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RESEARCH ARTICLE :

Molecular characterization of metal homeostasis related gene orthologs in nutri- rich foxtail millet accessions

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SUMMARY : Foxtail millet is drought tolerant and nutritionally reached functional food in tribal parts of world. The present study aim to focused the nutritious accessions through the molecular characterization and differential gene expression profiling of nutritionally rich (iron and zinc) accessions of foxtail millet. Accessions IC12059, was found to be more promising having high concentration of both zinc and iron content, whereas, IC120175, IC120213, IC97111, IC120179, IC1220207 and IC1220407 has high zinc content and IC97189, IC120150, IC120159, IC120239, IC120235, IC120355 and IC403579 were found to be high iron content amongst sixty six accessions. High iron containing accessions IC120239 (59.77 ppm), IC120235 (57.81 ppm), IC120355 (56.82 ppm) and one low iron containing IC344225 (9.69 ppm) were then explored for spatial expression profiling using genes belonging to ferritin, ZIP, YSL, His-rich NAC families and found high expression of ferritin and NAC gene in high mineral containing accessions. Histidine rich gene targeted studies showed 500 bp of isoform expressed in both high and low iron containing accessions, whereas, 297 bp isoform was found associated with high mineral containing foxtail millet accessions.

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BACKGROUND AND OBJECTIVES

Metals have binary role in biochemistry like some are essential and some are toxic in higher concentrations (Guengerich, 2009). Among metal elements Fe, Mn, Zn, Cu, Mo, Ni are considered plant micronutrients because they are necessary for physiological and metabolic processes in small amounts (Vincevica-Gaile *et al.*, 2012). Poor micronutrient content in soil and edible part of the plant is primary cause of micronutrient deficiency in world (Imtiaz *et al.*, 2010; Genc *et al.*, 2005 and Thompson, 2011) this micronutrient deficiency is expressed as "hidden hunger". Micronutrient deficiencies can affects more than a 1/3rd of the total world population (Palmgren *et al*., 2008). Fe is vital element in heamoglobin, and its deficiency leads to anaemia (Ekweagwu *et al*., 2008). Zinc is also used in more than 300 eukaryotic enzymes as cofactor and deficiency of zinc in human beings leads to the underdeveloped growth and lowered resistance which often contributes to diseases like pneumonia and malaria (Ekweagwu *et al*., 2008). Nutritional deficiency of essential micronutrients is serious health problem in world. Approximately 60 per cent people are iron deficient, 30 per cent are zinc deficient, calcium, magnesium, copper and manganese deficiencies are very common in many developed and developing countries (Welch and Graham, 2005; Grusak and Cakmak, 2005 and Thacher *et al*., 2006).

Foxtail millet (*Setaria italica*) contains high amounts of proteins and minerals (Pawar and Pawar, 1997). Millets are rich in micronutrients and proteins (Geervani and Eggum, 1989). Phenolics, tannins and phytates are the antinutritional factors which are called as antinutrients reduce the bioavailability of minerals. Millets are rich in antinutrients (Thompson, 1993). On the other hand, these antinutrients are plays important role to reduce the severity of colon and breast cancer in animals (Graf and Eaton, 1990). The bran of foxtail millet has highest radical scavenging activity as compare to whole flour suggesting that the antioxidant compounds are present in the bran (Suma and Asna, 2012). After these entire properties of these crops still the millets are the nutritionally rich neglected cereals from research as well as diet in both developed and developing countries.

Foxtail millet grains contain higher seed protein (14 - 16%), crude fat (5–8%) and minerals than finger millet (Ravindran, 1991 and Dwivedi *et al.,* 2012). Biological value of the digestible protein in foxtail millet is superior to major cereal crops such as rice and wheat as it contains seven of the eight essential amino acids (Zhang *et al*., 2012). The grains contain 2.5-fold more edible fibre than rice and its bran contains 9.4 per cent crude oil enriched with linoleic (66.5%) and oleic (13.0%) acids (Dwivedi *et al.,* 2012) and high fibre (42.56%) (Amadou *et al*., 2011).

Foxtail millet was called as nutri-cereal since they are rich in micronutrients like minerals and B-complex vitamins. Iron content of foxtail millet is 2.8-6.0mg/100g which is in rice found to be 1.8 - 5.8 mg/100g. phytochemical screening revealed the presence of flavonoids, phenolics, tannins along with other components which are potent antioxidants. High antioxidant activity was observed in the extracts from bran rich fraction compared to whole flour, suggesting the presence of antioxidant components in the bran rich layer (Suma and Asna, 2012). Additionally millets are also rich in health promoting phytochemicals and can be used as functional foods.

The micronutrient status of wild accessions is still unknown and no reports found on investigation of trace elements from edible parts. Hence, the wild collection of foxtail millet accessions from different geographical areas of India were analysed for grain micronutrient content and antioxidant activity. Prasad and Lepakshi are the commercial varieties and standard checks of foxtail millet. The micronutrient analysis of these two accessions revealed that the zinc content 15.62mg/kg and 43.73mg/kg, respectively and iron content 20.53mg/kg and 39.63mg/kg, respectively. In the set of 66 accessions the wide diversity for grain iron, zinc, calcium, magnesium content. Most of the accessions are found superior than the checks for micronutrient content.

Therefore, present investigation was carried out to screen high mineral (Cu, Fe, Mn and Zn) containing accessions collected from various tribal packets of India. Differential depositions of mineral in embryo and endosperm of developing grains has been known to play important role in generating genotypic variation in foxtail millet grain micronutrient content (Prom-u-thai *et al.*,2009). Differential gene expression for mineral accumulation was carried out using eight putative candidate genes belonging to five gene families *viz.,* OsYSLs, OsZIPs, OsNRAMPs, OsFROs and Ferritin (Gross *et al.,* 2003). Micronutrient characterization for grain micronutrients with good radical scavenging activity will prove to be a central phase in fetching important accessions useful in building interest of farmers in cultivation of foxtail millet on large scales which in turn will benefit the consumers.

RESOURCES AND METHODS

Experimental site :

The experiment was carried out during *Kharif* - 2014 at the experimental field of Department of Agricultural Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth. The soil was medium black with clay, fairly leveled and uniform in topography with appropriate drainage system.

Plant material :

The experimental material comprising 66 accessions of foxtail millet (*Setaria italica* L.) including 2 standard checks Lepakshi and Prasad were procured from National Bureau of Plant Genetic Resources (NBPGR), Akola. Also collection of two local accessions from Jalgaon (JLG) and Akola (AKL) districts of Maharashtra as enlisted in (Table A).

Experimental design :

The experiment was laid out in a Randomized Complete Block Design with three replicates. The plot was ploughed one month before sowing and followed by three harrowing to bring the land to a fine tilth. The spacing of row to row and plant to plant was of 45 × 10 cm. Seeds were sown using drill method during *Kharif*- 2016. The fertilizers were applied at the rate of 30 kg N, 75 kg P_2O_5 and 20 kg K_2O per hectare at the time of sowing. Fertilizers were applied in the form of urea, single super phosphate and murrate of potash. As flowering initiated, 2-3 irrigations were provided as per requirement. Harvesting was done as per maturity of the genotypes.

Sample preparation :

5 g seeds of each sample were dried in oven for 48 hours at 60^0C and were ground to superfine powder with bran. This powder was used for estimation of micronutrients analysis for the identification of potent accessions having more mineral content.

Estimation of iron, copper, manganese and zinc from foxtail millet accessions

Grain Cu, Fe, Mn and Zn densities were analysed in the laboratory of Soil science and soil chemistry (AICRP) on micronutrients at Dr. PDKV, Akola. Samples were digested according to di-acid digestion described by Wheal *et al*., 2011. Grain samples were finely ground and oven dried at 60°C for 48 h. Ground sample (0.2 g) with 7.0ml of 69 per cent conc. nitric acid (HNO_3) and 3ml of 30 per cent Perchloric acid ($HClO_4$) was digested by adding in 200ml digestion tubes. Temperature adjusted in three steps 80°C for 1 hour, 120°C for 2 hours, 200°C for 2 hours and 300°C for 1 hour. Final volume of digest is made to 50 ml by adding double distilled water and filtered through Whatman number 1 filter paper. Micronutrient densities were analyzed using Atomic Absorption Spectrometer (AAS) (Model- Varian Spectra

Table A: Foxtail millet accessions used in present investigation

Sr. No.	Accessions	Source
1.	IC 28439	Amreli, Gujarat
2.	IC41883	Betul, Madhya Pradesh
3.	IC41898	Akola, Maharashtra
4.	IC58243	Uttarakhand
5.	IC97086	Maharashtra
6.	IC97087	Maharashtra
7.	IC97109	Maharashtra
8.	IC97111	Madhya Pradesh
9.	IC97123	Parbhani, Himachal Pradesh
10.	IC97172	Maharashtra
11.	IC97174	Maharashtra
12.	IC97175	Kangra, Himachal Pradesh
13.	IC97177	Kangra, Himachal Pradesh
14.	IC97179	Basti, Himachal Pradesh
15.	IC97182	Basti, Himachal Pradesh
16.	IC97189	Maharashtra
17.	IC97295	Simour, Himachal Pradesh
18.	IC120148	Gulbarga, Karnataka
19.	IC120150	Gulbarga, Karnataka
20.	IC120158	Osmanabad, Maharashtra
21.	IC120159	Parbhani, Maharashtra
22.	IC120160	Buldhana, Maharashtra
23.	IC120163	Maharashtra
24.	IC120164	Unknown
25.	IC120165	Unknown
26.	IC120166	Unknown
27.	IC120175	Unknown
28.	IC120177	Unknown
29.	IC120179	Unknown
30.	IC120191	Unknown
31.	IC120192	Unknown
32.	IC120200	Unknown
33.	IC120201	Unknown
34.	IC120204	Unknown
35.	IC120207	Unknown
36.	IC120208	Unknown
37.	IC120210	Unknown
38.	IC120212	Unknown
39.	IC120213	Adilabad, Andhra Pradesh
40.	IC120221	Bidar, Karnataka
41.	IC120228	Osmanabad, Maharashtra
42.	IC120239	Unknown

Table A : Contd…………

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43.	IC120251	Sholapur, Maharashtra
44.	IC120234	Parbhani, Maharashtra
45.	IC120235	Parbhani, Maharashtra
46.	IC120244	Yavatmal, Maharashtra
47.	IC120250	Sholapur, Maharashtra
48.	IC120255	Gulbarga, Karnataka
49.	IC120346	Unknown
50.	IC120355	Unknown
51.	IC120394	Unknown
52.	IC120404	Andhra Pradesh
53.	IC120406	Andhra Pradesh
54.	IC120407	Andhra Pradesh
55.	IC120408	Andhra Pradesh
56.	IC200125	Amravati, Maharashtra
57.	IC325968	Narmada, Gujarat
58.	IC344224	Maharashtra
59.	IC344225	Maharashtra
60.	IC372606	Osmanabad, Maharashtra
61.	IC403579	Andhra Pradesh
62.	IC480117	Uttar Pradesh
63.	LEPAKSHI	Check/control variety
64.	PRASAD	Check/control variety
65.	AKL	Akola, Maharashtra
66.	JLG	Jalgaon, Maharashtra

AA 220). Micronutrient standard solutions of (2mg/kg, 4mg/kg, 6mg/kg, 8mg/kg) for Fe, Zn, Cu, Mn were used as reference. Iron is very sensitive element so, care was taken to avoid any contamination with dust particles or any extraneous matter (Stangoulis and Sison, 2008).

RNA extraction and semiquantitative RT-PCR:

Total RNA was isolated from flag leaf at mid-grain filling stages using TRIzol reagent (Invitrogen, USA). After DNaseI treatment (Ambion Inc, Austin TX USA), RNA integrity was checked by electrophoresis and quantified by using nanophotometer (Implen) and qualitative estimation has been done on 1 per cent agarose gel. Synthesis of first-strand cDNA was obtained from the total RNA using a cDNA Synthesis Kit (InVitrogen Japan). The reaction contained template RNA, 2.0 µl of Accu Script RT Buffer (10X), oligo(dT) primer (0.5 µg/µl), 0.8 µl of dNTP mix (25 mM each dNTP) and RNase- free water to total volume 16.5 µl in an air incubator. The reaction mixture was kept on ice till next step. The reaction was Incubated at 65°C for 5 minutes, cooled at room temperature to allow the primers to anneal to the RNA (approximately 5 minutes) and the following components were added to the reaction mixture, in order, for a final reaction volume of 20 µl; 2 µl of 100 mM DTT, 1 µl of AccuScript RT, 0.5 µl of RNase Block ribonuclease inhibitor (40 U/ µl). The tubes were

Table B : Primers sequence of metal homeostasis related genes

Gene	Reference gene	F/R	Nucleotide sequence	Length	Tm value
Ferritin	PREDICTED: *Setaria italica ferritin-1*, chloroplastic-like	Forward	CTCCCACAATCCACGCACTC	20	61.02
		Reverse	CCTCCCTCCACGTGTGTTTT	20	60.18
His-rich domain	*Setaria italica* cysteine and histidine-rich domain-containing protein RAR1-like	Forward	CTGCCAATACCATCCCTCCG	20	60.25
		Reverse	TTTTGTACCTTGGGCTGCGA	20	60.18
IRT	PREDICTED: *Setaria italica* Fe^{2+} transport protein 1-like	Forward	AGCTTAGCTGCCGGTATTCG	20	60.25
		Reverse	GGATCCACACAGTTACGCGG	20	61.08
NAC	PREDICTED: *Setaria italica* NAC domain-containing protein 100-like	Forward	CCTGACCTCGGGTCTGACTA	20	60.03
		Reverse	GATGCAGCACCACCCCTAAT	20	60.11
NRAMP	PREDICTED: *Setaria italica* metal transporter Nramp1-like	Forward	TCTGGAACCGTCTGCAACTC	20	59.97
		Reverse	AATGGGAGCTCAACGGACAG	20	60.04
Sipf40	*Setaria italica* zinc transporter ZTP29-like	Forward	AAGGCTGATGATGGTGGCTC	20	60.11
		Reverse	TGACACTGTGGAGGACTTGC	20	59.89
WRKY 40	PREDICTED: *Setaria italica* probable WRKY transcription factor 2-like	Forward	GCCTTTTGGAAGGAGGAATTTCG	23	60.37
		Reverse	CAGTCCTCCATGAGCGATCC	20	59.97
YSL9	PREDICTED: *Setaria italica* probable metal-nicotianamine transporter YSL9	Forward	CAGGAGCGGCGAAGGAG	17	59.83
		Reverse	CGGCCATCTCGTAGGTCTTC	20	59.97

placed in a temperature-controlled thermal block at 42°C and incubated for 60 minutes. The reaction was terminated by incubating the reaction at 70ºC for 15 minutes.

Validation of differentially expressed genes :

The product of first strand cDNA synthesis can be used directly for PCR. The volume of first strand cDNA synthesis reaction mixture should not comprise more than 1/10 of the total PCR reaction volume, 1 µL of the first strand cDNA synthesis reaction mixture was used as template for subsequent PCR in 20 µL total volume. RT-PCR for genes predicted in the Rf region was performed using PCR mastermix (2X) of puregene. PCR cycles were as follows: 94°C for 3 min, 94°C for 30 seconds, Tm 59°C-62°C for 45 seconds (annealing varies according to primers), 72°C for 2 min and a final extension at 72°C for 10 minute. Gene specific primers were designed for metal homeostasis related candidate genes YSl9, ferritin, His-rich, NRAMP etc using Primer-3 software (*http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www. cgi*) enlisted in (Table B) were used for expression profiling. Amplified products were visualized on a 1 per cent TAE agarose gel and photographed. The relative intensity of amplified fragments provided basis for quantification of level of expression of gene as high, moderate, low and negligible.

Sequencing and *in silico* prediction :

The amplified cDNA fragments from semi quantitative RT-PCR were purified using a Gel Extraction kit (Genaxy) and sequencing was done through Europhins Genomics, Bangalore, India. His-1 and His-2 sequences data were analyzed using BLAST program from NCBI against non redundant protein database. Clustalw sequence alignment tool of EBI (European Bioinformatics Institute) was used to align the sequences of his-1 and his-2 genes. The protein sequences of His isoform (297 bp) and (500 bp) were used as an input data for the PSI-Blast again protein data bank to identify its homologous structures. Swiss-Model online tool used for structure prediction.

OBSERVATIONS AND ANALYSIS

The results obtained from the present study as well as discussions have been summarized under following heads:

Identification of core set of accessions having high mineral content :

In present study the screening of foxtail millet accessions for high mineral content revealed that there was broad diversity in grain zinc, iron, manganese and copper content within the accessions. The grain iron content was found in range of 9.69-60.04mg/kg. While the grain zinc content was found in range of 1.14-53.98 mg/kg. The grain manganese content was recorded in the range of 1.20-29.80 mg/kg and the grain copper (Cu) content was recorded in the range of 0.40 mg/kg-28.8 mg/kg. The details are enlisted in (Table 1).

Iron is an exceptionally essential element to human health. Current studies focusing to increase the iron content in edible part of the grains of important staple food crops to overcome the problem of malnutrition. The range of iron content in whole grains of foxtail millet is 9.69mg/kg-60.04mg/kg (Table 1 and Fig 1). Highest iron containing accession was IC120159 (60.04mg/kg) followed by IC120239 (59.77mg/kg), IC120150 (59.15mg/kg), IC97189 (58.12mg/kg), IC120235 (57.81mg/kg), IC120355(56.82mg/kg),IC403579(56.6mg/kg). Whereas, the lowest iron containing accessions was found to be IC344225 (9.69mg/kg), JLG (10.88mg/kg) IC120407 (11.83mg/kg), IC120251 (12.78mg/kg), IC120234 (12.75mg/kg), IC97179 (11.95mg/kg), IC120200 (15.87mg/kg). Accessions IC120239 (59.77mg/kg), IC120235 (57.81mg/kg), IC120355 (56.82mg/kg) contain more iron as compare to the standard checks Lepakshi (39.63mg/kg) and Prasad (20.53mg/kg) as shown in (Table 1 and Fig 1).

Highest zinc containing accession was found to be IC120159 (52.98mg/kg) followed by IC120175 (52.67mg/kg), IC120213 (49.78 mg/kg), IC97111 (49.63mg/kg), IC120179 (48.75mg/kg), IC120207 (48.51mg/kg), IC120407 (48.9mg/kg). While the lowest zinc containing (7.14mg/kg), IC372606 (8.44mg/kg), IC120408 (12.82mg/kg), IC120255 (12.16mg/kg), IC120355 (13.57mg/kg). Accessions IC120175 (52.67mg/kg),IC120213 (49.78mg/kg), IC120207 (48.51mg/kg) contain more Zinc as compare to the average and standard checks Lepakshi (15.62 mg/kg) and Prasad (43.73 mg/kg).

In present investigation the average copper content of sixty six foxtail millet accessions was 10.81mg/kg. Suddenly, it is maximum as per the previous reports are concern. The range of grain copper content among foxtail

Table 1: Iron, zinc, copper and manganese densities of foxtail millet accesion

Sr. No.	Accession	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Cu (mg/kg)
1.	IC 28439	31.1 ± 0.18	37.2± 0.30	7.2 ±0.08	5.2±0.06
2.	IC41883	22.1± 0.79	34.2± 0.75	5.6±0.13	5.8±0.13
3.	IC41898	16.7± 0.97	33.8± 0.58	8.2±0.19	7.2±0.16
4.	IC58243	16.5± 0.82	43.1± 0.47	7.8±0.10	4.8±0.06
5.	IC97086	21.0± 0.26	46.8± 0.26	17.0±0.32	7.0±0.13
6.	IC97087	18.8± 0.56	24.6± 0.51	6.2±0.08	5.0±0.06
7.	IC97109	25.5± 0.62	40.9± 0.39	25.4±0.17	5.4±0.04
8.	IC97111	21.4± 0.47	49.6± 0.46	17.0± 0.27	5.4±0.09
9.	IC97123	19.4± 0.64	40.1± 0.70	14.6± 0.11	5.8±0.04
10.	IC97172	19.6± 0.47	42.1± 0.60	13.0± 0.51	6.0±0.23
11.	IC97174	18.0± 0.62	22.1± 5.46	29.8± 0.63	4.6±0.10
12.	IC97175	43.8± 0.70	45.5± 0.86	12.6± 0.14	5.6±0.06
13.	IC97177	42.1± 0.15	45.9± 0.74	15.4± 0.17	5.2±0.06
14.	IC97179	11.9± 0.16	38.6± 0.41	5.60± 0.04	9.0±0.06
15.	IC97182	19.6± 0.49	45.0± 0.55	5.20± 0.20	5.0±0.19
16.	IC97189	58.1± 0.90	33.9± 0.30	9.40± 0.11	5.8±0.07
17.	IC97295	21.0± 0.40	35.5± 0.54	8.60± 0.10	2.6±0.03
18.	IC120148	27.2± 0.19	26.1± 0.41	12.4± 0.28	3.6±0.08
19.	IC120150	59.2± 1.09	26.2± 0.27	27.6± 0.63	5.2±0.12
20.	IC120158	50.7± 0.60	32.4± 0.14	11.2± 0.13	28.8± 0.34
21.	IC120159	60.0± 0.41	54.0± 0.58	25.4± 0.48	28.4± 0.53
22.	IC120160	57.5± 0.59	46.4± 1.03	13.4± 0.17	4.8±± 0.06
23.	IC120163	55.7± 0.79	32.1± 0.51	6.8± 0.05	5.2±0.04
24.	IC120164	35.8± 0.59	25.9± 0.42	7.0± 0.11	4.8±0.08
25.	IC120165	37.9± 0.27	20.7± 0.48	6.0± 0.04	5.2±0.04
26.	IC120166	23.2± 0.50	50.8± 0.30	4.6± 0.18	15.0±0.58
27.	IC120175	29.8± 0.61	52.7± 0.30	5.4± 0.12	14.8±0.32
28.	IC120177	23.9± 0.20	40.7± 0.40	4.0± 0.05	18.2±0.21
29.	IC120179	48.4± 0.48	48.8± 0.32	6.0± 0.08	11.6± 0.13
30.	IC120191	20.2± 0.70	31.0± 0.44	4.6± 0.03	18.2±0.12
31.	IC120192	56.7± 0.66	29.0± 0.53	6.4± 0.25	22.2±0.86
32.	IC120200	15.9 ± 0.31	43.8± 0.55	9.0± 0.11	24.6±0.29
33.	IC120201	19.7± 0.40	42.8± 0.30	8.2± 0.09	22.6±0.25
34.	IC120204	30.3± 0.57	42.1± 0.35	4.0± 0.09	21.4±0.49
35.	IC120207	18.7± 0.35	48.5± 0.53	3.0± 0.07	22.6±0.51
36.	IC120208	16.8± 0.22	26.0± 0.17	1.8± 0.02	6.60±0.08
37.	IC120210	21.6± 0.51	41.0± 0.17	2.4± 0.05	0.40±0.08
38.	IC120212	43.9± 0.25	42.8± 0.37	2.0± 0.03	19.8±0.25
39.	IC120213	48.9± 0.57	49.8± 0.34	6.4± 0.03	15.6± 0.11
40.	IC120221	42.7± 0.36	37.4± 1.02	6.8± 0.11	14.5± 0.24
41.	IC120228	52.3± 0.14	31.1± 0.38	5.0± 0.04	14.8± 0.11
42.	IC120239	59.8± 0.34	27.7± 0.44	12.6± 0.49	13.0±0.51

Table 1 : Contd…………….

Table 1 : Contd……………..

43.	IC120251	12.8± 0.58	28.7± 0.40	26.8± 0.57	12.8±0.27
44.	IC120234	12.8± 0.61	28.8± 0.40	18.0± 0.20	15.6± 0.18
45.	IC120235	57.8± 0.40	46.1± 0.14	17.4± 0.20	14.8±0.17
46.	IC120244	36.8± 0.48	20.4± 0.41	24.7± 0.17	18.6±0.13
47.	IC120250	27.7± 0.45	47.9± 0.20	27.4± 1.06	17.6±0.68
48.	IC120255	25.6± 0.56	12.2± 0.24	15.2± 0.18	13.2±0.15
49.	IC120346	18.8± 0.41	24.9± 0.42	7.20± 0.08	14.8±0.17
50.	IC120355	56.8± 0.26	13.6± 0.99	3.40± 0.08	17.2±0.39
51.	IC120394	22.3± 0.47	46.6± 0.56	25.2± 0.57	22.8±0.52
52.	IC120404	19.9±0.17	26.1±0.81	19.6±0.23	8.8±0.10
53.	IC120406	24.8 ±0.72	46.9±1.68	20.0±0.38	5.8±0.11
54.	IC120407	11.8±0.56	48.9±0.68	17.0±0.22	9.2±0.12
55.	IC120408	25.5±0.54	12.8±0.25	7.6±0.05	7.2±0.05
56.	IC200125	47.8±0.17	28.4±0.53	27.2±0.44	5.4±0.09
57.	IC200125	16.0±0.26	47.8±0.38	22.8±0.17	4.2±0.03
58.	IC344224	50.0±0.62	25.9±0.17	2.4±0.09	13.8±0.54
59.	IC344225	9.7±0.56	27.9±0.16	6.4±0.14	4.8±0.10
60.	IC372606	29.9±0.20	8.4±0.89	2.8±0.03	9.0±0.10
61.	IC403579	56.6±0.48	24.8±0.57	2.6±0.03	6.2±0.07
62.	IC480117	15.9±0.56	7.14±0.65	4.8±0.03	5.8±0.04
63.	LEPAKSHI	20.5±0.72	15.6±0.35	3.2±0.12	8.2±0.32
64.	PRASAD	39.6±0.32	43.7±0.94	1.2±0.01	7.2±0.08
65.	AKL	16.8±0.34	40.6±0.45	1.6±0.01	6.8±0.08
66.	JLG	10.9±0.20	48.7±0.43	3.4±0.08	6.6±0.15
General man		31.1	35.3	10.9	10.81
S.E. (Mean)		0.29	0.51	0.52	0.655
C.D.at (P<0.005)		1.07	2.46	1.47	1.835
F-test		SIG.	SIG.	SIG.	SIG.

millet accessions was found to be 0.40mg/kg to 28.8mg/kg. The accession IC120158 has the copper content (28.8mg/kg) followed by IC120159 (28.4mg/kg), IC120200 (24.58mg/kg), IC120394 (22.86mg/kg), IC120207 (22.60mg/kg). Therefore, these accessions are useful to fetch the copper deficient regions of globe where the foxtail millet is major staple food crop the people. The mean copper content is to a great extent higher than the checks Lepakshi (8.20mg/kg), Prasad (7.20mg/kg) as shown in (Table 1 and Fig. 1).

The average grain manganese content is 10.88mg/kg and it ranges from 1.2 mg/kg to 29.8 mg/kg. The maximum grain manganese content was found in IC97174 (29.8mg/kg) followed by IC120150 (27.60mg/kg), IC120250 (27.40mg/kg), IC200125 (27.2mg/kg), IC120251 (26.8mg/kg), IC120159 (25.4mg/kg) as shown in (Table 1 and Fig. 1).

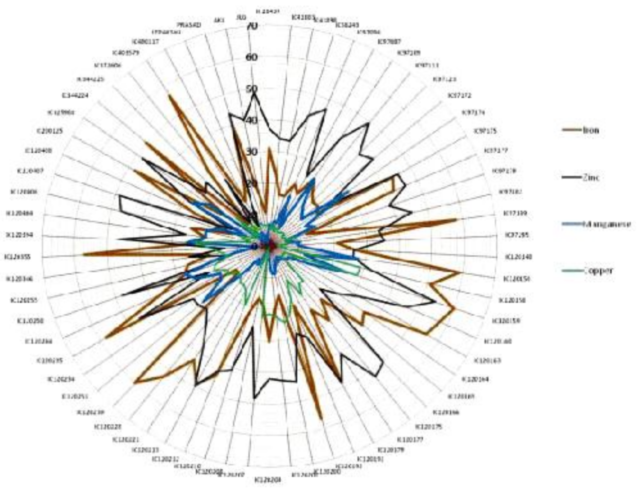

Fig. 1: **Graph showing the micronutrient (Fe, Mn, Zn and Cu) in foxtail millet accessions (mg/kg)**

Selection of promising high iron containing accessions for differential gene expression:

Expression profiling of flag leaf molecular analysis revealed differential expression of metal homeostasis related candidate genes among accessions and flag leaf tissues at mid grain filling stage. Four accessions selected for differential gene expression studies for iron homeostasis related candidate genes expressed in flag leaf. Out of four accessions three are high iron containing accessions and one is lowest iron containing accession as mentioned in (Table 2). Flag leaf total RNA analysis of these four accessions revealed that the regulation of seven foxtail millet metal transporter genes belonging to the ferritin, ZIP, YSL, His- rich, NAC families with respect to environmental and genetic factors influencing iron uptake.

Table 2 : Selected accessions for differential gene expression studies

Sr. No.	Accession	Iron content (ppm)
1.	IC120235	57.81
2.	IC120355	56.82
3.	IC344225	9.69
4.	IC120239	59.77

Spatial expression of different genes for iron homeostasis :

cDNA gene specific primers were used for the differential gene expression profiling and different genes known for mineral homeostasis have been used in the present investigation to see the polymorphism nature of expression. The size of amplicons found to be increased with increased iron accumulation and amplicons corresponding to each genes were described below:

Sipf40:

Our results showed that the Sipf40 expressed only in high iron containing accessions with amplicon size (708bp) and found to be not expressed in accession (IC344225) which has comparatively low iron content (9.69ppm) (Fig. 2A).

IRT proteins:

IRT1 is a member of the ZIP (ZRT IRT-like Protein) family (Maser *et al.,* 2001). IRT gene was expressed uniformly in flag leaf tissues of both high and low iron containing accessions. Our results indicate the conserved nature of IRT family proteins in foxtail millet. The size of amplicon in all accessions was found to be 180bp (Fig. 2A).

YSL9 an YSL super family protein :

Similarly, YSL9 an YSL super family protein was found to be poorly expressed among all the accessions under study. The differential expression of YSL9 gene

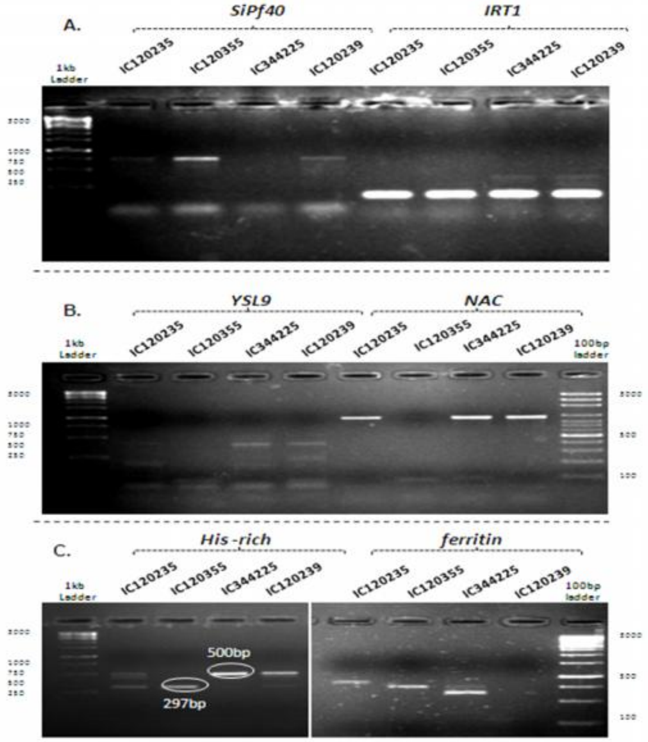

Plate : Flag leaf transcriptome analysis using gene specific primers I- *Sipf40, and IRT1,* II- *YSL9, and NAC* III- *His-rich, and ferritin His-rich* expressed differentially in both high and low iron containing accessions showing two different alleles of sizes (297bp) in high iron containing accession and (500bp) common in both high and low iron containing accessions

Fig. 2: Flag leaf transcriptome analysis using gene specific primers

Table 3 : Differential gene expression of 8 iron homeostasis related candidate genes

Sr. No.	Expression level	Genes	Total
1.	High expression	*NAC, Ferritin, IRT, His-rich*	4
2.	Genotypic variation	*His-rich, ferritin, YSL, Ferritin*	4
3.	Poor expression	*YSL, Sipf40*	2
4.	No expression	*Wrky, NRAMP*	2

in all accessions doesn't give any conclusion (Fig. 2B). The differential gene expression of iron related candidate genes mentioned in (Table 3). Hence, during grain filling stage number of genes expressed at different level of gene expression. Some are uniformly expressed (IRT, NAC) some are differentially expressed (His-rich, ferritin). Some are shows high level of expression (IRT, NAC, Ferritin, His-rich) with high genotypic variation and some are shows poor expression (Table 3).

NAC domain containing proteins :

Expression of NAC gene in both high and low mineral containing foxtail millet accessions except accession (IC120355), were in it was not expressed at all . The size of amplicons in all high and low iron containing accessions observed to be (989bp) (Fig. 2B). Our results of NAC gene do not seem to play an important role in mineral homeostasis in foxtail millet.

Histidine and cystein rich proteins :

His-rich domains have multiple binding sites providing attachment to the bivalent ions. In present investigation two amplicons of different sizes of his-rich proteins were found to be expressed in both high and low iron containing foxtail millet accessions. One 297bp is found to be differentially expressed in high iron containing accession (IC120355). Other high molecular weight (500bp) His-rich protein gene was found to be expressed in both high and low iron containing accessions (Fig. 2C).

Ferritin gene family :

Ferritin expression was found to differ with varying iron content, different size isoforms were found to be expressed in different mineral containing accessions. The splicing variants of different sizes were obtained during present investigation. The high molecular weight amplicon of size (880bp) was found in IC120235 (57.81ppm) followed by (747bp) in IC120355 (56.82ppm) and (625bp) found in IC344225 which lowest iron containing accession (9.69ppm). Further sequence characterization will be helpful in identifying exact molecular reason for splicing variants (Fig. 2C).

In silico prediction of his rich protein :

BLAST results indicate sequence similarity with Setaria italica Histidine rich protein RAR1 like protein with query coverage of 53 per cent and E-value 2e-76 and 100 per cent identity. Protein BLAST shows significant sequence similarity with cysteine and histidine-rich domain-containing protein RAR1-like (*Setaria italica*).

The proportions of zinc in kodo millet and foxtail millet was reported as 19.60mg/kg and 40.4mg/kg respectively (Banerjee and Chandel 2011). Foxtail millet showed the highest zinc content among all followed by Barnyard millet, little millet and Finger millet. Minor millets are significantly differs from each other for zinc content and foxtail millet content highest zinc among all small millets. In finger millet the zinc content is reported as 15mg/kg (Iren, 2004). Astonishingly, in present investigation we found the range of 7.14mg/kg to 53.98mg/kg for grain zinc content among the sixty six accessions of foxtail millet which is significantly high as compare to all reported data by various researchers. Similar results were obtained by (Velu *et al.*, 2007). He characterized the hybrids and some germplasm lines of pearl millet for grain micronutrient content. According to (Velu *et al.*, 2007) the deviation among the germplasm was recorded for grain micronutrient (for Fe 30.1mg/kg-75.7mg/kg and zinc 24.5-64.8mg/kg on dry weight basis) content at ICRISAT, India

Copper is important trace element in plant system and reported in very minute concentrations among the millets. In pearl millet the copper content is 6.2mg/kg, finger millet 0.3mg/kg, Proso millet 8.3mg/kg, foxtail millet 9.2mg/kg and in sorghum it is reported as much as 10.8mg/kg (Iren, 2004). Our results reflected that the values are more significant than the previous data reported by (Iren, 2004) the grain Mn content of pearl millet is 18.0mg/kg, finger millet 7.5mg/kg, Proso millet 21.9mg/kg and in sorghum it is 16.3mg/kg.

Transgenic tobacco plants over expressing ferritin either in the cytoplasm or in the plastids were generated iron over accumulation. A major consequence of ferritin accumulation in transgenic tobacco was the increased leaf iron concentration and root ferric reductase activity, a key step in dicotyledonous plant iron uptake (Holden *et al.,* 1992) and reported that over expression of ferritin in tobacco increases 3 folds of leaf iron accumulation. However, in present study the high molecular weight ferritin amplicon was found to be expressed in high iron accumulating accessions. Thus, the size of amplicons found to be increased with increased iron accumulation. The barley and wheat encoded proteins have more than

13 His-rich domains, whereas the putative rice orthologue has only 5 his-rich regions. His-rich motifs are well established metal binding motifs, therefore, we selected the His-rich proteins for molecular docking to study the protein ligand docking of iron as a ligand in his rich protein active site.

NAC family proteins play important role iron homeostasis in rice as reported by Chandel *et al*. (2011). Similar, findings reported in sorghum by Anuradha *et al*. (2012). NAC constitutes a plant- specific transcription factor family with a highly conserved N-terminal DNA-binding domain. Some NAC proteins have been implicated in developmental programs, metal homeostasis, drought stress tolerance, and pathogenesis stress (Uauy *et al*., 2006). IRT protein is major root Fe transporter in Arabidopsis as reported by (Vert *et al.*, 2002. Orthologs of IRT1 have also been characterized in tomato and rice; the mRNAs of both genes, like IRT1, accumulate in Fe-deficient roots (Eckhardt *et al*., 2001). Although, IRT1 is able to mediate the transport of multiple metals in yeast, including Fe, Mn, Zn and Cd, (Eide *et al*., 1998). Similar results were obtained by Chandel *et al.* (2011). IRT gene was expressed in roots in mid grain fill stage of rice grain development. Whereas, our study indicate that the IRT was also expressed in flag leaf but the expression was found in both high and low iron containing accessions. Hence, some regulatory proteins or transcription factors may express in flag leaf tissues along with IRT proteins and involved in iron homeostasis. However, some researchers reported that YSL family proteins are expressed in both strategy-I and strategy-II plants and transports the iron across the plasma membrane. At the tissue level, OsYSL2 is expressed in the companion cells of the phloem suggesting a role for OsYSL2 is the transport of Fe and Mn in the phloem (Koike *et al.*, 2004).

Aizat *et al.* (2011) was identified a gene from barley encoding a cell wall protein with multiple His-rich motifs interspersed with short arabinogalactan protein (AGP) domains and called it Hordium vulgare His-rich AGP (HvHRA1). Sequence analysis shows that His-rich AGPs are very rare in plants and that the number of His-rich and AGP domains differ between cereals and dicots. His-rich motifs are well established metal binding motifs; therefore they developed transgenic rice plants that constitutively over express barley HvHRA. Micronutrient analysis of brown and white rice showed that the grain nutrient yield for Fe, Zn and Cu was higher in two transform lines compare to their respective nulls. They investigate that His-rich arabinogalactan protein for micronutrient biofortification of cereal grain and reported 3-folds increased iron content by transferring the HvAGP (Hordium vulgare) into rice.

Hence, present study will found to be helpful to study the mineral sequestration in crop plants. The data is useful for breeders to establish suitable breeding programme for nutritional improvement of foxtail millet accessions.

Conclusion:

Due to richness of important micronutrients like iron and zinc along with previously discovered health benefits, minor millets can be one of the magic cereal foods to combat micronutrient malnutrition. Based on the finding of this study, it can be concluded that foxtail millet is superior cereal grain with good nutrient profile and hence will be worthy addition to one's diet. Enhancing micronutrient in the diet by using minor millet cereal foods in the diet to ensure adequate attainment of iron and zinc seems to be most suitable strategy to combat micronutrient malnutrition. Additionally, owing to their high nutritive value, foxtail millets can also serve as source of new genes or more valuable alleles of existing genes, which can be further utilized by transgenic program to improve micronutrient content in the dominated foods.

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