Stage Specific Comparative Transcriptomic Analysis To Reveal Gene Networks Regulating Iron And Zinc Content In Pearl Millet [*Pennisetum glaucum* (L.) R. Br.]

C. Tara Satyavathi (✉ csatyavathi@gmail.com)

ICAR-AICRP on Pearl Millet, Agriculture University

Rukam S. Tomar

Junagadh Agricultural University

Supriya Ambawat

ICAR-AICRP on Pearl Millet, Agriculture University

Jasminkumar Kheni

Junagadh Agricultural University

Shital M. Padhiyar

Junagadh Agricultural University

Hiralben Desai

Junagadh Agricultural University

S. B. Bhatt

Junagadh Agricultural University

M. S. Shitap

Junagadh Agricultural University

Ramesh Chand Meena

ICAR-AICRP on Pearl Millet, Agriculture University

Tripti Singhal

Indian Agricultural Research Institute, ICAR

S. Mukesh Sankar

Indian Agricultural Research Institute, ICAR

S. P. Singh

Indian Agricultural Research Institute, ICAR

Vikas Khandelwal

ICAR-AICRP on Pearl Millet, Agriculture University

Research Article

Keywords: Pearl millet, Malnutrition, Iron, Zinc, Metabolic pathways, EST-SSR, Transcriptomics

Posted Date: September 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-903625/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Version of Record: A version of this preprint was published at Scientific Reports on January 7th, 2022. See the published version at <https://doi.org/10.1038/s41598-021-04388-0>.

Abstract

Pearl millet is an important staple food crop of poor people which is rich in micronutrients like iron and zinc and amenable for focused breeding for these micronutrients along with high yield. Transcriptome sequencing using ION S5 Next Generation Sequencer generated 43.5 million sequence reads resulting in 83,721 transcripts with N₅₀ of 597 bp and 84.35% of transcripts matched with the pearl millet genome assembly. The genotypes having high Fe and Zn showed differential gene expression during different stages. Of which, 155 were up-regulated and 251 were down-regulated while during flowering stage and milking stage 349 and 378 transcripts were differentially expressed, respectively. Gene annotation and GO term showed the presence of transcripts involved in metabolic activities associated with uptake and transport of iron and zinc. In the present study, the 83,721 transcripts were also examined for identification of SSRs. A total of 4,327 SSRs were identified with dominance of tri-nucleotide SSRs in comparison to di-nucleotide SSRs. These EST-SSRs can be used in molecular breeding, genetic diversity analysis and determination of heterozygosity of the allelic loci. Information generated will help in gaining insights into iron and zinc metabolism and develop genotypes with high yield, grain iron and zinc content.

1. Introduction

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a highly nutritious and multipurpose cereal crop primarily cultivated on more than 27 million ha in the arid and semi-arid tropical regions of Africa and Asia. It is a chief source of dietary energy for the rural people in these areas. It is well adapted to the adverse conditions like drought, high salinity, low pH, low soil fertility and high temperature making it an excellent climate-resilient crop and put forward good security against crop failure in developing countries [1,2]. It is nutritionally better than rice and wheat due to presence of higher quantity of proteins, dietary fibers, iron, zinc, phosphorus, calcium, potassium, vitamin B and essential amino acids and delivers 80–90% of the calories to several millions of poor people across the world [3]. It supplies 19 to 63% of the total Fe intake and 16 to 56% of the total Zn intake from pearl millet based foods in Maharashtra, Gujarat and Rajasthan states of India [4]. In comparison to other cereals and vegetables, it is cheaper and more economical source of Fe and Zn. It has enormous genetic variability for the key mineral elements- iron and zinc when compared to other cereal crops [5].

Nutritional security is a key challenge for the growing population of the world which is primarily due to micronutrient deficient cereal based diet. Iron (Fe) and zinc (Zn) deficiencies are among the two most common and prevalent micronutrient deficiencies (MNDs) leading to ill health, high mortality, lower work productivity, learning disabilities in children and meager national economic development [6,7]. Among the 26 foremost risk aspects of the world, Fe deficiency ranks 9th and Zn deficiency ranks 11th [8]. This is a major issue mainly in developing countries, particularly for infants, adolescent children and pregnant women. In India, approximately 80% of pregnant women, 52% of non-pregnant women and 74% of children of 6–35 months suffer from anaemia caused due to deficiency of Fe [9]. Zinc (Zn) deficiency leads to diarrhoea, stunted physical growth and repressed immune system affecting around 50% of the world population [10]. More than three billion people are affected by MNDs of essential minerals and vitamins globally [11]. Breeding and developing crop varieties rich in these elements through biofortification is usually considered as a cost-effective approach to eliminate Fe and Zn deficiencies [12].

Pearl millet exhibits higher Fe density in comparison to the other major cereals and '*iniadi*' pearl millet germplasm is the main basis of breeding lines, improved populations, hybrid parents and composites containing high Zn and Fe densities. Research on pearl millet revealed that it has huge genetic variation for Zn and Fe densities with majorly additive gene action with better parent heterosis. This enormous genetic variation (30–140 mg/kg Fe and 20–90 mg/kg Zn) can be efficiently exploited for developing high yielding cultivars with enhanced Zn and Fe densities [1,2,13,14]. Pearl millet also possesses high bioavailability (absorption) of iron which can fulfill > 50% daily requirement of children or adult males. Approximately 50–100% of the daily allowance of iron is provided by one meal of biofortified high iron variety of pearl millet and proved helpful in eliminating iron deficiency in women, men and children [15].

Morphological and physiological responses are controlled by numerous genes and significantly prejudiced by the environment. Transcriptomics and metabolomics can prove useful to identify gene function and increase production of important compounds in pearl millet by understanding gene-to-metabolite pathways [16]. Development of next-generation sequencing has provided the opportunity for high-throughput sequencing and used as a valuable tool with vast potential and applications in plant biology including genome sequencing and transcriptome analysis [17]. The next-generation sequencing (NGS) technology is a boon as it overcomes the different inadequacies of microarrays making it most preferable method of transcriptome profiling in recent years.

Transcriptomic studies in pearl millet have been used to reveal the functions of salinity and heat stress-responsive genes such as PgDREB2A, PgNHX1, PgDHN, PgVDAC, PgHsp 16.97 [18–23]. A comprehensive transcriptome analysis for drought stress response has also been carried out in pearl millet [24]. The molecular mechanisms underlying salinity tolerance, physiological analyses and a comparative transcriptome analysis were revealed using salinity tolerant (ICMB 01222) and salinity susceptible (ICMB 081) lines under control and salinity conditions and around 11,627 differentially expressed genes (DEGs) were reported in both lines [25]. In another study, transcriptome sequencing was carried out to identify 'molecular portraits' in floral buds in cotton among the Cytoplasmic Genic Male Sterility (CGMS) line, its near-isogenic maintainer, and restorer lines. A total of 300, 438 and 455 genes were found to be differentially expressed in CGMS, Maintainer, and Restorer lines respectively [26].

In the present experiment, comparative transcriptome profiling of stage-specific spikes from pearl millet with high and low Fe and Zn was carried out to know about the genes that are expressed during developing spike and the transcripts whose expression is associated with Fe and Zn. Nowadays marker assisted selection (MAS) has become an essential tool for conventional breeding to achieve rapid genetic gain. Microsatellite markers are genomic markers, used extensively in many genetic studies. EST-SSRs were more conserved during evolution than non-coding sequences and are more

transferable than genomic SSRs. Transcriptome sequencing is a cheaper and faster approach to develop EST-SSRs when compared to other methods. Developing expressed sequence tags (ESTs) and genomic sequence information can be used to study polymorphism among the genotypes and improve the breeding efforts in pearl millet. This study provides valuable candidate genes that can be genetically targeted for yield along with improvement for Fe and Zn content in pearl millet.

2. Material And Methods

The methods adopted in this study are relevant and according to institutional, national and international guidelines and legislation.

2.1 Plant material and growth conditions

This experiment was conducted at PC Unit, ICAR-AICRP on Pearl Millet, Agriculture University, Jodhpur during *kharif* 2018. A total of four inbred lines viz. PPMI 953, PPMI 1108, PPMI 627, 5540 B were sown in field conditions following recommended agricultural practices. The four inbred lines were grouped in two categories- high and low, based on Fe and Zn content in the grains of the genotypes as measured by Atomic Absorption Spectrophotometer (AAS). Out of the four inbred lines, the two lines namely PPMI 953 and PPMI 1108 were having high Fe & Zn content and the other two viz., PPMI 627 and 5540 B were having low Fe & Zn content and were considered as control. The samples were collected at three stages *i.e.*, panicle initiation [28 Days After Sowing (DAS)], flowering stage (36 DAS) and milking stage (52 DAS) in 3 replicates for transcriptomic analysis. The samples were stored in RNeasy Lysis Buffer (Qiagen) solution in 15 ml tubes under sterilized conditions at 4°C in a freezer for later use. The transcriptome sequencing and analysis was carried out at Department of Biotechnology, Junagadh Agriculture University, Junagadh, Gujarat.

2.2 RNA extraction and high-throughput sequencing

Total RNA was extracted from each replicate of the 12 samples (Table 1) using RNeasy Plant Mini Kit (*Qiagen*). The quality of RNA was analyzed on 1.2% agarose gel electrophoresis and concentration was measured using a Qubit RNA HS Assay Kit (Invitrogen) along with the Qubit 2.0 Fluorometer (Invitrogen). mRNA was extracted using Dynabeads mRNA DIRECT Kit (ThermoFisher Scientific) from ~ 1 µg of total RNA input. After purification, mRNA was fragmented by RNase enzyme at 37°C in RNAase buffer provided in cDNA library preparation kit *Total RNA-Seq Kit v2* (ThermoFisher Scientific). These fragmented mRNA were hybridized and ligated with adapters followed by SPRI cleanup (Solid Phase Reversible Immobilization). The fragments were reverse transcribed using random hexamers and superscript II reverse transcriptase (Life Technologies) and further cleaned using Beckman colter agencourt ampure XP SPRI beads. The cDNA library was amplified using PCR for the enrichment of the adapter-ligated fragments. The individual libraries were measured using a Qubit 2.0 Fluorometer and validated for quality in E-Gel 2% Agarose (Invitrogen). Subsequently, these libraries of each sample were diluted up to 100pM concentration and subjected to emulsion PCR (Ion One Touch 2, Life Technologies). Later, enriched template positive ion sphere particles were loaded on 540 chip and were sequenced in ION S5 next generation sequencer platform (Life technologies).

Table 1
Sample details and concentration of Iron (Fe) and Zinc (Zn) in the grains of pearl millet genotypes

S. No.	Sample ID	Genotypes	Description	Fe (ppm)	Zn (ppm)
High Fe and Zn containing Pearl millet Genotype (HFZPG)					
1	1A	PPMI 953	Panicle initiation	103.23	77.53
2	1B	PPMI 953	Flowering stage		
3	1C	PPMI 953	Milking stage		
4	2A	PPMI 1108	Panicle initiation	93.71	56.95
5	2B	PPMI 1108	Flowering stage		
6	2C	PPMI 1108	Milking stage		
Mean				98.47	67.24
SD				5.68	11.80
Low Fe and Zn containing Pearl millet Genotype (LFZPG)					
7	3A	PPMI 627	Panicle initiation	50.33	30.51
8	3B	PPMI 627	Flowering stage		
9	3C	PPMI 627	Milking stage		
10	4A	5540 B	Panicle initiation	32.31	26.62
11	4B	5540 B	Flowering stage		
12	4C	5540 B	Milking stage		
Mean				41.32	28.57
SD				9.96	2.32
<i>t-test</i> significance (between H & L) at $P < 0.01$ <i>P</i> value				0.00000024	0.000013

2.3 Preprocessing of RNA-Seq data and transcriptome profile analysis

Initially, low-quality sequences were removed from the raw reads from all the individual data files. The reads having > 50% bases with low-quality scores and/or > 10% bases unknown (N bases) were removed from each raw data for accuracy of results using CLC genomics tool kit [27].

2.4 *De novo* transcriptome assembly, read mapping and sequence annotation

The reads were processed for sequencing adapter removal, further trimmed for quality and ambiguity. The *de novo* assembly was conducted using default parameters in the CLC Genomics workbench 20.01 (CLC bio, Aarhus, Denmark) to develop contigs/transcripts for all the samples. The transcript reads were further matched to the draft genome of pearl millet [28] to find out the percentage match. BlastX of assembled transcripts was done with the close relative of pearl millet i.e. *Foxtail millet* protein database with an e-value cut-off of $1E-6$, as the protein database of pearl millet was not available. Sequence annotation was carried out with the software BLAST2GO. Based on these results, gene ontology (GO) terms were assigned to the sequences [29].

2.5 Differential Gene Expression Analysis

Two groups were generated by combining the reads of High Fe and Zn containing Pearl millet Genotype (HFZPG) and Low Fe and Zn containing Pearl millet Genotype (LFZPG). For HFZPG, the reads of sample number 1A, 1B, 1C, 2A, 2B and 2C were combined and for LFZPG the reads of sample number 3A, 3B, 3C, 4A, 4B and 4C were combined. The reads were aligned back to the assembled main assembly to quantify the abundance of transcripts assembled and to determine the number of reads and reads per kilo million (rpkm) corresponding to each transcript. CLC RNA-Seq analysis was used for aligning the reads of each sample onto the assembled transcripts [30]. Assembler provides the annotated transcripts and their annotated length and coverage values for each sample. The resulting files of aligned reads were input in Differential Expression (DE) analysis, a tool for quantifying the abundances of a set of target sequences from sampled subsequence based on a model (GLM model) using the negative binomial distribution [31]. Gene expression levels were estimated by RPKM and FDR P values. Genes with RPKM fold changes > 2 or < -2, and FDR-corrected p values < 0.05 were regarded as Differential Expressed Gene's (DEGs). DEGs transcripts were annotated for pathway analysis and analyzed in MapMan tool for data visualization [32,33].

2.6 SSR discovery in transcripts

The assembled transcripts were screened using MicroSatellite (MISA) identification tool and SSRs were identified [34] based on unit size or minimum number of repeats (2–6, 3–5, 4–4, 5–3 and 6–3) and the maximum number of bases interrupting two SSRs in a compound microsatellite = 100. The SSR containing sequences were separated by in-house Perl script.

3. Results

3.1 Identification of Fe and Zn concentration

The Fe and Zn concentration in the grains of four pearl millet genotypes PPMI 953, PPMI 1108, PPMI 627 and 5540 B was analyzed by atomic absorption spectrophotometer. Fe concentration in high Fe containing genotypes PPMI 953 and PPMI 1108 was found to be 103.23 ppm and 93.71 ppm respectively which was significantly higher than low Fe containing genotype PPMI 627 (50.33 ppm) and 5540 B (32.31 ppm). Similarly, the Zn concentration in PPMI 953, PPMI 1108, PPMI 627 and 5540 B was found to be 77.53 ppm, 56.95 ppm, 30.51 ppm and 26.62 ppm, respectively (Table 1). A significant genetic variation in Fe and Zn concentration was found among high and low Fe genotypes. The iron concentration in the genotype PPMI 953 is sufficient enough to fulfill the desired iron concentration of daily human need [35]. Ample work has been carried to enhance iron and zinc content in pearl millet [36–43], however little or negligible work has been carried out to understand the mechanism or pathways involved in high Fe and Zn containing genotypes.

3.2 RNA sequencing and assembly

The samples for RNA-sequencing were collected from four pearl millet genotypes (two genotypes for high Fe and Zn, while two for low Fe and Zn) at three different stages *i.e.* panicle initiation stage, flowering stage and milking stage. The samples were collected in three replicates for each stage. The total RNA was isolated from each replicate and were pooled before sequencing to make a total of 12 samples. The sequencing was carried out in Ion S5 next generation sequencing platform with a chemistry of 200 bp. The total number of raw reads varied from approximately 9–20 million reads for the samples of different stages. The raw reads were further subjected to adaptor trimming and bases quality check. The percentage of high quality (HQ) reads varied from 76.6–91.44% and the number of HQ reads varied from ~ 7 to 15 million (Table 2). Mapping of HQ reads with draft pearl millet genome showed 82.39–86.71% of reads mapped. Further, the HQ reads of all the 12 samples were subjected to master assembly by combining all the reads of all the samples. A total of 83,721 contigs were generated with an average contig length of 519 bp and N₅₀ of 597 bp (Table 3).

Table 2
Reads, de-novo assembly and mapping statistics of 2 high and 2 low-Fe & Zn containing genotypes of pearl millet

Sr. No	Sample ID	Genotypes	Stages	Total number of raw reads	Total number of high quality reads & percentage after quality control	Percentage of aligned reads with genes of pearl millet <i>de novo</i> assembly
High Fe and Zn containing Pearl millet Genotype (HFZPG)						
1	1A	PPMI 953	Panicle initiation	14,765,667	12,689,248 (84.94%)	85.22
2	1B	PPMI 953	Flowering	13,000,597	11,118,391 (86.06%)	83.92
3	1C	PPMI 953	Milking	10,124,113	7,775,336 (76.6%)	82.39
4	2A	PPMI 1108	Panicle initiation	17,116,512	14,773,081 (86.06%)	84.15
5	2B	PPMI 1108	Flowering	13,456,265	11,534,461 (85.72%)	85.74
6	2C	PPMI 1108	Milking	9,961,928	7,996,499 (80.27%)	82.62
Low Fe and Zn containing Pearl millet Genotype (LFZPG)						
7	3A	PPMI 627	Panicle initiation	14,296,802	12,102,565 (84.65%)	86.71
8	3B	PPMI 627	Flowering	17,410,659	14,959,020 (85.92%)	84.48
9	3C	PPMI 627	Milking	11,035,612	8,60,2071 (77.95%)	83.52
10	4A	5540 B	Panicle initiation	12,865,870	10,878,273 (84.55%)	85.64
11	4B	5540 B	Flowering	20,433,511	15,922,075 (91.44%)	85.13
12	4C	5540 B	Milking	12,289,745	10872938 (88.47%)	82.79

Table 3
de Novo assembly statistics of
master assembly

Features	Contig length
N ₇₅	353 bp
N ₅₀	597 bp
N ₂₅	1,204 bp
Minimum	200 bp
Maximum	7,492 bp
Average	519 bp
Count	83,721
Total	43,459,438 bp

3.3 Differentially Expressed genes in High and Low Fe and Zn genotypes

Differentially Expressed Genes (DEGs) were defined as the fold change of the normalized (RPKM) expression values of at least 2 transcripts/contigs in both direction of $\log_2$ ratio ≥ 1 and p value ≤ 0.05 . During panicle initiation stage, a total of 406 transcripts were differentially expressed of which 155 were up-regulated and 251 were down-regulated in high Fe and Zn containing genotypes in comparison to low Fe and Zn containing genotypes. During flowering stage, 349 transcripts were differentially expressed of which 227 were up-regulated and 122 were down regulated. Similarly, during milking stage, 184 transcripts were up-regulated and 194 transcripts were down-regulated in HFZPG in comparison to LFZPG (Table 4). The GO terms with their gene description and other functional activities of all the up and down regulated transcripts have been described in Supplementary Table S1 to S6. Among the differentially expressed genes, 62 transcripts were expressed during all the three stages of panicle development, while 239 transcripts were specifically expressed during panicle initiation and 163 and 261 transcripts were specifically expressed during flowering and milking stage, respectively (Fig. 1A). During panicle initiation and milking stage, 18 transcripts were expressed commonly, while during panicle and flowering stage 87 transcripts were expressed commonly during both the stage.

Table 4
Differentially Expressed Genes (DEGs) in pearl millet genotypes
during different stages of panicle developmental and in High Fe and
Zn containing pearl millet genotypes.

Stages	Total	Up-regulated	Down-regulated
1 Panicle Initiation	406	155	251
2 Flowering	349	227	122
3 Milking	378	184	194
Total	1,133	566	567

When the up-regulated genes were characterized, it was found that there were 32 transcripts which were up-regulated during all the three stages of panicle development. However, 68 transcripts up-regulated only during panicle initiation stage and 120 and 122 transcripts up-regulated only during flowering and milking stages (Fig. 1B). While among the down-regulated genes, 30 transcripts were down-regulated during all the three stages of panicle development (Fig. 1C).

3.4 Functional annotation of DEGs

To identify the putative function, pearl millet's differentially expressed genes were compared against the *Foxtail millet* protein database using BLASTx search. Only 1,133 transcripts of HFZPG which includes 566 up-regulated and 567 down-regulated were used for functional annotation and assigning GO-based classification (Fig. 2). Cellular components (CC) GO terms *i.e.* plasma membrane, intracellular, cell periphery, protein-containing complex, transcription factor complex, membrane-bounded organelle, organelle membrane, cytoplasm, membrane, cytosol, extracellular region, apoplast, membrane, etc. were found to be enriched, indicating their participation in the uptake of micronutrients. Biological process (BP) GO terms likewise oxidation-reduction process, regulation of transcript, macromolecule localization, establishment of localization, metabolic process, organic substance metabolic process, biosynthetic process, small molecule metabolic process *etc.* were associated with up-regulated genes. The presence of nucleoside-triphosphate phosphatase as up regulated enzyme has been reported to be involved in different metal ions accumulating metabolic process [44]. Molecular functions (MF) GO terms like ion binding, small molecule binding, amide binding, lipid binding, transporter activity, transferase activity, transmembrane transporter activity, etc. were highly enriched in up-regulated genes. The further classification of ion binding components of MF indicated

up-regulation of metal binding, zinc ion binding, iron ion binding, calcium ion binding, etc, which may have contributed significantly in the uptake and transport of iron, zinc and other metals (Fig. 3). Fe involved in chlorophyll synthesis, contributing to the maintenance of chloroplast structure and its function [45]. The presence of metal ion binding, iron ion binding, zinc ion binding and calcium ion binding in HFZPG genotypes indicates their role in accumulation of Fe and Zn in pearl millet genotypes (Fig. 3 and supplementary Table S7).

3.5 Pathway analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [46–48] were analyzed to find out the differences in metabolic processes between HFZPG and LFZPG genotypes (Fig. 4). Pathway results infer that in HFZPG, starch/sucrose metabolism and glycolysis/gluconeogenesis pathways were highly enriched as compared to other pathways in LFZPG. Pathways like amino sugar & nucleotide sugar and fixation of photosynthetic organism were found common in both the groups of genotypes. MapMan pathway analysis at milking stage of high Fe genotypes as depicted in Fig. 4, indicates up-regulated and down regulated genes in different locations of cell and their level of expression in red to dark blue color range. Genes involved in photorespiration process and carbon metabolism were found to be more up and down regulated (Fig. 5).

3.6 DEGs involved in transportation and uptakes of mineral in HFZPG

Transportation and accumulation of minerals (Zn, Fe, Cu, Ca, Cu, Cd etc.) by plant from soil involves many complex regulatory processes directed by group of genes responsible for enhancing mineral uptake by the roots and their transportation to the most suitable place of accumulation. Different types of protein families involved in transportation of minerals from root/shoot to the other parts of the plant have been identified. In the present study, different protein families have been identified which are involved in different process of mineral uptake at various stages of panicle development. During different stage of panicle development i.e. panicle initiation, flowering and milking different protein families responsible of transportation and uptake of minerals were either up-regulated or down-regulated in the HFZPG (Table 5 and Supplementary Table S7 and S8). A total of 21 protein families were identified to have function associated with minerals uptake in pearl millet. Among these 21 protein families, all the identified protein family were up-regulated during different stage of panicle development except for ABC transporter family protein and ZC3H15 family which were down regulated during milking stage. ABC transporter family proteins having functions like ATP binding, ATPase activity and transmembrane movement of substances, were found to be up-regulated during panicle initiation stage (↑panicle initiation) and down regulated during milking stage (↓milking stage). This indicates that the mineral transport process is active during panicle initiation stage and further deactivated during flowering and milking stage. Three transcripts related to multicopper oxidase family were up-regulated during panicle initiation and were responsible for copper ion binding, iron ion transport and iron ion homeostasis. There were few protein families which were up-regulated only during panicle initiation *viz.*, ABC transporter family proteins, Multicopper oxidase family, cytochrome P450 family and Peroxidase family, while one protein family was upregulated only during flowering stage *i.e.* terpene synthase family, while 7 protein families were up-regulated only during milking stage. Protein families like Catalase family, major facilitator superfamily, E3 ubiquitin-protein ligase RZFP34 and ZC3H15/TMA46 family were up-regulated during all the three stages of investigation.

Table 5
List of transcripts along with their protein family having a functional association with minerals uptake in High Fe and Zn containing pearl millet genotypes (HFZPG)

Transcript ID	Protein Family	Gene Description	Predicted Functions	Stage of panicle development when expressed
1 4B(single)_trimmed_contig_73886	ABC transporter family proteins	ABC transporter F family member 1	ATP binding; ATPase activity, coupled to transmembrane movement of substances	↑Panicle Initiation ↓ Milking stage
2 4B(single)_trimmed_contig_5720 4B(single)_trimmed_contig_372084B(single)_trimmed_contig_16869	Multicopper oxidase family	L-ascorbate oxidase homolog; laccase-15	Ferroxidase activity; copper ion binding ; plasma membrane; iron ion transport ; lignin catabolic process; apoplast; hydroquinone: oxygen oxidoreductase activity; iron ion homeostasis ; obsolete oxidation-reduction process	Panicle Initiation
3 4B(single)_trimmed_contig_7271	Catalase family	Catalase isozyme	Catalase activity; heme binding ; metal ion binding	↑Panicle Initiation ↑Flowering stage ↑Milking stage
4 4B(single)_trimmed_contig_55418 4B(single)_trimmed_contig_55418	Flavoprotein pyridine nucleotide cytochrome reductase family	Cytochrome b5	Cytoplasm; integral component of membrane; heme binding; intracellular membrane-bounded organelle; metal ion binding	↑Panicle Initiation ↑Flowering stage
5 4B(single)_trimmed_contig_5470 4B(single)_trimmed_contig_1419	Heavy metal-associated isoprenylated plant protein family	Heavy metal-associated isoprenylated plant protein	Metal ion transport ; metal ion binding	↑Panicle Initiation ↑Flowering stage
6 4B(single)_trimmed_contig_66144	Major facilitator superfamily	SPX domain-containing membrane protein Os02g45520	Reverse transcriptase zinc-binding domain	↑Panicle Initiation ↑Flowering stage ↑Milking Stage
7 4B(single)_trimmed_contig_81256 4B(single)_trimmed_contig_58468	Cytochrome P450 family	Cytochrome P450 87A3	Heme binding ; iron ion binding ; monooxygenase activity; oxidoreductase activity	↑Panicle Initiation

	Transcript ID	Protein Family	Gene Description	Predicted Functions	Stage of panicle development when expressed
8	4B(single)_trimmed_contig_48605	Peroxidase family	Peroxidase 31	Heme binding; metal ion binding peroxidase activity; extracellular region; response to oxidative stress; obsolete oxidation-reduction process; cellular oxidant detoxification	↑Panicle Initiation
9	4B(single)_trimmed_contig_10249 4B(single)_trimmed_contig_8196		E3 ubiquitin-protein ligase RZFP34	Zinc ion binding; ubiquitin protein ligase activity	↑Panicle Initiation ↑Flowering stage ↑Milking Stage
10	4B(single)_trimmed_contig_9268 4B(single)_trimmed_contig_72417 4B(single)_trimmed_contig_12423 4B(single)_trimmed_contig_16541	ZC3H15/TMA46 family	Zinc finger CCCH domain-containing protein 15 homolog	Metal ion binding	↑Panicle Initiation ↑Flowering stage ↓ Milking stage
11	4B(single)_trimmed_contig_11940 4B(single)_trimmed_contig_50921	Mitogen-activated kinase	Mitogen-activated protein kinase kinase kinase 3 isoform X2	Metal ion transportation; Signal transducer, downstream of receptor, with serine/threonine kinase activity	↑Flowering stage ↑Milking Stage
12	4B(single)_trimmed_contig_2313	Zinc-containing alcohol dehydrogenase family	Probable cinnamyl alcohol dehydrogenase 8D	Zinc ion binding; lignin biosynthetic process; cinnamyl-alcohol dehydrogenase activity; obsolete oxidation-reduction process	↑Flowering stage ↑Milking Stage
13	4B(single)_trimmed_contig_75011	Terpene synthase family	Alpha-humulene synthase	Lyase activity; metal ion binding; magnesium ion binding	↑Flowering stage
14	4B(single)_trimmed_contig_11410	Ferredoxin–NADP reductase type 1 family	Ferredoxin	Electron transfer activity; chloroplast; electron transport chain; metal ion binding; iron binding	↑Milking Stage
15	4B(single)_trimmed_contig_59340 4B(single)_trimmed_contig_42031 4B(single)_trimmed_contig_71669	YSL (TC 2.A.67.2) family	Probable metal-nicotianamine transporter YSL7	Oligopeptide transporter; May be involved in the transport of nicotianamine-chelated metals	↑Milking Stage

Transcript ID	Protein Family	Gene Description	Predicted Functions	Stage of panicle development when expressed
16 4B(single)_trimmed_contig_19764	Glycosyl hydrolase 13 family	Alpha-amylase type A isozyme	alpha-amylase activity; calcium ion binding ; carbohydrate metabolic process; alpha-amylase activity	↑Milking Stage
17 4B(single)_trimmed_contig_74989	Zinc finger transcription factor family	Zinc finger CCCH domain-containing protein 33	Metal ion binding ; Transcription factor activity, sequence-specific DNA binding; transcription regulatory region DNA binding; Stress response	↑Milking Stage
18 4B(single)_trimmed_contig_68815	CONSTANS family	Zinc finger protein CONSTANS-LIKE 3	Zinc ion binding ; DNA binding; DNA-binding transcription factor activity; identical protein binding	↑Milking Stage
19 4B(single)_trimmed_contig_37316	Multicopper oxidase family	Putative laccase-9	Ferroxidase activity ; copper ion binding ; plasma membrane; iron ion transport ; lignin catabolic process; apoplast; hydroquinone: oxygen oxidoreductase activity; iron ion homeostasis ; obsolete oxidation-reduction process	↑Milking Stage
20 4B(single)_trimmed_contig_65409	Serine-threonine kinase family	Family of serine hydrolases 3-like	Metal ion binding	↑Milking Stage
21 4B(single)_trimmed_contig_6082	Iron/ascorbate-dependent oxidoreductase family	flavanone 3-dioxygenase 2	Metal ion binding ; dioxygenase activity; obsolete oxidation-reduction process	↓Panicle Initiation

3.7 Genes related to Fe and Zn biosynthesis in HFZPG

In plants, for translocation of minerals from one part to another part, various kind of accumulators have been identified. Transport of Fe and Zn from soil to root and then to shoot is affected by many factors like pH, water availability, organic substances, which may affect transport of Fe and Zn to its target destinations. Many studies have been carried out on accumulator which may play role in mineral transport and also in stress tolerance response (STR) [49]. Functional annotation of high Fe and Zn containing genotypes with foxtail millet database revealed transcripts belonging to different families of genes to be involved in higher Fe and Zn accumulation (Supplementary Table S7 and S8). The functional annotation of the DEG's indicated few transcripts to be belonging to the families having function related to metal binding and metal ion transport. The protein families identified in the high Fe and Zn containing genotypes having function specifically related to Fe and Zn were multicopper oxidase family, major facilitator superfamily, cytochrome P450 family, zinc-containing alcohol dehydrogenase family, ferredoxin–NADP reductase type 1 family and CONSTANS family (Table 5).

3.8 SSR discovery in transcripts

A total of 3878 SSR loci harboring 4,087 SSR motifs were discovered in 83,721 transcript sequences of pearl millet (Table 6 and Supplementary Table S9). The number of sequences having more than one SSR and compound SSRs were 209 and 219, respectively. The number of SSR containing tri-nucleotides motif were most abundant (61.73%) followed by di-nucleotides repeats (28.75%). The frequencies of di and tri nucleotide repeats were higher in pearl millet [50] as compared to the other repeats. The number of tetra, penta and hexa-nucleotides repeats were found to be 127, 28 and 15, respectively among the transcripts of pearl millet. Among the di-nucleotides, AG/CT was more frequent (14.79) followed by AC/GT (9.36%), AT/AT (4.48%) and CG/CG (2.15%). In tri-nucleotides, the frequency of CCG/CGG was highest (18.37%) followed by AGC/CTG (14.54%), AGG/CCT (8.78%), ACC/GGT (6.01%) and ACG/CGT (5.66%). While among the tetra-nucleotides, the frequency of ATCC/ATGG was highest, in penta-nucleotide the frequency of AACAC/GTGTT was highest and in hexa-nucleotide the frequency of AAGATG/ATCTTC was highest (Fig. 6).

Table 6
Features of microsatellites identified by MISA in pearl millet.

Feature	Value
Total number of sequences examined	83,721
Total size of examined sequences (bp)	43,459,438
Total number of identified SSRs	4,087
Number of SSR containing sequences	3,878
Number of sequences containing more than one SSR	209
Number of SSRs present in compound formation	219
SSR loci with Di-nucleotide repeat	1,175 (28.75%)
SSR loci with Tri-nucleotide repeat	2,523(61.73%)
SSR loci with Tetra-nucleotide repeat	127 (3.11%)
SSR loci with Penta-nucleotide repeat	28 (0.69%)
SSR loci with Hexa-nucleotide repeat	15 (0.37%)

4. Discussion

Micronutrient malnutrition due to iron and zinc deficiencies is a serious public health problem in developing countries. Globally malnutrition has been in the prime focus of researchers across the world. The development of cereal crops rich in micronutrients is one of the major approach to overcome this problem. In India alone, about 74% of children and 80% of the pregnant women suffer from iron and zinc deficiency. At present, knowledge of the genes involved in transportation and accumulation of Fe and Zn in the seeds of pearl millet is not known and hence a study on genes responsible for Fe and Zn has become important. Various strategies for iron uptake, iron transport and iron storage in many agricultural crops mainly in rice, wheat, common bean, maize reported earlier by many researchers. Movement of iron from soil to its destination place is a complex mechanism and require transportation system which is performed by different genes that regulate the mineral uptake [51].

Transcriptome analysis using Next generation sequencing (NGS) technologies is a robust and efficient method for exploring the pattern of gene expression in host. Transcriptome sequencing or RNA-seq surpasses cloning and is helpful in exploring the complexity of transcriptome by allowing RNA analysis through cDNA sequencing at massive scale [52]. It has the potential to identify important secondary metabolic pathways, various transcripts associated with diseases and helpful for discovering novel genes [53] and developing molecular markers [54]. RNA sequencing (RNA-Seq) is widely used among the different transcriptome analysis methods as it can efficiently detect unknown genes and novel transcripts and has much potential to study gene expression and their regulating pathways [55]. This approach has been successfully applied in many important crops, including sesame [56], chickpea [57], finger millet [58], foxtail millet [59], peanut [60], maize [61] and cotton [62, 63].

In this study, we used transcriptome sequencing for identification of transcripts involved in Fe and Zn accumulation in the pearl millet genotypes. Two sets of pearl millet genotypes having high and low Fe and Zn in their grains were taken. The samples were collected at three stages of spike development *i.e.* panicle initiation, flowering stage and milking stage. mRNA was isolated and was subjected to sequencing using Ion S5 next generation sequencer. The analysis of the transcripts was carried out individually for each stage and later combining all the transcripts into two group *i.e.* high Fe and Zn containing pearl millet genotype (HFZPG) and low Fe and Zn containing pearl millet genotype (LFZPG).

The pearl millet developing spikes transcriptome sequence generated 43 million sequence reads resulting in 83,721 transcripts with N₅₀ of 597 bp. When matched with the pearl millet *de novo* assembly, 84.35% of transcripts matched with the genome assembly. Further, when the differentially expressed gene were analyzed, it was found that the maximum number of genes were differentially expressed during the panicle initiation stage of panicle development indicating that the accumulation and transportation of Fe and Zn might be taking place during the panicle initiation stage of panicle development. The group of genotypes having high Fe and Zn showed that during panicle initiation stage a total of 406 transcripts were differentially expressed of which 155 were up-regulated and 251 were down-regulated while during flowering stage and milking stage 349 and 378 transcripts were differentially expressed, respectively. The gene annotation and GO term assigning of up-regulated genes showed the presence of transcripts involved in

metal ion binding, ATP binding, DNA binding, heme binding, iron ion binding, zinc ion binding, catalytic activity, transferase activity, transferring acyl groups, oxidoreductase activity, peroxidase activity, calcium ion binding, copper ion binding, nutrient reservoir activity, etc. indicating their role in uptake and transport of iron, zinc and other metals. The genes like multicopper oxidase family, major facilitator superfamily, cytochrome P450 family, Zinc-containing alcohol dehydrogenase family, ferredoxin–NADP reductase type 1 family and CONSTANS family having activities related to iron and zinc accumulation were also found to be up-regulated in the transcripts of high Fe and Zn containing genotypes.

SSR markers are cost effective and most suitable molecular marker systems due to various characteristics such as high reproducibility and polymorphism, abundance and co-dominant nature. It can be exploited for diversity analysis of germplasm, linkage map construction and identification of molecular markers for MAS [64, 65]. In the present study, 83,721 transcripts were examined for identification of SSRs. A total of 4,327 SSRs were categorized with the dominance of trinucleotide SSRs in comparison to dinucleotide. Previous studies in *Arabidopsis* and rice have also detected higher number of trinucleotide SSRs in comparison to dinucleotide SSRs in ESTs as compared to genomic sequences [66]. The SSRs identified here in the transcripts of genes engrossed in iron and zinc transport will serve as a promising and cost-effective resource for genotyping a large number of pearl millet genotypes enriched with zinc and iron content and identifying the markers associated with these traits.

The iron we consume from plant-based foods undergoes several processing steps with the help of many plant proteins before it forms the final product in form metal cofactor or in storage form [67]. Enzymes such as proton ATPases, ferric reductases intervene with the solubilization of iron hydroxides in the soil. Further, Fe is transported from the apoplast to the symplast and numerous transporters are needed to distribute it within the plant via xylem and phloem. Specific transporter proteins are required for its transportation across intracellular membranes and biosynthetic enzymes helps in integrating it into heme or iron-sulfur clusters. Later, iron is stored in ferritin in the plastids or in the vacuoles. Hence, any of these proteins/ genes can be utilized as biofortification targets and iron uptake entails many genes [68, 69]. But, only a few them have been identified as biofortification targets. Plants possess two main approaches for iron uptake – chelate based approach in grasses and reductive approach in non-grasses. Iron concentration was observed to increase by 1.7-fold in rice leaves while it was 1.1-fold in grains due to over expression of IRT1, a divalent metal transporter which is central to reductive iron uptake [70]. Thus, iron accumulates in the vegetative tissues in the absence of extra sink capacity in seeds. Infact, iron concentration increased in the endosperm up to 4-fold in polished rice when IRT1 is overexpressed along with PvFER1 [71]. Similarly, collective over expression of Arabidopsis IRT1 and FER1 lead to a 5.5-fold in cassava [72]. During reductive iron uptake mechanism, small molecules such as coumarin-derivatives are secreted in some species while flavins in others [69], but till now it has not been confirmed if biosynthesis genes may be used for biofortification. Derivatives of NA-mugineic acid and deoxymugineic acid (DMA) are secreted in the rhizosphere in grasses and cereals and chelate Fe^{3+} . The Fe-chelator complexes are transported into the cell by YSL15 in rice and YS1 in maize [73]. But, overexpression of IDS3 (Iron Deficiency Specific Clone 3, encoding 2'-deoxymugineic acid 2'-dioxygenase) and YSL15 by increasing DMA resulted into modest increases in iron concentration in rice seeds [74, 75].

Conclusion

We successfully used transcriptome sequencing for identification of transcripts involved in Fe and Zn accumulation in the pearl millet genotypes and it is the first report for identification of metabolic pathways involved for Fe and Zn content in pearl millet. This will be further useful in developing hybrids/varieties rich in Fe and Zn content in pearl millet which will aid in mitigating hidden hunger and enhance nutritional security. The SSRs identified in the present study especially in the transcripts of genes involved in iron and zinc transport would be an excellent cost-effective resource for genotyping a large number of pearl millet genotypes with respect to seed iron and zinc content.

Declarations

Acknowledgements

We sincerely acknowledge Indian Council of Agricultural Research (ICAR), New Delhi for providing financial assistance under CRP Biofortification Project to carry out this research. The service provided by Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat for transcriptomic analysis is also acknowledged.

Author Contributions

This work was carried out in collaboration among all authors. Author CTS, RST, SA designed and executed the study and wrote the whole manuscript. JK, SMP, HD, SBB and MSS performed the transcriptomic and statistical analysis. Author TS, SMS, SPS were involved in field experiment and preliminary studies on Fe & ZN estimation at IARI, New Delhi. Authors RCM, VK supported in conducting the experiment at PC Unit, ICAR-AICRP on Pearl millet, Jodhpur. All co-authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Ethical statement

All the experiments conducted under this study are relevant and according to institutional, national and international guidelines and legislation.

References

1. Singhal, T., Satyavathi, C.T., Kumar, A., Sankar, S.M., Singh, S. P., Bharadwaj, C., J. Aravind, J., Anuradha, N., Meena, M.C. & Singh, N. Genotype x environment interaction and genetic association of grain iron and zinc content with other agronomic traits in RIL population of pearl millet. *Crop Pasture Sci.* **69**, 1092–1102 (2018).
2. Anuradha, N., Satyavathi, C.T., Meena, M.C., Sankar, S.M., Bharadwaj, C., Bhat, J., Singh, O., Singh, S.P. Evaluation of pearl millet [*Pennisetum glaucum* (L.) R. Br.] for grain iron and zinc content in different agro climatic zones of India. *Indian J. Genet. Plant Breeding* **77** (1), 65–73 (2017).
3. Saleh, A.S.M., Zhang, Q., Chen, J. & Shen, Q. Millet grains: nutritional quality, processing, and potential health benefits. *Compr. Rev. Food Sci. Food Saf.* **12**, 281–295 (2013). doi: 10.1111/1541-4337.12012.
4. Parthasarathy, R.P., Bithal, P.S., Reddy, B.V.S., Rai, K.N. & Ramesh, S. Diagnostics of sorghum and pearl millet grains-based nutrition in India. *International Sorghum and Millets Newsletter* **44**, 93–96 (2006).
5. Krishnan, R., Meera, M.S. Pearl millet minerals: effect of processing on bioaccessibility. *J. Food Sci. Technol.* **55**, 3362–3372 (2018). doi: 10.1007/s13197-018-3305-9.
6. Welch, R.M. & Graham, R.D. Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* **55**, 353–364 (2004). doi: 10.1093/jxb/erh064.
7. Bailey, R.L., West, K.P. Jr. & Black, R.E. The epidemiology of global micronutrient deficiencies. *Ann. Nutr. Metab.* **66**, 22–33 (2015). doi: 10.1159/000371618.
8. Ezzati, M., Lopez, A.D., Rodgers, A., Vanderhoorn, S. & Murray, C.J.L. Selected major risk factors and global and regional burden of disease. *Lancet* **360**, 1347–1360 (2002).
9. Kramer, C.V. & Allen, S. Malnutrition in developing countries. *Paediatr. Child Health* **25**, 422–427 (2015).
10. Gibson, R.S., Hess, S.Y., Hotz, C. & Brown, K. H. Indicators of zinc status at the population level: a review of the evidence. *Br. J. Nutr.* **99**, S14-S23 (2008). doi: 10.1017/S0007114508006818V.
11. Chasapis, C.T., Loutsidou, A.C., Spiliopoulou, C.A. & Stefanidou, M.E. Zinc and human health: an update. *Arch. Toxicol.* **86**, 521–553 (2012). doi: 10.1007/s00204-011-0775-1.
12. Howarth E Bouis, Christine Hotz, Bonnie McClafferty, J V Meenakshi, Wolfgang H Pfeiffer. Biofortification: a new tool to reduce micronutrient malnutrition. *Food Nutr. Bull.* **32**, S31-S40 (2011). doi: 10.1177/15648265110321S105.
13. Anuradha, N., Satyavathi, C.T., Bharadwaj, C., Sankar, M., Singh, S.P., Pathy, T.L. Pearl millet genetic variability for grain yield and micronutrients in the arid zone of India. *J. Pharmaco. Phytochem.* **7**(1), 875–878 (2018).
14. Satyavathi, C.T., Sankar, M.S., Singh, S.P., Bhowmick, P., Bhat, J., Singh, O. & Anuradha, N. Stability analysis of Grain Iron and Zinc content in Pearl millet (*Pennisetum glaucum* (L.) R. Br.). *Int. J. Tropical Agri.* **33** (2), 1387–1394 (2015).
15. Kodkany, B.S., Bellad, R.M., Mahantshetti, N.S., Westcott, J.E., Krebs, N.F., Kemp J.F. & Hambidge, K.M. Biofortification of pearl millet with iron and zinc in a randomized controlled trial increases absorption of these minerals above physiologic requirements in young children. *J. Nutr.* **143**, 1489–1493 (2013). doi:10.3945/jn.113.176677.
16. Hirai, M.Y., Yano, M., Goodenowe, D.B., Kanaya, S., Kimura, T., Awazuhara, M., Arita, M., Fujiwara, T., Saito, K. Integration of Transcriptomics and Metabolomics for Understanding of Global Responses to Nutritional Stresses in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*. **101**, 10205–10210 (2004) doi: 10.1073/pnas.0403218101.
17. Kulski, J.K. Next-Generation Sequencing -An Overview of the History, Tools, and “Omic” Applications (2016) 10.5772/61964.
18. Agarwal, P., Agarwal, P.K., Joshi, A.J., Sopory, S.K. & Reddy, M.K. Overexpression of PgDREB2A transcription factor enhances abiotic stress tolerance and activates downstream stress-responsive genes. *Mol. Biol. Rep.* **37** (2), 1125–1135 (2010).
19. Verma, D., Singla-Pareek, S.L., Rajagopal, D., Reddy, M.K. & Sopory, S.K., Functional validation of a novel isoform of Na⁺/H⁺ antiporter from *Pennisetum glaucum* for enhancing salinity tolerance in rice. *J. Biosci.* **32** (3), 621–628 (2007). <https://doi.org/10.1007/s12038-007-0061-9>.
20. Reddy, P.S., Reddy, G.M., Pandey, P., Chandrasekhar, K. & Reddy, M.K. Cloning and molecular characterization of a gene encoding late embryogenesis abundant protein from *Pennisetum glaucum*: protection against abiotic stresses. *Mol. Biol. Rep.* **39** (6), 7163–7174 (2012). <https://doi.org/10.1007/s11033-012-1548-5>.

21. Desai, M.K., Mishra, R.N., Verma, D., Nair, S., Sopory, S.K. & Reddy, M.K. Structural and functional analysis of a salt stress inducible gene encoding voltage dependent anion channel (VDAC) from pearl millet (*Pennisetum glaucum*). *Plant Physiol. Biochem.* **44** (7–9), 483–493 (2006).
22. Singh, J., Reddy, P.S., Reddy, C.S. & Reddy, M.K. Molecular cloning and characterization of salt inducible dehydrin gene from the C4 plant *Pennisetum glaucum*. *Plant Gene* **4** 55–63, (2015) <https://doi.org/10.1016/j.plgene>.
23. Sankar, S.M., Satyavathi, C.T., Barthakur, S., Singh, S.P., Bharadwaj, C. & Soumya, S.L.. Differential modulation of heat inducible genes across diverse genotypes and molecular cloning of a sHSP from Pearl millet [*Pennisetum glaucum* (L.) R. Br]. *Front. Plant Sci.* (2021) doi:10.3389/fpls/2021.659893
24. Jaiswal, S., Antala, T.J., Mandavia, M.K., Chopra, M., Jasrotia, R.S., Tomar, R.S., Kheni, J., Angadi, U.B., Iquebal, M.A., Golakia, B.A., Rai, A. & Kumar, D. Transcriptomic signature of drought response in pearl millet (*Pennisetum glaucum* (L.) and development of web-genomic resources. *Sci. Rep.* **8**(1), 3382 (2018).
25. Shinde, H. et al. Comparative de novo transcriptomic profiling of the salinity stress responsiveness in contrasting pearl millet lines. *Environ. Exp. Botany* **155**, 619–627 (2018).
26. Hamid, R., Marashi, H., Tomar, R.S., Shafaroudi, S.M., Sabara, P.H. Transcriptome analysis identified aberrant gene expression in pollen developmental pathways leading to CGMS in cotton (*Gossypium hirsutum* L.). *PLoS One* **14**, (2019).
27. Strickler, S., Bombarely, A. & Mueller, L. Designing a transcriptome next-generation sequencing project for a nonmodel plant species. *American J. Botany* **99**, 257–266 (2012) 10.3732/ajb.1100292.
28. Varshney, R.K., Shi, C., Thudi, M., Mariac, C., Wallace, J., Qi, P. et al. Pearl millet genome sequence provides a resource to improve agronomic traits in arid environments. *Nat. Biotech.* **35**, 969–976 (2017).
29. Conesa, A.S., Gotz, J.M., Garcia-Gomez, J., Terol, M. & Talon, M.R. Blast 2GO: a universal tool for annotation, visualization and analysis in functional genomics research, *Bioinformatics* **21** (18), 3674–3676 (2005).
30. Langmead, B. & Salzberg, S. Fast gapped-read alignment with Bowtie. *Nat. Methods*, **9**, 357–359 (2012) <https://doi.org/10.1038/nmeth.1923>.
31. Robinson, M.D., McCarthy, D.J. & Smyth, G.K. Edger: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatic* **26** (1), 139–140 (2010).
32. Usadel, B., Obayashi, T., Mutwil, M., Giorgi, F.M., Bassel, G.W., Tanimoto M., Amanda et al. Co-expression tools for plant biology: opportunities for hypothesis generation and caveats. *Plant Cell Environ.* **32**(12), 1633–1651 (2009).
33. Hamid, R., Tomar, R.S., Marashi, H., Shafaroudi, S.M., Golakiya, B.A. & Motahhareh Mohsenpour, M. Transcriptome profiling and cataloging differential gene expression in floral buds of fertile and sterile lines of cotton (*Gossypium hirsutum* L.). *Gene* **660**, 80–91 (2018).
34. Tulsani, N.L., Hamid, R., Jacob, F., Umretiya, N.G., Nandha, A.K., Tomar, R.S. & Golakiya, B.A. Transcriptome landscaping for gene mining and SSR marker development in Coriander (*Coriandrum sativum* L.). *Genomics* **112**(2), 1545–1553 (2020).
35. Institute of Medicine. Food and Nutrition Board. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc: a Report of the Panel on Micronutrients. Washington, DC: National Academy Press; 2001.
36. Lockyer, S., White, A. & Buttriss, J.L. Biofortified crops for tackling micronutrient deficiencies-what impact are these having in developing countries and could they be of relevance within Europe. *Nutr. Bull.* **43**, 319–357 (2018).
37. Singhal T, Satyavathi CT, Singh SP, Kumar A, Sankar SM, Bharadwaj C, Mallick M, Bhat J, Anuradha N. Singh N Multi environment quantitative trait loci mapping for grain iron and zinc content using biparental recombinant inbred line population in pearl millet. *Frontiers in Plant Science* (2021) doi:10.3389/fpls/2021.659789
38. Singhal, T., Satyavathi, C.T., Singh, S.P., Sankar, S.M., Anuradha, N., Bharadwaj, C., Kumar, A., Mallick, M. & Singh, N. Identification of new stable and high iron rich fertility restorers in Pearl millet. *Indian J. Genet. Plant Breed.* **79**(3), 552–562 (2019) doi: 10.31742/IJGPB.79.3.4
39. Satyavathi, C.T., Singh, S.P., Sankar, M.S., Prabhu, K.V. & Gupta, H.S. PPMI 904 (IC0617290; INGR16004), a Pearl Millet (*Pennisetum glaucum* L.) Germplasm with High Iron Content of 91 mg/kg High Zinc Content of 78 mg/kg. *Indian J. Plant Genet. Resources* **31** (1), 105–106 (2018).
40. Anuradha, N, Satyavathi, C.T., Bharadwaj, C., Bhat, J. & Pathy, T.L. Correlation studies on quality and other economic traits in pearl millet. *Int. J. Chem. Studies* **6**(5), 2041–2043 (2018).
41. Kumar, S., Hash, C.T., Nepolean, T., Mahendrakar, M.D., Satyavathi, C.T., Singh, G., et al. Mapping Grain Iron and Zinc Content Quantitative Trait Loci in an Iniadi-Derived Immortal Population of Pearl Millet. *Genes*. **9** (248), (2018). doi:10.3390/genes9050248

42. Anuradha, N., Satyavathi, C.T., Bharadwaj, C., Sankar, S.M. & Pathy, T.L. Association of agronomic traits and micronutrients in pearl millet. *Int. J. Chemical Studies* **6**(1), 181–184 (2018).
43. Anuradha, N., Satyavathi, C.T., Bharadwaj, C., Nepolean, T., Sankar, S.M., Singh, S.P., Meena, M.C., Singhal, T. & Srivastava, R.K. Deciphering genomic regions for high grain iron and zinc content using association mapping in pearl millet. *Front. Plant Sci.* **8**, 412. (2017) doi: 10.3389/fpls.2017.00412.
44. Shanmugam, V.L. J.C. & Yeh, K.C. Control of Zn uptake in *Arabidopsis halleri*: a balance between Zn and Fe. *Front. Plant Sci.* **4**, 281 (2013) doi: 10.3389/fpls.2013.00281
45. Rout, G.R. & Sahoo, S. Role of iron in plant growth and metabolism. *Rev. Agric. Sci.* **3**, 1–24 (2015)
46. Kanehisa, M. and Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
47. Kanehisa, M. KEGG bioinformatics resource for plant genomics and metabolomics. *Methods Mol. Biol.* **1374**, 55–70 (2016).
48. Kanehisa, M., Sato, Y., and Morishima, K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* **428**, 726–731 (2016).
49. Mishra, V.K., Gupta, S., Chand, R., Yadav, P.S., Singh, S.K. et al. Comparative transcriptomic profiling of High- and Low- grain Zinc and Iron containing Indian wheat genotypes. *Curr. Plant Bio.* **18**, 100105 (2019).
50. Senthilvel, S., Jayashree, B., Mahalakshmi, V., Kumar P.S., Nakka, S., Nepolean, T & Hash, C.T. Development and mapping of Simple Sequence Repeat markers for pearl millet from data mining of Expressed Sequence Tags. *BMC Plant Biol.* **8**, 119 (2008) <https://doi.org/10.1186/1471-2229-8-119>.
51. Ludwig, Y. & Slamet-Loedin, I.H. Genetic Biofortification to Enrich Rice and Wheat Grain Iron: From Genes to Product. *Front. Plant Sci.* **10** (2019) 833. doi: 10.3389/fpls.2019.00833.
52. Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A. et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376–380 (2005) <https://doi.org/10.1038/nature03959>.
53. Jaiswal, S., Jadhav, P.V., Jasrotia, R.S. et al. Transcriptomic signature reveals mechanism of flower bud distortion in witches'-broom disease of soybean (*Glycine max*). *BMC Plant Biol.* **19**, 26 (2019).
54. Vatanparast, M. et al. Transcriptome sequencing and marker development in winged bean (*Psophocarpus tetragonolobus* Leguminosae). *Sci. Rep.* **6**, 290 (2016) <https://doi.org/10.1038/srep29070>.
55. Hrdlickova, R., Toloue, M. & Tian, B. RNA-Seq methods for transcriptome analysis. *Wiley Interdiscip. Rev. RNA* **8** (1), e1364 (2017) <https://doi.org/10.1002/wrna>.
56. Jaiswal, S., Tomar, R.S., Vadukool, K., Chopra, U.M., Rathod, V.M., Parakhia, M.V., Iqbal, M.A., Rai, A. & Kumar, D. Transcriptome profiling of Indian sesame (*Sissemum indicum* L.) and discovery of genetic region markers. *Bharatiya Krishi Anusandhan Patrika*, **35** (3), 151–158 (2020).
57. Garg, R., Patel, R.K., Jhanwar, S., Priya, P., Bhattacharjee, A., Yadav, G., Bhatia, S. et al. Gene discovery and tissue-specific transcriptome analysis in chickpea with massively parallel pyrosequencing and web resource development. *Plant Physio.* **156** (4), 1661–1678 (2011) <https://doi.org/10.1104/pp.111.178616>.
58. Kumar, A., Gaur, V.S., Goel, A. & Gupta, A.K. De novo assembly and characterization of developing spikes transcriptome of finger millet (*Eleusine coracana*): a minor crop having nutraceutical properties. *Plant Mol. Biol. Rep.* **33**, 905–922 (2015).
59. Jo, Y., Lian, S., Cho, J.K., Choi, H., Kim, S.M., S.L. et al. De novo transcriptome assembly of *Setaria italica* variety Taejin. *Genome Data* **5**(8), 121–122 (2016) doi: 10.1016/j.gdata.
60. Zhang, Y, Xia, H., Yuan, M., Zhao, C., Li, A. & Wang, X. Cloning and expression analysis of peanut (*Arachis hypogaea* L.) CHI gene. *Elect.c J. Biotech.* **15** (1), 5 (2012) doi: 10.2225/vol15-issue1-fulltext-6.
61. Yue, R., Lu, C., Qi, J., Han, X., Yan, S., Guo, S. et al. Transcriptome analysis of cadmium-treated roots in maize (*Zea mays* L.). *Front. Plant Sci.* **7**, 1298 (2016).
62. Hamid, R., Jacob, F., Marashi, H., Rathod, V. & Tomar, R.S. Uncloaking lncRNA-mediated gene expression as a potential regulator of CMS in cotton (*Gossypium hirsutum* L.). *Genomics* **112**, 3354–3364 (2020).
63. Hamid, R., Marashi, H., Tomar, R.S., Shafaroudi, S.M. & Sabara, P.H. Transcriptome analysis identified aberrant gene expression in pollen developmental pathways leading to CGMS in cotton (*Gossypium hirsutum* L.), *Plos One* **14**, e0218381 (2019).
64. Kalia, R.K., Rai, M.K., Kalia, S. et al. Microsatellite markers: an overview of the recent progress in plants. *Euphytica* **177**, 309–334 (2011). <https://doi.org/10.1007/s10681-010-0286-9>.

65. Miah, G., Rafii, M.Y., Ismail, M. R., Puteh, A. B., Rahim, H.A., Asfaliza, R. & Latif, M.A. Blast resistance in rice: a review of conventional breeding to molecular approaches. *Molecular Biology Reports* **40**, 2369–2388 (2012).
66. Lawson, M.J. & Zhang, L. Distinct patterns of SSR distribution in the *Arabidopsis thaliana* and rice genomes. *Genome Biol* **7**, R14 (2006).
67. Kawakami, Y. & Bhullar, N.K. Molecular processes in iron and zinc homeostasis and their modulation for biofortification in rice. *J. Integr. Plant Biol.* **60**, 1–32 (2018).
68. Brumbarova, T., Bauer, P. & Ivanov, R. Molecular mechanisms governing *Arabidopsis* iron uptake. *Trends Plant Sci.* **20**, 124–133 (2015)
69. Connorton, J.M., Balk, J. & Rodríguez-Celma, J. Iron homeostasis in plants—a brief overview. *Metallomics* **9**, 813–823 (2017).
70. Lee, S., & An, G. Over-expression of OsIRT1 leads to increased iron and zinc accumulations in rice. *Plant Cell Environ.* **32**, 408–416 (2009).
71. Boonyaves, K., Wu, T.Y., Grissem, W. & Bhullar, N.K. Enhanced grain iron levels in rice expressing an iron-regulated metal transporter, nicotianamine synthase, and ferritin gene cassette. *Front. Plant Sci.* **8**, 130 (2017).
72. Narayanan, N., Beyene, G., Chauhan, R.D. *et al.* Biofortification of field-grown cassava by engineering expression of an iron transporter and ferritin. *Nat Biotechnol* **37**, 144–151 (2019) <https://doi.org/10.1038/s41587-018-0002-1>.
73. Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S.L., Briat, J.F., Walker, E.L. Maize yellow stripe1 encodes a membrane protein directly involved in Fe (III) uptake. *Nature* **409**, 346–349 (2001).
74. Masuda, H., Suzuki, M., Morikawa, K.C. *et al.* Increase in Iron and Zinc Concentrations in Rice Grains via the Introduction of Barley Genes Involved in Phytosiderophore Synthesis. *Rice* **1**, 100–108 (2008) <https://doi.org/10.1007/s12284-008-9007-6>.
75. Lee, S., Chiecko, J.C, Kim, S.A., Walker, E.L., Lee, Y., Lou, M., An, G. Disruption of OsYSL15 leads to iron inefficiency in rice plants. *Plant Physiol.* **150**, 786–800 (2009).

Figures

A

B

C

Figure 1

Characterization of Differentially Expressed Genes (DEGs) in pearl millet genotypes during different stages of panicle developmental i.e. Panicle initiation, Flowering and Milking. (A) Over-all distribution of differentially expressed genes during different stage, (B) Distribution of up-regulated genes among the different stage of panicle development and (C) Distribution of down-regulated genes among the different stage of panicle development

Figure 2

GO enrichment analysis of DEGs were classified into (A) up-regulated cellular components, molecular function and biological process; (B) down-regulated cellular components, molecular function and biological process.

Figure 3

Classification of ion binding component of molecular function which were up-regulated during the three different stages of panicle development i.e. panicle initiation, flowering stage and milking stage.

Figure 4

Different metabolic pathways and associated genes encoding enzymes that participated in different metabolic pathways

Figure 5

A representation of transcript abundance from the high Fe genotype milking stage versus Low Fe genotype milking stage experiment displaying differential gene expression involved in metabolism (a) and anthocyanin biosynthesis (b). Each colored square represents the mean log2 expression data for the pearl millet. The logarithmic colour scale bar ranges from -1.5 (dark blue, representing a threefold down regulated gene).

Figure 6

Frequency distribution of different SSRs and SSR motifs.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableS1S6.xlsx](#)
- [SupplementaryTableS7S8.xlsx](#)
- [SupplemnetaryTableS9.xlsx](#)