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Identification and Validation of Putative Candidate Gene Markers For Grain Iron Content in Recombinant Inbred Lines of Rice (*Oryza sativa*.L)

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Abstract: **Genetic variability studies and molecular level understanding of Fe translocation and homeostasis paved the way for combining the desirable grain characters of improved varieties with high iron content in grains. The objectives of the experiment were to evaluate grain Fe concentration and candidate gene markers associated with this trait among 160 recombinant inbred lines population derived from a cross between IRRI 38 and Jeerige sanna, of rice and validate the putative markers in another biparental RILs mapping population. Grain iron concentration in RILs ranged from 7.20 ppm to 24.00 ppm with an average of 11.30 ppm. Out of 41 candidate gene markers, 11 showed polymorphism between parents. It included 8 putative candidate gene markers, 2 candidate gene based SSR primers and one SSR marker. Single marker analysis of 11 polymorphic markers on RILs mapping population showed that four gene specific markers viz.OsNRAMP5g, OsMTP1a, OsYSL4e and OsZIP8a and one candidate gene based SSR primer; iRMm9-1 is associated with grain Fe concentration. Validation of these markers on 69 segregants of another mapping population showed that only two markers OsYSL4e and OsZIP8a are significantly associated with grain Fe concentration with phenotypic variance of 11.9% and 9.2% respectively. Thus the high priority candidate genes for high Fe in grains identified in this study are *OsYSL4* and *OsZIP8* located on 5 and 7 chromosomes respectively.**

Keywords**: Candidate Gene, Iron, Markers, Micronutrient and Rice**

1.INTRODUCTION

Rice is life for thousands of people, being the major source of carbohydrate and protein. Rice provides 21% of global human per capita energy and 15% of per capita protein. Rice also imparts minerals, vitamins, and fiber, although all constituents except carbohydrates are reduced by milling. To some extent, this reflects on Asia's large population as micronutrient malnutrition even in relative terms since rice is consumed as the most important commodity in their daily lives (http://www.knowledgebank.irri.org).Micronutrient malnutrition has turned out to be a persisting problem in India, and as the recent data suggest, some forms of micronutrient malnutrition are reaching their peak in the present century. The intake of micronutrients in daily diet by over 70% of Indian population is far from satisfactory and largely less than 50% RDA. The consequences of micronutrient malnutrition are unacceptably high morbidity and mortality. Iron, zinc and Vitamin A deficiency when combined constitute the second largest risk factor in the global burden of diseases. Anemia, an indirect indicator of iron deficiency affects more than 1.6 billion people worldwide or approximately 25% of the population (De Benoist *et al*; WHO 2008). Utilization of genetically enriched staple food with these micronutrients has been recognized as an efficient and cost-effective strategy for reducing micronutrient malnutrition in the developing countries.

Rice could be developed as a source of iron due to its wide availability, but has low micronutrient content, since these were improved mainly for yield. Increasing iron concentration in the edible portion of rice grain either by, molecular and conventional breeding or genetic modification may help to alleviate the incidences of iron deficiency anemia in the rice consuming countries (Sison *et al*. 2006). A number of studies have demonstrated about considerable genetic variability underlying differences in concentration of iron in different rice varieties, pointing that rice lines would be selected on the basis of grain iron content or grain iron content of their parentals for breeding programs aiming at iron and zinc biofortification (Belarmino *et al*. 2013). These resources also provide information to identify QTLs/genes for iron and zinc accumulation in rice grains that may facilitate cultivar improvement through marker-assisted breeding.

Candidate gene (CG) based association studies have proven extremely powerful in studying the genetic architecture of complex traits (Patel and Patel. 2013). CGs are sequenced genes of known biological function putatively involved in the expression of a given trait or trait variation, based on its map localization (Pflieger *et al*. 2001). CGs involved in the regulation of a metabolic pathway could serve as potential targets for identifying gene-specific molecular markers. The idea is to detect molecular polymorphism within the CG that in turn corresponds to phenotypic variation. Simple sequence

repeats of varying length (di-, tri- and tetra-nucleotide repeats) were identified in the introns as well as exons of the candidate genes for iron transporters of maize by Chauhan (2006). Banerjee *et al*. (2010) reported several SNPs and SSRs within metal transporter genes that can be used for developing gene specific markers. The advent of molecular marker technology speeded up the process of gene identification and a total of forty-three genes belonging to five different protein families: Yellow Stripe like (YSL), Ferric Reductase Oxidase (FRO), [ZRT (Zinc regulated transporter) - IRT (Iron regulated transporter) like Protein (ZIP) Guerinot, 2000], Natural Resistance Associated Macrophage Protein (NRAMP) and ferritin involved in iron homeostasis were identified in rice (Gross *et al*. 2003). Chandel *et al*. (2011) characterized two QTLs, qFE-1 and qFE-9 governing iron content and identified eight candidate genes involving in uptake, transport and accumulation of iron and zinc in rice grains using various gene-identification parameters and bioinformatics tools. *OsYSL1* and *OsMTP1* (Metal Tolerance Protein) are two candidate genes underlying QTLs reported for high iron accumulation in rice seeds (Anuradha *et al*. 2012).

Numerous studies have established the positive correlation between micronutrient content and aroma (Gregorio, 2002; Welch and Graham, 2004; Graham 2011; Thongbam *et al*. 2012) that can be exploited to screen for high Fe and Zn levels in rice grains for breeding. Many local upland rice varieties containing high iron in grains were reported by Prom-u-thai and Rerkasem (2001). In this context, we selected 160 recombinant inbred lines population of rice developed from the cross IRRI 38 x Jeerige sanna for the study. IRRI 38 is an early drought resistant line and Jeerige sanna is a local aromatic (Singh *et al*. 2000) upland rice variety popular in Coorg district of Karnataka, India having certain medicinal values (The Hindu, 2013). The present investigation intended to evaluate Fe concentration in rice grains among Recombinant Inbred Lines (RILs) population, assessment of polymorphism across the RILs using putative candidate gene markers and validation of significant markers in another biparental mapping population.

2.MATERIALS AND METHODS

Plant Material

A population of 160 recombinant inbred lines (RILs) derived from a cross between IRRI 38 and Jeerige sanna using single seed descent method was used in the study. RILs were grown under aerobic condition in an augmented block design in the field of University of Agricultural Science, Bangalore, India during wet seasons of 2011 and 2012. RILs and checks were raised by direct seeding with a spacing of 30 cm between the rows and 15 cm between the plants in 16 blocks. Standard agronomic practices and plant protection measures were adopted uniformly to raise the crop. Plants were grown in available rainfall conditions but irrigation was done once in five days in the absence of rainfall.

Sample preparation and estimation of iron concentration

All panicles from five plants in the middle row were harvested manually (to avoid any metal contamination) and seeds were bulked for iron analysis. Dehusking was done manually and whole grains each with embryo intact were selected for iron estimation. The grains were washed quickly with 0.1N HCl and then with sterile double distilled water to remove any surface contaminants. Washed seeds were dried in hot air oven at 70^{o} C for 72 h. The iron content of seed samples (5grams) was estimated by energy dispersive X-ray florescence spectrometry (EDXRF) at M. S. Swaminathan Research Foundation, Chennai. EDXRF was performed using Oxford Instruments X-Supreme 8000 fitted with a 10 place auto sampler (Paltridge *et al*. 2012). Measurement conditions were as recommended by the manufacturer for analysis of Fe in a cellulose matrix. Calibration of instrument was done using standard samples of high, medium and low Fe containing genotypes. Total analysis time for each sample was 180 s which included 60 s acquisition times for the separate Zn and Fe conditions as well as 66 s 'dead time' during which the EDXRF established each measurement condition. Scans were conducted in sample cups assembled from 21 mm diameter Al cups combined with polypropylene inner cups sealed at one end with 4 µm Poly-4 XRF sample film. Cups containing samples were gently shaken to evenly distribute grains. In 21 mm cups, minimum depths equated to ≥ 4 g of grain for Zn analysis, and ≥ 2 g of grain for Fe analysis, so sample mass was fixed at 4 g. According to the manufacturer, the X-Supreme 8000 scans a circle of 21 mm diameter with the sample spinner on.

Identification of candidate genes and co-localized ESTs for iron transporters in rice

Eight putative candidate genes involved in iron accumulation in grains were selected based on prior knowledge: *OsYSL1, OsYSL4, OsMTP1, OsNAS3* (Nicotinamine synthase), *APRT1* (Adenine Phosphoribosyl transferase), *OsNRAMP1, OsNRAMP5* and *OsZIP8* (Chandel and Banerjee 2011 and Anuradha *et al*. 2012). Genomic sequences for these genes were downloaded from nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov). Three to five sets of forward and reverse primers were designed for each gene by splitting the whole genomic sequence into segments of 1000bps each in order to detect polymorphism. Thirty one gene specific primers were designed for iron related putative candidate genes using primer-3 web version4.0.0- a primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primerblast/primertool.cgi). Primer designing was done by putting the nucleotide sequences in FASTA format and the specifications for primer designing included 20-25 bp of primer length, Tm range of 50-60 °C and a range of 100-1100 bp of product size, based on the size of genes. All other options were kept as default value. The details of the markers employed and the chromosomes in which they locate dareen listed in Table:I.

International Journal of Agriculture Innovations and Research
Volume 3, Issue 3, ISSN (Online) 2319-1473

Table: I-Details of targeted gene, chromosomes in which they located, primers designed based on these genes and its properties:

Sl. No.	Markers/Genes	Chr. No.	Forward Primer (5'-3')	Reverse primer (5'-3')	Ann. Temp. (^{0}C)	Expected product size (bp)	Source/Reference
1	gRMm1-1	1	GTCGTCATGATCTGGGACT	AATGAATGGGGTGATAGAAAT	58	173	Chandel *et al*, 2011
2	cRMm1-1	1	TAGCGACAACCAGGTCAG	AGACGGAGTGGAAACAGAG	59	170	Chandel *et al*, 2011
3	OsYSL1a	1	TAAACCAAAGATGGCAGACC	GAACATGCCGGTTGATGATT	56	995	Primer-3
4	OsYSL1b	1	GCTCGAGGCGTACAAGGAG	ATCGACGGCGAGCTTCTG	58	984	Primer-3
5	OsYSL1c	1	AGGAACCAGGTGTTCCTGAG	ACCTCCTGGATCTGGCTTCT	52	926	Primer-3
6	OsYSL4a	5	GCAAACACTACCCCAAAAGC	CATGCGGGTCTCTGACATTA	56	999	Primer-3
7	OsYSL4b	5	AGCTCTGCGGGTGAACATAC	GCACGAAAAACGGAGCAGT	63	890	Primer-3
8	OsYSL4c	5	TATTAGTGAGGGCGATCCAC	CACAACCACTGCCTGAAGAA	50	934	Primer-3
9	OsYSL4d	5	TCTAGAACGGTCACACAGAAAA	GTAGCTATTAATTTGGGGGACA	60	917	Primer-3
10	OsYSL4e	5	TATGCATGCGGTGGATGA	GTACTTTGGGAGGCCCTTCA	55	851	Primer-3
11	OsYSL4f	5	CACCTCTACATCCCGAGCAT	CAACCAAAGCTGGCTCACAT	58	523	Primer-3
12	OsMTP1a	5	TCTCTCCTCCCCATCTCCAA	GCTGGTTACAGCGAAAGCAC	60	961	Primer-3
13	OsMTP1b	5	GGGGACCTTCTTGTTGTTGG	GCCATTCTCTCCCTTGCATC	60	934	Primer-3
14	OsMTP1c	5	CGGTCGAAGTGGTTGGAG	GATCAGCGTCTGCGTCCT	64	969	Primer-3
15	OsMTP1d	5	TGCCATGTGACAATCACTCA	ATTATTTCCCATGCCAGTGC	55	519	Primer-3
16	OsNAS3a	7	TCACCAGTTGGAGCTAATCG	GCGATGATGACGGAGTTGAT	56	904	Primer-3
17	OsNAS3b	7	CACCAGAAGATGGAGGACAT	CGCAACAGAGACAATGGTTG	63	386	Primer-3
18	OsNRAMP1a	7	GAGAGGCAAGGCAAACTCAG	GCGACAATGGTAGGGCAAGT	66	973	Primer-3
19	OsNRAMP1b	7	TTGTACGTCTTTGAGACTTTGACTG	GGTTGAAAGCAAATCCTGTACCTAT	59	984	Primer-3
20	OsNRAMP1c	7	GGGTTCTCATTGCTGGCTCT	ATGCAGTACCCTGAGGAGGA	64	976	Primer-3
21	OsNRAMP1d	7	CTAGGAAAGTCAAGTGCGACAG	CAGGAGAATCCAACAATCTGC	59	911	Primer-3
22	OsNRAMP1e	7	GTTCGTCATCATCGGGATAAAC	TTGCCTAGCTACAACATTCTGC	57	501	Primer-3
23	OsNRAMP5a	7	CCACCATTCTCTTCTTCGTC	GCACTGGGGATTAAAAGTGG	55	903	Primer-3
24	OsNRAMP5b	7	GATTGGACTCATCTTCGCACT	TGCAACTGCTACACCACTGA	56	988	Primer-3
25	OsNRAMP5c	7	GCTTTGCTGATCGGGATTAGTT	GGACTAGATCGAGTTCATGTCCTA	56	997	Primer-3
26	OsNRAMP5d	7	TAATTTCGGGCTCCAGTACC	CTGCACCGTGAAATGGACTA	56	1048	Primer-3
27	OsNRAMP5e	7	ATATCCCATCGCACCGTAAA	ATAAATCCGCACGATCGACT	55	859	Primer-3
28	OsNRAMP5f	7	GATTAGCAAAATGTCACTGACTAGC	CGATGTCTGTAAAGATCAAGCAAG	56	865	Primer-3
29	OsNRAMP5g	7	GATCATTACGTATGTCGTTGTATCTC	AATTACAAGGATACATGAGCCACCT	56	992	Primer-3
30	OsZIP8a	7	ATGAGGACGAACACCACCAC	CGGAGGGAGGGAGTAGTAATG	67	880	Primer-3
31	gRMm9-2	9	AACCGGGTTTCTTACCTG	CCCAAGCTGCTAAACAGT	57	153	Chandel *et al*, 2011
32	cRMm9-1	9	GGAACTCGTACAGCAACG	CGTGTACCACGAGAATCC	52	128	Chandel *et al*, 2011
33	cRMm9-2	9	CGAGGAAAAGCTTGTTAAAGT	GTCAACATCCGTTACAATCAC	58	136	Chandel *et al*, 2011
34	utrRMm9-2	9	GTTTACTCCCCAGAAATCAAT	ATCACAAGGGTGAGTAGGACT	60.2	176	Chandel *et al*, 2011
35	iRMm9-1	9	GTGACGAGCGAGCGGATG	TGTTCAACAGATTCTTCTTCG	51	205	Chandel *et al*, 2011

International Journal of Agriculture Innovations and Research
Volume 3, Issue 3, ISSN (Online) 2319-1473

36	APRT1a	12	CTCTCTCCCGCTTTTTGCTT	CAGTGATCCCTTTGTCCTTG	59	630	Primer-3
37	APRT1b	12	GTTGTGAAATATCAGGTGTTGAAGC	TGCAGATCAATTACCCAACACATAC	57	944	Primer-3
38	APRT1c	12	GGAAGTTTCCTTTTGGCTGT	CCCCATGAATAATTGGCACT	58	603	Primer-3
39	RM300	2	GCTTAAGGACTTCTGCGAACC	CAACAGCGATCCACATCATC	55	121	Gramene
40	RM8226	6	TTAGGATACGGCTTCTAGGC	CGTAATTGTTGCATATGGTG	55	251	Gramene
41	RM325A	6	GACGATGAATCAGGAGAACG	GGCATGCATCTGAGTAATGG	55	201	Gramene

To obtain the spatial and temporal expression pattern of these genes in different tissue libraries, the gene sequences were analyzed in-silico for co-localised ESTs (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer). The locus ID of each gene was used as query to search for the ESTs mapped over these genes. ESTs corresponding to a tissue library provided information about putative site of expression of the genes in which it was identified. It is expressed in terms of TPM (transcript per million) value, which is an indirect measure of level of corresponding gene expression (Chandel *et al.* 2011).

Genotyping

Young leaf samples from five plants in the middle row of 160 RILs and parents were collected and bulked for DNA isolation. DNA was isolated using CTAB (cetyl trimethyl ammonium bromide) extraction method described by Doyle and Doyle (1990). The purity and concentration of the isolated genomic DNA sample was quantified using Nanodrop (Biotech Epoch). A set of 41 markers (Table: I) including 31 candidate gene primers, seven candidate genes based SSR primers and three SSR markers were used to identify polymorphism between IRRI 38 and Jeerige sanna.

DNA amplification was carried out in a 20µl reaction volume containing 50ng template DNA, 2.5mM of each dNTP, 2.5mM of $MgCl_2$, 1x PCR buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$ pH 8.8 at 25^0 C,), 5 pico molars each of forward and reverse primer and 1 unit of Taq polymerase. PCR amplification was carried out in a Master Cycler Gradient Eppendorf PCR using the following temperature profile: initial denaturation at 95^0 C for 5min followed by 38 cycles at 95^0 C for 1 min, 60^0 C (varies for different primers) for 1 min, 72^0 C for 1 min and a final extension at 72^0 C for 7 mins followed by cooling at 4^0 C. The PCR product (10µl) was then mixed with 3µl of 10x gel loading dye (0.2% bromophenol blue, 0.2% xylene cyanol dye and 30% glycerol in a TE buffer) and electrophoresed in a 3% metaphor agarose gel at a constant voltage of 100V for 2-3h. The polymorphic markers were used to screen RILs mapping population.

Statistical Analysis

The association of grain Fe concentration with molecular markers was analyzed with single marker analysis and regression analysis with SPSS16.0 (SPSS Inc.).

3.RESULTS AND DISCUSSION

Iron concentration in brown rice of RILs

Fe concentration analysis showed that most of the RILs had more or equal values as that of parents. IRRI 38 exhibited 8.00 ppm of Fe concentration in brown rice while in Jeerige sanna it was found to be 13.60 ppm. The grain Fe concentration showed a wide range of variation among the 160 RILs mapping population analyzed. It varied from 7.20 ppm to 24.00 ppm with a mean value of 11.30 ppm in both years. The distribution of Fe concentration in RILs is shown in Fig: 1. Out of 160 RILs, nine lines showed Fe concentration >16 ppm with relatively better yield whereas about 91% showed Fe concentration <16 ppm in brown rice. The genotypes with high iron concentration can be selected as donors for further breeding programs or released as varieties after multi-location trials.

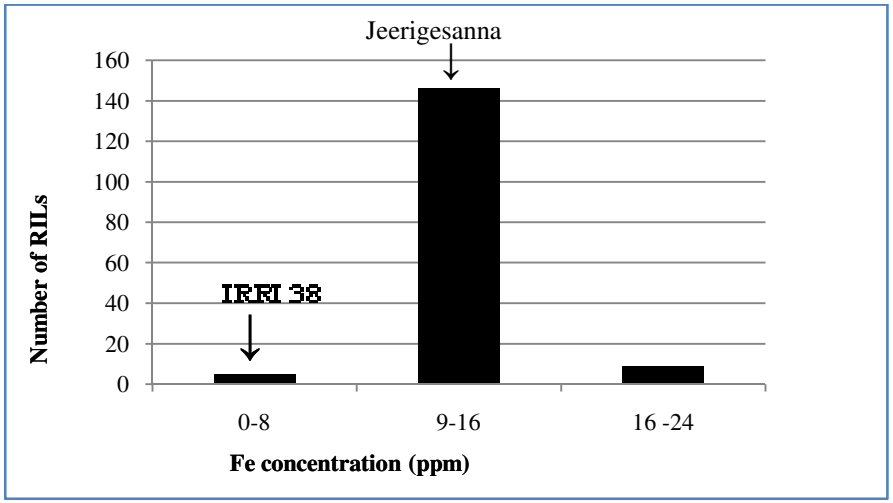


Fig: I- Distribution of Fe concentration in unpolished rice of 160 recombinant inbred lines (RILs) derived from a cross between IRRI 38 and Jeerige sanna. The Fe concentration in parents is indicated by arrows.

Similar results were also reported by Anuradha *et al.* (2012) for high Fe concentration of 14.8 ppm in brown rice of improved cultivars. Prom-u-thai and Rerkasem (2001) analyzed grain Fe concentration in rice genotypes and found that it varies between 7–19 mg Fe kg^{-1} in brown rice. In another study, Prom-u-thai *et al.* (2007) obtained 10 to 20 mg kg^{-1} and 3–11 mg kg^{-1} of Fe in brown and white rice respectively among different rice cultivars of diverse genetic backgrounds. Pintasen *et al.* (2007) indicated that grain Fe concentration generally varied much more with genotype than with environment and genotype x environment interactions. Several studies suggests that variation in grain Fe concentration of same or different accessions may depends on sample lots harvested, position of grain on the panicle, presence or absence of embryo in grains or time of harvest (Chandel *et al.* 2010; Anuradha *et al.* 2012). The variation among plants in their ability to absorb iron is not always consistent and is affected by changing conditions of soil and climate and by the stages of plant growth.

Identifying polymorphic loci between parents

Parental polymorphism survey between IRRI 38 and Jeerige sanna was performed using 41 markers located on rice chromosomes 1, 2, 5, 6, 7, 9 and 12 derived from the genomic regions associated with iron

metabolism. Out of 41, eleven markers showed polymorphism between the parental genotypes, i.e. 8 putative candidate gene primers out of 31, two out of seven candidate gene based SSR primers and one SSR marker out of three, exhibited polymorphism between parents giving an overall polymorphism of 27%. All 160 RILs were genotyped using eleven polymorphic markers (Fig:2).

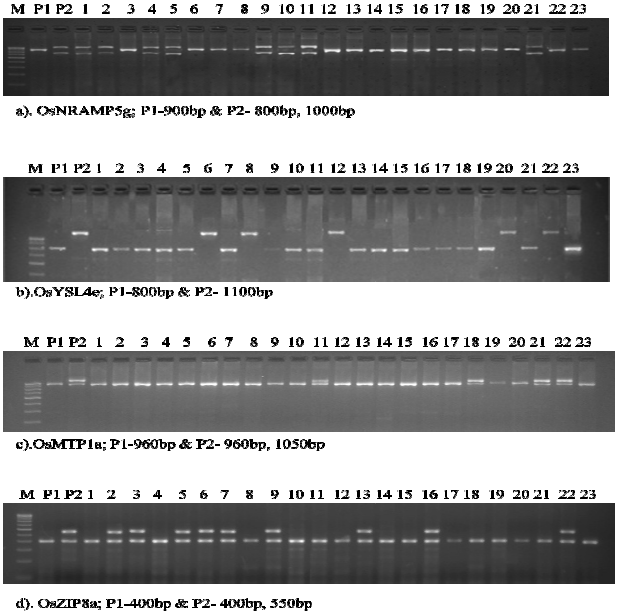


Fig: II- Gel picture of PCR analysis showing polymorphic bands in parents and RILs for different candidate gene markers; a). OsNRAMP5g,b). OsYSL4e, c). OsMTP1a & d). OsZIP8a. (M- 100bp DNA Marker, P1- IRRI 38, P2- Jeerige sanna and 1to 23- RILs of cross IRRI 38 X Jeerige sanna).

Single Marker Analysis and Validation

The advent of plant molecular biology and bioinformatics tools hastened the discovery of genes involved in specific pathways associated with various phenotypic traits. The Fe concentration in seeds is such a complex polygenic trait governed by a set of genes involved in a series of processes: uptake from the soil by the roots, translocation and redistribution within the plant, import and deposition in the seeds etc (Chauhan 2006). Several candidate genes underlie QTLs encoding iron transporters and correspond to different classes of protein families were identified so far. One objective of the present study was aimed at establishing an association between candidate gene markers and Fe concentration in unpolished rice grains, by adopting single marker analysis**.** Among eleven polymorphic markers screened on 160 RILs, four gene specific markers (OsNRAMP5g, OsMTP1a, OsYSL4e and OsZIP8a) and one candidate gene based SSR primer, iRMm9-1 reported by Chandel *et al.* (2012) showed association for grain Fe concentration explaining a maximum phenotypic variation ranging from 3% to 14%. OsZIP8a located on chromosome 7 and OsYSL4e located on chromosome 5 explained high phenotypic variance (14% and 9.6% respectively), significant at 1% (p=0.001 and 0.003 respectively) (Table: II). SSR markers did not show any significant difference among the RIL population.

Table: II- Markers linked to Fe concentration in unpolished rice of RIL population using single marker analysis:

Sl. No.	Marker	p(F)	R^2 (%)
1	OsNRAMP5c	0.984	0.0
2	OsNRAMP5f	0.222	1.9
3	OsNRAMP5g	0.029*	3.4
4	OsMTP1a	0.035*	4.2
5	OsYSL4b	0.202	2.9
6	OsYSL4c	0.231	2.5
7	OsYSL4e	0.003**	9.6
8	OsZIP8a	0.001**	14.0
9	RM8226	0.159	2.3
10	gRMm1-1	0.791	0.3
11	iRMm9-1	0.065	1.8

In an earlier approach, Anuradha *et al.* 2012 also reported the candidate gene, APRT within the QTL *qFe12.1* showing a high phenotypic variation of 71% for grain Fe concentration. Our results are also consistent with Neelamraju *et al.* (2012) experiments, that identified *OsYSL1*, *OsMTP1*, *OsAPRT* and *OsNAS3* underlying QTLs accounted for >30% phenotypic variation in MadhukarXSwarna RIL population. Ishimaru *et al.* (2012) discussed about the role of *OsNRAMP5* in increased Fe uptake from soil during flowering and seed development. It functions jointly with other transporters to transport Fe from root xylem. The fact that *OsNRAMP5* is the only gene that affect rate of Fe remobilization from flag leaves

to developing grains (Banerjee *et al*. 2010) also supports our findings. *OsMTP1* has been reported as high priority candidate gene for high Fe concentration in seeds in addition to their role in one or more steps of Fe uptake and transport within the plant (Anuradha *et al*. 2012). According to Chandel *et al*. (2011) iRMm9-1 primer belongs to Class II or potentially variable SSR marker (consisting of ≥ 12bp < 20bp) and are designed from the intron region of the candidate gene. Temnykh *et al*. (2001) reported that GC-rich trinucleotide repeats were most abundant in protein-coding portions of ESTs which is in accordance with iRMm9-1 that consists of SSR motif of (GC) 3.

Validation of these five markers on 69 segregants of another mapping population with a different genetic background showed that only two markers OsYSL4e and OsZIP8a are significantly associated with grain Fe concentration with phenotypic variance of 11.9% and

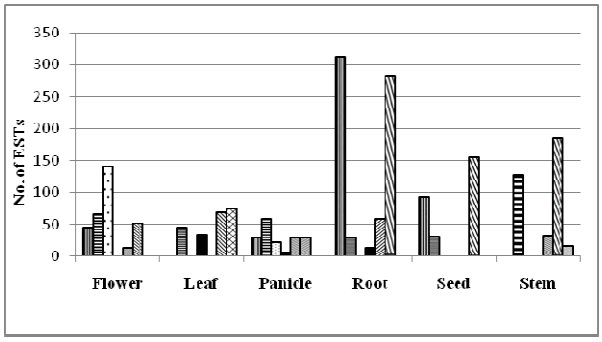
9.2% respectively (Table: III). The *OsYSL* genes, components of Strategy II of metal transport found in cereals, encoding oligopeptide phytosiderophore transporter proteins (Gross *et al*. 2003) has an important role in increased uptake and transport of Fe ions into developing rice grains. Several studies on differential expression analysis of metal homeostasis related candidate genes in reference to grain Fe/Zn contents were reported (Chandel *et al*. 2011., Ishimaru *et al*. 2012) showing the correlation of genes viz. *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsYSL6*, *OsYSL12*, *OsYSL4*, *OsZIP8*, *OsZIP10*, *OsFER1* etc with high grain Fe content.

Table: III-Single-marker analysis of significant candidate gene markers for grain Fe concentration in segregants of a biparental RILs mapping population:

Sl. No.	Marker	p(F)	R^2 (%)
1	OsNRAMP5g	0.634	2.6
2	OsMTP1a	0.225	3.1
3	OsYSL4e	0.041	11.9
4	OsZIP8a	0.018	9.2

Candidate Gene Analysis and Identification of co-localised Expressed Sequence Tags underlying putative candidate genes

It was found that eight candidate genes used in the present study were belonged to six classes of protein families (http://www.ncbi.nlm.nih.gov/proteinclusters/) viz. 1. Oligopeptide transporter protein, 2.Natural resistance associated macro-phage protein, 3. Cation diffusion facilitator family transporter, 4. Nicotianamine synthase protein, 5. Phosphoribosyl transferase and 6. Zinc transporter. In silico analysis of distribution of expressed sequence tags (ESTs) underlying the target genes was also performed to predict the approximate spatial expression pattern in different tissue libraries. A total of 2044 ESTs were identified in seven genes with maximum 312 ESTs in gene encoding natural resistance associated macrophage protein (Os07g0257200) and minimum 7 ESTs in zinc transporter genes (Os07g0232800). Out of seven iron accumulating genes analyzed, ESTs in seed tissue library were identified in four genes (OsNRAMP5, OsMTP1, OsNAS3 and APRT1). The total number of ESTs observed for each gene and their distribution in different tissue libraries is shown in Fig: 3. The ESTs identified in two genes OsMTP1 (Os05g0128400) and APRT1 (Os12g0589100) were found to be expressed in almost all tissues including flower, leaf, panicle, root, seed and stem suggesting their major role in iron homeostasis in rice. The ESTs corresponding to the genes OsNRAMP5 and OsNAS3 were found to express mostly in flower, panicle, seed and root explains their specific expression in reproductive parts of plant. It was observed that ESTs constituted in majority of the genes studied was more correlated to root tissue indicating that these genes participate in uptake and transport of metal ions from the soil into the plant cells. Similar results were also reported by Chandel *et al*. (2011) about co-localization and differential expression of ESTs underlying metal homeostasis related candidate genes. Gross *et al*. (2003) reported OsNRAMP5 and OsNRAMP1 as having largest number of related ESTs and are found on the same chromosomal locus.



OsNRAMP5; *OsMTP1*; *OsYSL4*; *OsNAS3*; *APRT1*; *OsZIP8* and Ion channel nompc, putative, expressed

Fig: III- *EST count in different tissue libraries for genes controlling iron in rice.*

4.CONCLUSION

The present study indicated that there is significant genetic variability exists among RILs mapping population for grain iron concentration that can be utilized to increase Fe concentration substantially in rice grains by means of plant breeding. The identification of genes that could enhance micronutrient uptake and accumulation will provide us with more opportunities to breed diverse, fortified varieties. Molecular analysis showed that two markers OsYSL4e and OsZIP8a are significantly associated with grain Fe concentration with phenotypic variance of 9.6% and 14% respectively. Thus it can be concluded that two high priority candidate genes for grain

Fe concentration are *OsZIP8* and *OsYSL4* that are located on chromosomes 7 and 5 respectively.

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