traits are strongly correlated to each other. Similarly, *qSSFW-6*, *qSSDW-6* were mapped on chromosome 6 in Fe stress experiment, while *qRSDW-6* was detected under stressed/control environment on same chromosome.

qSSDW-4 and *qRSDW-4* were detected on same chromosome 4 across stress and ratio condition, likewise, chromosome 10 had two QTL, *qSRFW-10* and *qRRFW-10* across Fe stress and stressed/control ratio. Current study identified two stable QTL, *qCRDW-3* for CRDW in control and *qSRDW-3* for SSDW in Fe stress on chromosome 3 on same position and four stable QTL for SDW across stress and stressed/control ratio. These stable QTL could be isolated and cloned for further implications in plant breeding. Fe toxicity tolerance in difficult to measure because it is complex soil nutritional constraint and Fe toxicity tolerance is

complex polygenic trait and effect of toxicity is sometime not clearly visible at seedling traits (Das *et al.*, 2021). Secondary tolerance index like SL, SFW, SDW, RFW and RDW are easy to measure and can help plant breeder to perform selection for Fe toxicity tolerance. These above-mentioned QTL would be useful to develop the resistant lines against Fe toxicity tolerance.

Conclusions

Fe tolerance is important trait to study in rice. Many putative QTL were identified in current study controlling many seedling traits. QTL, *SSDW-4*, *SSDW-6*, *RSDW-4*, *RSDW-6* were stable across both stress and stress/ratio environment. In this study we used novel BRILs population and high-density map for QTL mapping. This is the reason this study is different from other studies. We used many secondary tolerance indices like SL, SFW, RFW, SDW and RDW for estimation of Fe tolerance. Three new QTL, *qRSFW-1*, *qRRFW-10* and *qRRDW-1* were identified in our study and these QTL can be used to develop Fe tolerant cultivars using MAS and QTL pyramiding. These findings shed light on comprehensive understanding of Fe tolerance mechanism in rice to sustain rice growth on Fe affected soils.

Authors' Contributions

AR planned and conducted research work, collected data, prepared manuscript. GMW and HK planned research work and data analysis. AMS helped in collection of data. MA provided technical assistance. RH helped in some lab work. JB provided BRILs population. ZW supervised the research.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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