Genome-wide expression analysis identifies core components during iron starvation in hexaploid wheat

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Running Title: Fe starvation response in hexaploid wheat roots

Abstract
Iron is one of essential micronutrient for all organisms. Its deficiency causes a severe loss in crops yield. Nevertheless, our current understanding on major crops response to Fe deficiency remains limited. Herein, we investigated the effect of Fe deprivation at both transcriptomic and metabolic levels in hexaploid wheat. A genome-wide gene expression reprogramming was observed with a total of 5854 genes showing differential expression in roots of wheat subjected to Fe-starved medium. Subsequent, analysis revealed a predominance of strategy-II mode of Fe uptake, with induced genome bias contribution from the A and B genomes. In general, the predominance of genes encoding for nicotianamine synthase, yellow stripe like transporters, metal transporters, ABC transporters and zinc-induced facilitator-like protein was noticed. Our transcriptomic data were in agreement with the GC-MS analysis that showed an enhancement of accumulation of various metabolites such as fumarate, malonate, succinate and xylofuranose, which could be linked for enhancing Fe-mobilization. Interestingly, Fe starvation causes a significant temporal increase of glutathione-S-transferase both at transcriptional and enzymatic activity, which indicate the important role of glutathione in the response to Fe starvation in wheat roots. Taken together, our result provides new insight on wheat response to Fe starvation and lays foundation to design strategies to improve Fe nutrition in crops.

**Key words:** iron starvation, *Triticum aestivum*, glutathione metabolism, transcriptome, gene expression, genome bias.

**Introduction**
Iron (Fe) is among the essential micronutrients in plants that participate as a catalytic cofactor in several key processes including photosynthesis, respiration, chlorophyll biosynthesis and nitrogen fixation (Kim and Rees, 1992; Morrissey and Guerinot, 2009; Li et al., 2017). The bioavailability of Fe in soil is strongly dependant on its solubility, aerobic and calcareous soil condition, pH and the presence of natural ligands secreted by plant roots (Marshner H., 1995; Morrissey et al., 2009; Thomine et al., 2013). To circumvent the above challenges for Fe uptake by the roots, plants have adapted majorly two modes of uptake strategies. Strategy-I, mostly predominant in Eudicot species, primarily relies on the enrichment of rhizospheric regions with protons (H⁺) and other reducing agents (Hell and Stephan, 2003; Santi et al., 2005; Santi and Schmidt 2009; Kobayashi and Nishizawa 2012). In contrast, the graminaceous species follow the strategy-II mode of uptake, which involves the transport of Fe in the complex-chelated form (Mori et al., 1999; Kobayashi and Nishizawa, 2012; Connorton et al., 2017). The primary components involved in chelation are the phytosiderophores (PS) secreted by plant cells in the rhizospheric region mainly by efflux transporters (Morrissey and Gueirnot, 2009; Kobayashi and Nishizawa, 2012). One of the main components of these secreted siderophores involved in Fe chelation is referred as mugineic acids (MAs). The transporters involved in the secretion of the MAs are identified to be a transporter of mugineic acid; TOM proteins (Nozoye et al., 2011). The complex chelated form of Fe-PS is taken up by the specific root transporter referred as yellow stripe-like transporter proteins (YSL) (Curie et al., 2001; Gross et al., 2003, Yordem et al., 2011). Subsequently, Fe is transported in the plant organelles by multiple partners including specialized long distance, tissue specific transporters as reported in monocots (e.g rice, maize) and eudicots (e.g. Arabidopsis) (Kim et al., 2006; Waters et al., 2011). In other monocots such as maize and rice, presence of genetic components for both strategy-I and II were reported (Ishimaru et al., 2006; Inoue et al., 2009; Lee et al., 2009; Zanin et al., 2017). At the metabolome level analytical approaches utilizing GC-MC and LC-MS were explored to study the components of Fe starvation in plants (Palmer et al., 2014; Kabir et al., 2012). However, the metabolic activity predominant for Fe uptake in roots of wheat crop remains unelucidated.

Microarrays have been successfully used to investigate the global transcriptional changes in Arabidopsis plants grown in Fe starved conditions (Thimm et al., 2001; Buckhout et al., 2009; Yang et al., 2010). Transcriptome analysis of Fe-starved plants is characterised by an important change in gene expression of several transcriptional regulators such as transcription factors (TFs) (Colangelo et al., 2004; Kobayashi et al., 2009; Long et al., 2010;
Li et al., 2016; Connorton et al., 2017) or key genes related to phytohormone homeostasis (Schmidt et al., 2000; Hindt and Guerinot, 2012). Fe starvation also leads to changes in the abundance of transcripts related to plant metabolism and genes involved in signalling pathway to modulate nutrient uptake. Additionally, genes involved in ethylene/auxin signalling or linked with certain other macronutrients like nitrogen, sulphur and phosphorus are also significantly expressed (Romera et al., 1994; Zheng et al., 2009; Romera, 2011; Borlotti et al., 2012; Zuchi et al., 2015; Lin et al., 2016; Zanin et al., 2017; Garnica et al., 2018). Therefore, transcriptome is powerful approach to help understand the network for how plants respond to Fe. The development of transcriptome technology (RNA sequencing), availability of genome sequence for hexaploid wheat (genome A, B, D) (International Wheat Genome Consortium, 2014) combined with metabolomic approaches will help in gaining knowledge on wheat response to nutritional stress (e.g. –Fe) (Borrill et al., 2018).

Hexaploid wheat is one of the important crops and is also a good, affordable source of nutrition. Fe deficiency strongly affects plant growth and productivity (Yousfi et al., 2009; Bocchini et al., 2015). Our current knowledge on how wheat regulates Fe homeostasis remains limited; and the question how wheat responds to Fe starvation at transcriptional and metabolomic levels remains to be answered. In our study, using RNA-seq analysis, we report the first transcriptome data in root of hexaploid wheat in response to Fe starvation. Our metabolite profiling was in agreement with the transcriptome data. Collectively our results provide the first insight on molecular and biochemical responses of hexaploid wheat under Fe starvation.

Materials and methods

Plant materials, starvation conditions and plant sampling

Bread wheat variety ‘C-306’ was used for all the experimental purpose. Once the endosperm started browning it was removed from the developing seedlings. After overnight stratification at 4°C, wheat seeds were germinated for 5 days in distilled water. A total of thirty-two seedlings were used for each treatment. The Seedlings were then transferred to Phytabox™ and grown in the Hogland’s nutrient solution. After 7 days, nutrient solutions were replaced on the basis of different treatments. For Fe starvation (-Fe) 1 µM Fe (III) EDTA was used as Fe source. For control plants (+Fe) concentrations of nutrients were unchanged in above-mentioned Hoagland’s solution containing (20 µM Fe (III) EDTA). Treated seedlings were grown in the described medium for 20 days in growth chamber set at 20°C ± 1°C, 50–70%
relative humidity and photon rate of 300 μmol quanta m$^{-2}$ s$^{-1}$ with 16 h day/8 h night cycle. For sampling, roots and shoots were collected at different time points after starvation (5, 10, 15 and 20 days). On the basis of distinct phenotype, samples collected at 20 days after starvation (DAS) were used for transcriptome analysis. A total of four biological replicates (each containing 10-12 seedlings) were used. Subsequently, RNA extractions were performed independently for each of the pools. Prior to RNA sequencing, quality of RNA was checked and extractions derived from two similar replicates were pooled together thereby generating two experimental samples for each of the respective conditions. Remaining samples were snap frozen in liquid nitrogen and stored at -80°C. To distinctively observe primary root and 1st order lateral root, individual plants were moved onto a 150mm wide petriplate filled with distilled water and characteristics were manually examined. Each treatment consisted of 6-8 wheat seedlings with two experimental replicates to ascertain root characteristics.

**RNA extraction and Illumina sequencing**

Total RNA was extracted from the treated root samples along with the control by Trizol (Invitrogen) as per the manufacturer’s instruction. The quality and quantity were checked on the 1% denaturing RNA agarose gel and Nanodrop respectively. Subsequently, all the RNA used for library preparations were checked for their RNA integrity number ≥ 8.5 using Bioanalyzer (Agilent, USA). The quality control (QC) passed RNA samples were then processed for library preparation. The Paired-ended (PE) libraries were prepared from the total RNA using illumina TruSeq stranded mRNA library prep kit as per the instructions (Illumina Inc., USA). The generated mean of the libraries fragment size distribution was 559 bp, 584 bp, 546 bp and 604 bp for the samples. The generated libraries were sequenced on the NextSeq 500 using 2 X 150 bp chemistry. The raw reads were processed further before the sorted high-quality reads were mapped to the reference genome.

**RNA-Seq analysis**

Adapter clipping and quality trimming of the raw reads were performed using Trimmomatic-0.35. The sequenced raw reads were processed to obtain high quality clean reads. Ambiguous reads (reads with unknown nucleotides “N” larger than 5%) and low-quality sequences (more than 10% quality threshold QV<20 phred score) were removed. A minimum length of 100 nts after trimming was applied. Finally, high-quality (QV>20), paired-end reads were used for reference based read mapping. The genome of *Triticum aestivum* L. was taken as reference for analysis. The Gene Feature Format files were downloaded from Ensembl Plants.
The reads were mapped to the reference genome using TopHat v2.1.1 with default parameters. Cufflinks v2.2.1 program assembled the transcriptome data from RNA-seq data and quantified their expression. Mapped reads were subjected to Cufflinks, followed by Cuffmerge and Cuffdiff. Log2 Fold Change (FC) values greater than one was considered up-regulated whereas less than 1 were considered as down-regulated. These genes were further categorized on the basis of statistical significance (p<0.05) and the False Discovery Rate (FDR 0.05) after Benjamin-Hochberg corrections for multiple testing for their significant expression.

Comprehensive gene annotation of wheat sequences was done using KOBAS 3.0 (Xie et al., 2011) annotate module by alignment with Rice and Arabidopsis sequences (BLASTP, cutoff 1e-5). MapMan was used to visualize the pathways involving wheat differentially expressed genes (DEGs). Pathway enrichment analysis was performed using KOBAS standalone tool. MeV was used to construct heatmaps for selected DEGs using the normalized expression values of genes. The data generated from this study has been deposited in the Gene Expression Omnibus of NCBI and will be accessible with the GEO series accession number after deposition.

**Gene annotation filtering and functional enrichment analysis**

Significant sets of DEGs under iron starvation were further mapped using GO and Mapman. The GO annotation was downloaded from ensembl plants (https://plants.ensembl.org/biomart/martview). Mercator (Lohse et al., 2013) was used to build MapMan mapping file for TGACv1 sequences and DEGs were visualized in MapMan v3.1.1 tool (http://mapman.gabipd.org/web/guest/mapmanstore; Usadel et al., 2009). For functional categorization of DEGs that were positively and negatively correlated with iron starvation, BINGO version 3.0.3 (Maere et al., 2005) was used to perform GO enrichment analysis with hypergeometric test and considered significant terms with an FDR value below 0.05. For gene ontology mapping, GO_full.obo ontology file was downloaded from GO consortium. Mapman classification was used to categorize DEGs into transcriptional factors. The results were visualized as network using EnrichmentMap version 2.2.1 (Merico et al., 2010) and gene expression overview in various pathways were visualized in Mapman tool.

**Homoeolog specific expression analysis for genome bias**

To identify wheat homoeologous triplets; ensembl biomart TGACv1 was used to extract all possible homoeologous relations. This led to 86,830 pairwise homoeologous relations. An in-
house script was used to select only the triplets where contribution from each genome was 1:1:1 (A:B:D), thus generating 16,850 triplets. Further, triplets resulting from potential translocation events were not considered, i.e., only homoeolog triplets from same chromosome (eg., 1A, 1B, 1D triplet is accepted, whereas, 2A, 3B, 3D is rejected) were taken for analysis. Finally, 15,604 triplets (15,604*3 = 46,812 genes) were used for studying genome induction biasness in response to iron stress. Paired end reads were aligned to the reference (selected scaffolds from genome that harbour 15,604 triplets) using TopHat v2.1.1 with a specific argument (--b2-very-sensitive) (Powell et al., 2017), which leads to more stringent alignments as required for homoeologs. The Cufflinks pipeline was used to obtain FPKM values and differentially expressed genes.

Further, relative abundance levels and expression bias for homoeologs was studied by considering the expression of FPKM \( \geq 1 \) in both the control as well as Fe starved conditions. For this, the normalised relative expression for each homoeolog within a triad was calculated. For example, the relative expression from A will be represented as:

\[
\text{Relative expression}_A = \frac{\text{FPKM}_A}{\text{FPKM}_A + \text{FPKM}_B + \text{FPKM}_D}
\]

Thus, relative expression levels of A, B and D homoeologs within each triad were calculated similarly. Seven homoeolog expression bias categories were defined as described earlier by Ramirez-Gonzalez et al., (2018). In total, seven categories that includes defined as one balanced category and six unbalanced homoeolog-suppressed/homoeolog-dominant (from either of the genomes) were listed during the ideal relative expression of A, B and D. Eucledian diatance (using cdist function from rdist package, R3.3.2) was calculated between normalised relative expression for each triad and the seven ideal categories. The shortest distance was used as a deciding factor to group the triads into the seven respective categories.

**Quantitative real time-PCR (qRT-PCR)**

For validation of the gene expression, qRT-PCR analysis was performed. An aliquot of the total RNA (2 µg) isolated from the above experiments were used for expression validation using qRT-PCR method. Genomic DNA present in trace amount was removed by DNaseI treatment using a Turbo DNA-free kit (Invitrogen, ThermoFisher, USA). Further, cDNA was synthesized from two micrograms of DNA-free RNA using Superscript III first strand (Invitrogen) with random hexamer primers following the manufacturer’s guidelines. For qPCR reaction, gene-specific primers of each gene were designed from conserved regions of all three homeolog sequences (Supplementary Table S1). qRT-PCR was performed using
QuantiTect SYBR Green RT-PCR mastermix (Qiagen, USA) with programs recommended by the manufacturer in the ABI 7700 sequence detector (Applied Biosystems, USA). ADP-ribosylation factor (ARF) and Actin were used as internal controls. Two independent experimental replicates with four technical replicates were performed for each sample. The relative amount of gene expression was calculated using $2^{-\Delta\Delta CT}$ method.

Metabolite extraction and GC-MS profiling
Extraction of total metabolites was performed similarly as previously described (Wang et al., 2018). Roots of plant grown under -Fe and +Fe were sampled in triplicate manner and dried for 1 week. Each of 50 mg crushed samples was extracted ( precooled; 300 µl methanol, 30 µl 2 mg ml$^{-1}$ nonadecanoic acid methylester and 30 µl 0.2 mg ml$^{-1}$ sorbitol) for 15 minutes (70°C, 1000rpm). Further, at room temperature 200 µl chloroform was added and shaken for 5 min (37°C, 1000rpm). To obtain phase separation, 400 µl H$_2$O was added to each sample, vortexed and centrifuged (10 min, 13,000 rpm). The upper polar phase nearly 200 µl was finally aliquoted for complete drying.

For GC-MS analysis, metabolites were subjected to methoxyamination and trimethylsilylation. Dried polar phase were shaken for 1.5 hr at 30°C in 40 µl of MeOX (40 mg ml$^{-1}$ methoxyaminhydrochloride in pyridine) followed by 30 min shaking at 37°C in 80 µl BSTFA mixture (70 µl N,O-bis(trimethylsilyl)trifluoroacetamide + 10 µl alkane mix). The derivatized metabolites were analyzed with a GC instrument (Agilent technologies 7890, USA) coupled with mass spectrometry. Measurement from an injection volume of 1 µl was taken in split-less mode in DB-5 column (30 m × 0.25 mm, 0.25 µm film thickness, Agilent) using helium as carrier gas. Metabolites were separated as described by Wagner et al., 2013. Qualitative analysis of chromatograms was performed in MassHunter Qualitative analysis Sp1 workstation (Agilent, USA). Identification and annotation of each compound was supervised manually using AMDIS software and NIST08 database (http://www.nist.gov/srd/mslist.html). Data were normalized to sample weight and internal control (sorbitol). Statistical analysis was performed as described earlier (Quanbeck et al., 2012). Log2 ratio of metabolite abundances in -Fe was plotted against +Fe. Delta method approximation was used to calculate standard errors (se) of log-ratio, se log-ratio = 1/ln $2\sqrt{[(\text{SE}_T/\text{T})^2 + (\text{SE}_C/\text{C})^2]}$, where SE$_T$ and SE$_C$ are standard errors of average -Fe and +Fe metabolite abundances.

Measurement of glutathione-S-transferase (GST) activity
Activity measurement of GST was performed in the wheat roots subjected to -Fe for 10, 15 and 20 days of treatment along with the control plants (no stress) as mentioned in plant materials. The estimation was done using Glutathione-S-transferases assay kit (Sigma, USA). Briefly, 100 mg of tissue was used for total protein extraction. Equal amount of total protein (25 μg) was used as a source of enzyme and 1-Chloro-2,4-dinitrobenzene (DNB) was used as a substrate. The resulting GS-DNB conjugate was measured at 340 nm wavelength during the time course of the reaction. The direct increase in absorption was measured and GST activity was calculated as described in the manufacturer instruction kit.

Elemental analysis was performed using Inductive Coupled Plasma-mass spectroscopy and nitrate estimation

Elemental analysis was performed using Inductive Coupled Plasma-MS (ICP-MS). Metal analysis was performed as described earlier (Bhati et al., 2016; Aggarwal, 2018). Briefly, the mature seeds were grounded to fine powder and subsequently subjected to the microwave-digested with HNO₃ (SuraPure™, Merck). Respective metal standards were also prepared for analysis. Three independent replicates were performed for each time point.

Nitrate content in wheat root was measured according to method described previously (Cataldo et al., 1975). Briefly, 1 g of fresh tissue was homogenized in 6 ml of deionised water and centrifuged at 30,000g for 15 min. The 100 μl of supernatant was added to 400 μl of salicylic acid (w/v dissolved in conc. H₂SO₄.). After mixing well the reaction was kept at room temperature for 20 mints. 2N NaOH (9.5ml) was then added slowly to raise the pH above 12. The samples were allowed to cool and readings were taken at 410nm in spectrophotometer.

Results

Fe starvation affects wheat growth capacity and nutrients uptake

Fe starvation is known to affect plant growth capacity. In order to determine the effect of Fe starvation, wheat plants of one-week old seedlings, grown on complete medium (presence of Fe) were transferred to Fe starvation media for additional days. After 20 days of starvation (DAS) the wheat seedlings started showing phenotypic symptoms including visible chlorosis and therefore detailed study was performed for this time point. In response to Fe starvation, plants showed decrease in the shoot biomass with an enhanced chlorosis phenotype and shortening of root system compared to wheat grown in presence of Fe (Fig. 1A and B). Root
of Fe-starved wheat showed decrease in number of lateral roots and significant reduction in primary root length in comparison to control plants (Fig. 1C and D). Earlier studies have suggested that changes in root system and Fe supply not only affect the Fe accumulation capacity but also impacts the uptake of other nutrients such as Zn, Cd etc. (Sperotto et al., 2012; Shukla et al., 2017). Therefore, we assessed the effect of Fe starvation stress on the uptake of Zn, Mn, Cu, and Mg in wheat undergoing Fe stress (Table 1). Our data indicated increased uptake of nutrient elements such as Zn, Mn, Cu, and Mg in roots but accumulation in shoots was either unaltered or decreased. This suggests that roots are an important tissue for understanding the molecular networks for how wheat could respond to Fe starvation.

Differential expression analysis, homoeolog (A, B and D) induction and expression bias during –Fe response

The effect of Fe starvation on the root transcriptome was investigated, that has not been investigated till date. To perform this, RNAseq technology was used to identify the changes in the transcripts of wheat roots, where plants were grown in presence or absence of Fe (20 DAS). Our analysis resulted in 87 million quality filtered reads, with an average of nearly 22 million reads from each sample (more than 87% reads had a quality score greater than Q30). Filtered reads from the four libraries had a mapping rate ranging from 81.7% to 85.4% when mapped against release-37 of the wheat genome using TopHat (Supplementary Table S2). As quality check, a strong correlation within the two biological replicates from each condition was observed, while a clear variation was seen between the two conditions (Fig. 2A). We thereafter analysed the expression values as FPKMs (fragment per kilobase of transcript per Million mapped reads), calculated by using Cufflinks software. Differentially expressed genes (DEGs) were then identified by calculating logFC (log2 fold change) and performing statistical tests between FPKM values from control and stressed samples using CuffDiff. 50,610 genes had an FPKM of greater than or equal to 1 in at least one of the two conditions were considered to be “expressed transcript”. In all, 7221 genes had logFC > 0 and 8010 had logFC < 0 (Fig. 2B). On setting up a criterion of logFC of more than 1 for up-regulated genes, and that of less than -1 for down-regulated genes and an FDR < 0.05, a total of 3478 genes were highly expressed, whereas, 2376 were down-regulated under –Fe condition in wheat roots (Fig. 2C). Interestingly, 45 genes were also induced exclusively during starvation condition when compared to the control samples (Supplementary Table S3).

Our data allowed us to analyse the chromosomal distribution of the DEGs under –Fe condition. While all chromosomes contributed the DEGs, the highest number of genes was
mapped on chromosome 2 of wheat genome (Fig. 3A). Equal representation of transcripts was observed for the chromosome 7 and 5. For the remaining chromosomes, 1, 3, 4 and 6 showed 15, 13, 12 and 10 % distribution of DEGs, respectively. In polyploidy crops like wheat, homoeolog induction bias could impact plant response to various stresses (Liu et al., 2015; Powell et al., 2016). To determine the extent of induction bias (A, B and D) in wheat during –Fe starvation, homoeolog specific expression analysis was performed. Starting from a list of 8,473 gene triplets present/expressed (Supplementary Table S4), ‘accepted triplets’, most of homoeolog triplets (8,349) showed no significant biasness in expression (A = B = D). Out of these, 8,321 triplets appeared to be unaffected by -Fe stress, while 22 and 6 homoeologous triplets were up- and down-regulated respectively. Homoeolog expression bias was observed in 124 homoeolog triplets. Eighty-seven triplets had only one of the homoeologs differentially expressed (up- or down-regulated). Forty-seven in the category ‘1UP’ with A > B = D, B > A = D and D > A = B, 40 in category named ‘1DOWN’ having A < B = D, B < A = D or D < A = B. Table 2, provides the list of genes with significant induction predominance occurring from the A and B genomes in response to Fe starvation (Table 2). Few of the prominent transcripts exclusively induced by these two genomes include transcripts related to MYB TFs, metal transporters, zinc transporters, RING-H2 type proteins, genes belonging to major facilitator superfamily proteins etc.. Additionally, 37 triplets had two of the homoeologs differentially expressed while the third showed normal expression even under Fe stress (Fig. 3B). ‘2UP’ category includes AB > D, AD > B and BD > A while ‘2DOWN’ includes AB < D, AD < B and BD < A (Fig. 3B). This suggests that during Fe starvation, the additive homoeolog contribution from either A or B was highest.

For polyploid genomes such as wheat, the interaction of its sub-genomes is known to affect the final phenotype or contribute towards trait development (Borrill et al., 2015). To check the homoeolog/sub-genome expression biasness under –Fe condition, effect on the expression of transcripts form the genomes was performed by comparing the relative normalised expression for each homoeolog within a triad. This resulted seven combinations including one balanced category and six homoeolog specific dominance or suppression. Our analysis reflected that most of the triads were falling under the balanced category (Table 3 and Fig.3C). This category was represented by 77% and 77.89% of the total triads for control and Fe starvation condition. Triads with unbalanced expression varied in the range of 0.94 to 7.09 for all the 6 sub-categories across the two conditions (Table 3). Our analysis revealed that maximum genome specific biasness was observed for A and B on both the conditions. Interestingly, D genome was least suppressed with a representation of 5.15 % of the total
triads taken in consideration in control when compared to 4.93 % in Fe starvation. 89 %
triads showed conserved balanced/unbalanced contribution across both conditions. Overall, a
higher relative abundance of D genome (Control: 33.94 %; Fe starvation: 33.95 %) was noted
as compared to the A (Control 33.06 %; Fe starvation: 33.00 %) and B (Control: 33.04 %; Fe
starvation: 33.95 %) genomes.

Identification of genes differentially regulated during –Fe in wheat
To identify the transcripts those are differentially expressed in roots in response to Fe
starvation, top 50 genes either up-regulated or down-regulated were shortlisted (Fig. 4). Expression of most of the highly up-regulated transcripts ranged from 12 to 4.8 log2 fold
change (Supplementary Table S3) indicating their higher fold accumulation under Fe
starvation compared to the control conditions. Categorically, these highly induced genes
conge to the sub-family of nicotinamine synthase (NAS). On the similar lines, deoxymugineic acids (DMA) biosynthesis genes were highly induced under Fe starvation
(Fig. 4, left panel). Genes belonging to the Major Facilitator Superfamily including an ABC
transporter, zinc-induced facilitator like transporters (ZIFL), sulphate transporters were also
induced under Fe limiting condition. Others transcripts encoding for thaumatin like proteins
etc. were also induced. The down-regulation of genes ranged from -7.1 to -2.85-fold at the
level of log2 scale (Supplementary Table S3). Interestingly, multiple genes encoding for
nitrate transporters were highly down-regulated under Fe limiting condition (Fig. 4, right
panel). Two genes encoding for cytochrome-P450 also showed down-regulation. Among the
others, genes encoding for Histone deacetylase, peptidases A1 containing domain protein,
cinnamyl alcohol dehydrogenase, dirigent protein were also highly down-regulated. In
addition to this, forty-five genes showed exclusive transcript abundance under Fe starvation
(Supplementary Table S3). No transcript for any of these genes was detected in the control
root samples, suggesting their high specificity for Fe response. Some of the transcripts
responding to Fe starvation encodes for metallothionine, metal transporters, vacuolar iron
transporters (VIT1) and nicotianamine synthase 2 (NAS2). This suggests that in wheat few
components involved in Fe uptake could actually respond exclusive to Fe starvation.

Identification of transcriptional regulatory genes during Fe starvation
To address an important question on how the –Fe signal is connected to the transcriptional
machinery in wheat, genes encoding TFs were identified. Earlier the TFs involved in the
response to Fe starvation were identified in model plant Arabidopsis including POPEYE
(PYE), basic helix-loop helix (bHLH), FIT, ethylene insensitive-3 (EIN3), EIN3-like1 (EIL1) 
(Long et al., 2010, Mai et al., 2016, Bauer et al., 2011, Ivanov et al., 2012). Consistently, our 
transcriptome analysis showed that all homoeologs for PYE encoding a bHLH transcription 
factor and BRUTUS-like (BTS) that is a putative hemerythrin E3 ligase protein show higher 
expression in wheat roots subjected to Fe starvation (Supplementary Table S5). The analysis 
of regulatory genes those are highly influenced by Fe starvation in wheat let to identification 
of 41 significantly up-regulated TFs family members (>log2FC) (Supplementary Table S5). 
The TFs family members belong to categories like APETALA2 ethylene-responsive element 
binding proteins (AP2/EREBP), WRKY, C2H2, Zinc finger proteins (including C3HC4), 
NAM, bHLH hemerythrin and U-box (Fig. 5A). Out of these, transcripts encoding for 
AP2/EREBP followed by WRKY and bHLH were the most abundant in wheat. Genes 
encoding for AP2-EREBP, WRKY and C2H2 type of TFs also showed high predominance 
(Supplementary Table S5 and Supplementary Fig. S1). However, TFs like MADS, PHD and 
homeobox (HB) domain containing proteins largely represented high number of down- 
regulated genes. Finally, to ascertain regulatory functions under iron starvation, enrichment 
mapping of significant DEGs those were classified into transcriptional factors category was 
performed. Interestingly, genes involved in regulation of gene expression were clustered with 
primary and nucleotide metabolism related genes in both positive and negative correlation. 
This functional cluster was in positive correlation with TFs involved in gene ontology (GO) 
categories such as “vacuolar transport” and “DNA topoisomerase III activity”. However, two 
separate clusters “cellular response to auxin stimulus and signalling” and “cell-fate 
specification” were in negative correlation with “regulation of gene expression” and; 
“primary and nucleotide metabolism” (Fig. 5B). This suggested important regulatory role of 
TFs involved in auxin signalling and cell-fate specification in Fe starvation response control, 
thereby controlling the network of Fe homeostasis.

Functional enrichment network of Fe starved related genes

Gene ontology annotations and classification of DEGs was performed to get the overview of 
processes those are representation of cellular, molecular and biological functions. Analysis 
was further extended to cluster analysis using Cytoscape plugins, BINGO and Enrichment 
Map (Maere et al., 2005; Merico et al., 2010). A total of 5854 DEG’s were found to be 
differentially-expressed in response to Fe starvation; significant GO categories were assigned 
to all DEGs. The DEGs annotated for GO terms were visualized using WEGO tool (Fig. 6A). 
For molecular function, twenty terms were categorized, most were catalytic activity,
heterocyclic compound binding, organic cyclic compound binding, ion binding. Twenty-four biological processes mostly representing metabolic process were identified (Supplementary Table S6). For cellular component, 14 terms were categorized, most common being membrane, membrane part and intrinsic component of membrane (Fig. 5A). Overall, catalytic activity and binding activities were most significantly enriched GO terms in –Fe condition (Fig. 6B). Further mapping of DEGs to databases such as, Kyoto encyclopedia of genes and genomes (KEGG) pathway (Xie et al., 2011) and MapManv (Thimm et al., 2004) revealed enrichment related to phenyl-propanoid biosynthesis, amino acid biosynthesis and carbon metabolism, and glutathione metabolism (Fig. 6C and Supplementary Fig. S2). The role of glutathione in response to Fe starvation is intriguing and deserves further investigations.

GO category “integral to membrane” found to be associated with pathways clustered into “metal ion and trans-membrane transport” and “photosynthesis” related GO categories (Fig. 6A). Clustered pathways involved in “response to nutrient levels” and “Sulphur amino acid metabolism” was also enriched (Fig. 7A). Few other up-regulated clustered pathway genes involved fall into the category “response to phosphate starvation”. In the enriched network of down-regulated DEGs, genes involved in iron and other metal homeostasis were associated with two different clusters, mainly included genes related to lipid, ketone and carboxylic metabolism, in addition to those involved in nitric acid and salicylic acid response (Fig. 7B). Second cluster contains transcripts related to purine and adenine nucleotide binding and pyrophosphatase activity. Thus, whole set of significant up-regulated and down-regulated DEGs were clustered in distinct cellular processes suggesting differential transcriptional response under Fe starvation.

Prolific expression of genes involved in strategy-II mode of Fe uptake in wheat

In general, the predominance of pathway genes encoding the components for strategy-II mode Fe uptake was observed during starvation. Especially, genes encoding for NAS, metallothionein, probable metal transporters and other gene families of transporters were highly induced under Fe starvation in the roots (Supplementary Table S6). All the genes encoding for S-adenosylmethionine, a precursor of mugineic acid biosynthesis and its pathway genes were highly induced in roots under Fe starved condition. In addition to extremely elevated expression of numerous TaNAS transcripts, a few genes were exclusively detected under Fe stress. All the homoeologs of genes encoding for nicotianamine aminotransferase-TaNAAT and TaDMAS localized on chromosome 1 and 4 respectively were highly expressed under Fe starvation. Similarly, YSL genes also showed significant higher
transcript accumulation under Fe starvation condition. Especially, all the homoeologs of *TaYSL9* and *TaYSL1A* showed high expression under starvation condition (Supplementary Table S7). Therefore, under Fe starvation, the DMAS biosynthesis genes were highly induced. On the similar lines, genes encoding for NRAMP also showed high transcript abundance in roots subjected to Fe starvation (Table S7). The validation of the expression of few strategy-II uptake genes was also done by quantitative real time-PCR (qRT-PCR). Our results for qRT-PCR validate our inference from the RNAseq analysis. Almost all of the strategy-II genes tested for their expression showed a very high fold expression in Fe starved roots as compared to the control (Supplementary Fig. S3). Highest expression was obtained for ZIFL4, DMAS1, NAAT1, NAS1 those are the prime components for strategy-II mediated uptake.

Interestingly, transcripts of few genes involved in the strategy-I mode of Fe uptake were also present (Supplementary Table S8). An important component of strategy-I pathway that includes H⁺-ATPase (AHA) subfamily genes was not differentially expressed under Fe limiting conditions (Supplementary Table S8). This was in agreement with the qRT-PCR analysis where, either no change or down-regulation of AHA genes was observed (Supplementary Fig. S3) in wheat roots. Metallo-reductases are important components of strategy-I represented by ferric-chelate reductase (FRO). Most of the transcripts encoding for wheat FROs do not show significant changes in starved roots as compared to control except for one transcript. Surprisingly, iron regulated transporters (IRTs; TRIAE_CS42_7DS_TGACv1_622068_AA2032200; TRIAE_CS42_4AL_TGACv1_289466_AA0971640) were significantly expressed in Fe starved wheat roots, suggesting its involvement in wheat under metal stress. The high expression of *TaIRT1* was also confirmed by qRT-PCR (Supplementary Fig. S3).

Glutathione-S-transferases are involved in the response to Fe starvation in wheat

Glutathione-mediated conjugation of multiple metabolites plays important role during the metal stress (Zhang et al., 2013). Our transcriptome data revealed the enhanced expression of multiple glutathione-S-transferases (GST) in Fe-starved wheat root as compared to control condition (Supplementary Table S9). To correlate the expression response with its enzymatic activity, temporal response of GST was measured in wheat roots under –Fe conditions. Using the GST functional assay, the activity was determined in wheat roots of plants grown for 10, 15 and 20 days after Fe starvation. Our activity assays showed significant increase in the GST activity under –Fe condition, which peaked at 10 and 20 days after the beginning of Fe
starvation compared to control plants grown in presence of Fe (Fig. 8A). Therefore, our data
demonstrate that the increase of GST transcripts abundance is correlated with a significant
increase in glutathione activity. This result is indicative for an important role of glutathione
in response to Fe starvation in wheat.

Fe starvation causes an accumulation of organic acids and polyhydroxy acids in wheat
To obtain a comparative insight of the metabolite profile of wheat roots of plants grown in
absence or presence of Fe; GC-MS profiling analysis was performed. Metabolites were
extracted from the roots of three replicate pools of plants each containing eight seedlings.
Qualitative processing of each chromatogram for peak area and identification was performed
in MassHunter version B.05.00 software coupled with NIST11 compound library. The
compound annotation was determined by comparing individual resolved peaks to library
searches based on mass spectra and compounds chromatographic retention indices.
Interestingly, analysis resulted in identification of 54 metabolites and further 39 annotated
metabolites were analysed for their response ratio (Supplementary Table S10). To compare
the change under Fe-starvation, metabolite abundances (-Fe roots/control roots) were
calculated and expressed in Log2 fold change values. Fe-starvation significantly affected
accumulation of 22 metabolites that includes organic acids, polyhydroxy acids, amino acids
and some of the sugars, fatty acids and phosphates (Fig. 8B). Amongst treatment-specific
changes, few organic acids such as fumaric acid, acetic acid and malonic acid showed higher
level of accumulation in Fe starved roots as compared to control. In contrast, citric acid,
malic acid, valeric acid and aconitic acid were significantly lowered in Fe starved roots when
compared to control samples. In Fe-starved roots, the accumulation of amino acids mainly, L-
Valine showed significant increase while hydroxyl butyric acid and pyroglutamic acid was
lowered in comparison with control roots. Polyhydroxy acids like gluconic acid and glyceric
acid were also significantly high in Fe starved roots, whereas level of hexonic acid and
arabinoic acid was found to be low. Taken together, our results showed that during Fe
starvation wheat roots undergo reprogramming for metabolic changes to maintain the Fe-flux.

Discussion
How plants maintain nutrient homeostasis is a fascinating question in plant biology. In this
direction, A. thaliana, with its fully sequenced small genome has provided some basic
information. Developing our knowledge on nutrient homeostasis in crops, especially those
having complex genome, such as hexaploid wheat is more challenging. This is due the
complexity of the genome and unavailability of genomic sequence until recently. Nevertheless, lately the development of genome sequence information and genomics-based techniques reduced these barriers. In this context, the current study was undertaken to gain insight into the response of hexaploid wheat exposed to Fe starvation that is known to severely affect crop production. In this study we report the first transcriptome of wheat (cultivar C306) in response to Fe starvation. Our analysis revealed that wheat a) utilizes primarily strategy-II mode of Fe uptake, b) accumulates transcripts encoding for methionine-salvage pathway coupled with enhanced GST activity and c) accumulates specific metabolites including malonic acid, xylofuranose, so as to efficiently mobilize soil Fe. Our systematic analysis of expression data revealed that transcripts show induction biasness for A and B genomes of wheat in response to –Fe condition. Overall, this work provides first insight for the molecular changes occurring in roots of Fe-starved wheat.

Wheat subjected to Fe starvation show physiological defect such as decrease in the root growth. This phenotype was consistent with the previous report showing negative impact on the root growth of wheat seedlings under Fe stress (Garnica et al., 2018). Our analysis also suggested down-regulation of few nitrate transporters those were previously referred as TaNRT2.2 and TaNRT2.3 (Buchner and Hawkesford, 2014). These observations suggest that Fe starvation represses nitrate transporters, which in turn leads to lower accumulation of nitrate level in roots (Supplementary Fig. S4). Although impact of Fe starvation on the nitrate metabolism has not been studied in detail, yet similar decrease in nitrate levels were also observed in cucumber shoots subjected to –Fe condition (Borlotti et al., 2012). This observation further reinforces the existence of an interaction between nutrients in plants, which is recently gaining attention (Rouached and Rhee, 2017).

Due to the low availability of micronutrients in soil, plants are equipped in recruiting two major modes of strategy for Fe uptake (Kobayashi and Nishizawa, 2012). Cereals such as maize predominantly utilize strategy-II mode of uptake, unlike rice, which is characterized for utilizing the combination of both the strategy (I and II) for uptake (Li et al., 2014; Zanin et al., 2017). Nevertheless, the comprehensive study to identify the molecular players involved during Fe limiting conditions in wheat was not yet attempted. Our RNAseq based analysis, strongly suggested an increase in the transcript abundance of genes for strategy-II mode of Fe uptake. Worth to mention, with the exception of conserved genes like IRT1 (iron regulated transporter) prime genes for strategy-I uptake mechanism like FRO (Ferric reduction oxidase), proton H-ATPase (AHA-like) also present during starved condition but at very low abundance. These results signify that wheat utilizes strategy-II mediated uptake of
Fe through roots and the IRTs might be conserved in its function for Fe transport across the plant kingdom.

The series of events during strategy-II uptake mechanism used by some plant species involves the secretion of certain specific metabolites that facilitate conjugation of Fe to form Fe-complexes. Transport of these Fe-complexes occurs via membrane transporters including ZIFLs (Nozoye et al., 2011; Nozoye et al., 2015). Wheat ZIFL gene shows high transcript accumulation and is closest homolog of OsTOM1 from rice, thereby speculating its role in Fe acquisition. Utilizing gain- and loss of function approaches OsTOM1 has been demonstrated to be a DMA effluxer for its role in enhancing mobilization and thereby improving Fe uptake (Nozoye et al., 2011). Altogether, the high similarity and response under Fe starvation, it is tempting to propose wheat ZIFL (ZIFL4) as a functional transporter of DMA. Although, other ZIFL were also significantly expressed but functional wheat transporter for DMA needs to be deciphered. Upon careful analysis of the RNAseq data, it was observed that multiple such efflux transporters are represented in the list that are highly abundant under Fe starvation. During our study, we identified TaDMAS1 encoding for deoxymugineic acid synthase that was highly responsive for Fe starvation (Supplementary TableS7). Earlier, TaDMAS1 was reported as a gene that was broadly expressed across the tissue and was regulated during Fe-starved condition. These observations support the notion that TaDMAS1 has potential to enhance the seed iron storage capacity (Beasely et al., 2017). Previous studies have shown that the phytosiderophores release mechanism in wheat is effective when the three genomes A, B and D come together indicating synergistic action to induce its biosynthesis and release. During our analysis, genome induction biasness for A and B genome of the hexaploid-wheat was observed. Interestingly, these genomes were also suggested to be important during starvation of other micronutrients like Zn (Tolay et al., 2001). These observations provide clue for the importance of A and B genomes during micronutrient starvation in hexaploid wheat when compared to their progenitor. In depth analysis confirms the minimal suppression for the expression bias for the D-genome derived transcripts as compared to A or B. These observations support the previous analysis wherein, suppression of D genome was significantly less frequent in multiple tissue (Ramirez-Gonzalez et al., 2018). Therefore, such studies are important to pin-point actively expressed/induced homoeolog that could be target for genetic improvement. However, the genome biasness and its association with the secretion of siderophores, genotypic variability in a complex wheat genome is yet to be investigated.
On the similar lines, multiple genes for the salvage pathway were highly up-regulated including NAS1, NAS2 and NAAT1 (Supplementary Table S3 and Supplementary Table S7). The contribution of these transcripts arises from different genomes suggesting that all the genomes of wheat are capable of responding to the starvation condition. Based on our RNAseq analysis it is likely that the gene encoding for TaYS1A and TaYSL9 could be the putative transporter for Fe-siderophore complex. Previous, studies have indicated the high response of TaYS1A under Fe starvation in roots and shoots (Supplementary Fig. S3 and Kumar et al., 2018). Out of the closest orthologs of wheat-YS1A, HvYS1A and ZmYS1A, HvYS1A has been shown to be involved in specific transport of Fe whereas, ZmYS1 was reported to have broad substrate specificity (Schaaf et al., 2004). Worth to mention that our RNAseq data revealed a conserved function of genes from monocots and dicots during the Fe regulation and known important Fe regulators such as PYE and BRUTUS also showed differential expression in wheat roots (Ivanov et al., 2012). Same for TFs spanning to gene families such as MYB (Rubio et al., 2001), bHLH (Colangelo et al., 2004; Jakoby et al., 2004; Ogo et al., 2007), C2H2, NAC, AP2-ERB (Kim et al., 2012), and WRKY (Devaiah et al., 2007) were characterized in Arabidopsis and rice for their involvement in nutrient uptake which were found to be highly represented in root of Fe-starved wheat. Overall, based on the information obtained from our studies future research focus should be to gain better insights of the molecular functions of these genes using tilling population in wheat and/or heterologous system.

Members of ABC-B transporters were also reported to participate in oxidative stress that was linked with metal stress including Fe starvation (Kispal et al., 1997; Schaedler et al., 2014). During our analysis we have identified an ABC-B subfamily transporter referred as TaABCB25 that showed very high expression during Fe starvation (Fig. 4). The closest ortholog for wheat ABCB25 transporters were identified in rice (OsABCB25), Arabidopsis (AtABCB27) and yeast (Atm1) (Supplementary Fig. S5A). The temporal-induction of TaABCC25 was also confirmed in roots subjected to Fe starvation (Supplementary Fig. S5B). Interestingly, wheat ABCB25 ortholog in yeast referred as Atm encode a mitochondrial ABC transporter (Atm1p) is involved in the transport of metals bound to glutathione derivatives into vacuoles and also involved in maturation of Fe-sulphur cluster (Kispal et al., 1997; Sipos et al., 2002). Similarly, Arabidopsis thaliana ABCB27 was shown to have the ability to transport glutathione conjugate metabolite precursors for the Fe-Sulfur cluster assembly (Schaedler et al., 2014). Based on the presence of domain structure these ABCB transporters from rice, Arabidopsis and wheat qualify as half-size transporter (Supplementary Fig. S5C).
In graminaceous species, glutathione related activity was observed to be highly up-regulated under Fe deficient condition supporting our observation in the current study (Bashir et al., 2007). Enhanced GST activity in wheat under Fe starvation and abundance of TaABCB25 in roots, warrants further study of this gene for its role in mobilizing micronutrient uptake. Glutathione and GST plays an essential role during Fe starvation related responses in Arabidopsis and also quench reactive molecules to protect the cell from oxidative damage and prevent chlorophyll loss (Ramirez et al., 2013; Shanmugan et al., 2015; Kumar and Trivedi, 2018). Our analysis also suggests that high transcript and enzyme activity of GST could be linked with the primary metabolism by accumulating fumarate. In Arabidopsis, it has been shown that GST catalyses Glutathione-dependent isomerization of maleyl-acetoacetate into fumaryl-acetoacetate eventually leading to accumulation of fumarate and acetoacetate (Dixon et al., 2002). Altogether, this supports the notion that wheat ABC transporters might play a significant role in mobilization of metabolite/organic acid or siderophores during Fe limiting conditions.

A schematic representation of data provides a summary of the metabolic and molecular events that occur during Fe starvation in wheat roots (Fig. 9). In conclusion, in this study, the core components for Fe starvation response in hexaploid wheat have been identified to provide a better understanding on the molecular events that participate during Fe starvation in wheat roots. The information here will help to design strategy not only to improve plant response to Fe starvation in wheat, but also to foster Fe uptake and accumulation, which is required to boost productivity and grain nutritional quality through Fe biofortification programs. Complementary approaches, analytical and transcriptome analysis reinforce the importance of primary metabolism for reprogramming organic acids and amino acids thereby responding to Fe homeostasis in wheat. In particular, we provide line of evidence for the role of GST in the response to –Fe in roots. Taken together, data presented in this study will help in deepening our insight and design strategy to improve plant response to Fe starvation, enhance Fe uptake and accumulation, which are required to improve the productivity and grain nutritional quality through Fe biofortification programs.

Acknowledgement
Authors thank Executive Director for facilities and support. This research was funded by the NABI-CORE grant to AKP and partial support from DST-SERB grant (PDF/2016/001355) to PG. GK and AK acknowledge NABI-JRF Fellowships. Technical help from Jagdeep for the GC-MS analysis is highly appreciated. DBT-eLibrary Consortium (DeLCON) is
acknowledged for providing timely support and access to e-resources for this work. The wheat genome resources developed by International Wheat Genome Sequencing Consortium is highly appreciated. We would like to thank Abhijeet Panwar (CDAC-Pune) for his help in the preparation of the Sankey plot.

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Table 1: Metal concentration in roots and shoots of wheat seedlings subjected to –Fe stress.

| Treatments | ROOTS | | | | | SHOOTS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Fe | Zn | Mn | Mg | Cu | Fe | Zn | Mn | Mg | Cu |
| +Fe+P | 106.73 ± 14.2 | 14.6 ± 3.2 | 15.8 ± 2.0 | 542 ± 70 | 7.4 ± 1.1 | 72.9 ± 1.7 | 26.8 ± 0.9 | 17.4 ± 0.5 | 1031 ± 2.3 | 10.4 ± 0.6 |
| -Fe+P | 66.8 ± 17 | 40.4 ± 12 | 38.2 ± 8.9 | 912 ± 213 | 17.9 ± 3.7 | 28.6 ± 1.6 | 22.3 ± 1.05 | 22.1 ± 1.9 | 1013 ± 48 | 5.1 ± 0.3 |

Table 2: List of genes showing bias expression contribution from either A or B genome.

| Gene | logFold change | RAP-DB description |
|---|---|---|
| A genome |
| TRIAE_CS42_3AL_TGACv1_197291_AA0665720 | 3.01 | Myb transcription factor domain containing protein. |
| TRIAE_CS42_2AL_TGACv1_094922_AA0304900 | 2.8 | Similar to Prolyl endopeptidase (EC 3.4.21.26) (Post-proline cleaving enzyme) (PE). |
| TRIAE_CS42_5AL_TGACv1_379416_AA1256390 | 2.44 | Similar to Solute carrier family 35, member F1. |
| TRIAE_CS42_2AL_TGACv1_094606_AA0300340 | 2.18 | RmlC-like jelly roll fold domain containing protein. |
| TRIAE_CS42_3AS_TGACv1_211026_AA0683370 | 3.36 | Protein of unknown function DUF1399 family protein. |
| TRIAE_CS42_7AL_TGACv1_557470_AA1781720 | 3.47 | Heavy metal-transporting P1B-ATPase, Root-to-shoot cadmium (Cd) translocation |
| TRIAE_CS42_6AL_TGACv1_471682_AA1512850 | 2.26 | Similar to CRT/DRE binding factor 1. |
| TRIAE_CS42_6AS_TGACv1_485332_AA1543320 | 1.85 | Zinc/iron permease family protein. |
| TRIAE_CS42_2AL_TGACv1_092944_AA0267670 | 2.43 | Heavy metal transport/detoxification protein domain containing protein. |
| TRIAE_CS42_5AL_TGACv1_375378_AA1220660 | 2.21 | Similar to SUSIBA2 (WRKY protein). |
| TRIAE_CS42_1AL_TGACv1_000708_AA0017440 | 2.57 | Chlorophyll a-b binding protein 2, chloroplast precursor (LHClII type 1 CAB-2) (LHCP). |
| Genomic Location | LogLik | Gene Name and Description |
|------------------|--------|---------------------------|
| TRIAE_CS42_2AL_TGACv1_093154_AA0273510 | 1.86 | C2 domain containing protein. |
| TRIAE_CS42_5AL_TGACv1_374025_AA1188170 | 2.66 | Nodulin-like domain containing protein. |
| TRIAE_CS42_3AL_TGACv1_197522_AA0666570 | 2.22 | Lipase, GDSL domain containing protein. |
| TRIAE_CS42_7AL_TGACv1_556100_AA1755640 | 2.34 | Bifunctional inhibitor/plant lipid transfer protein/seed storage domain containing protein. |
| TRIAE_CS42_4AS_TGACv1_306527_AA1009640 | 2.2 | Multi antimicrobial extrusion protein MatE family protein. |
| TRIAE_CS42_7AS_TGACv1_570336_AA1188170 | 1.98 | Major facilitator superfamily protein. |
| TRIAE_CS42_2AL_TGACv1_093456_AA0280470 | 2.15 | Glycolipid transfer protein domain domain containing protein. |
| TRIAE_CS42_1AL_TGACv1_002205_AA0039730 | -2.09 | Similar to IAA8 (Fragment). |
| TRIAE_CS42_4AL_TGACv1_290815_AA0039730 | -2 | Lipase, class 3 family protein. |
| TRIAE_CS42_5AL_TGACv1_373539_AA1197930 | -2.24 | Cinnamyl alcohol dehydrogenase (EC 1.1.1.195). |
| TRIAE_CS42_6AL_TGACv1_472629_AA1524260 | -2.36 | Similar to Lipoxygenase L2 (EC 1.13.11.12). |
| TRIAE_CS42_7AL_TGACv1_558250_AA1791610 | -1.89 | RAG1-activating protein 1 homologue domain containing protein. |
| TRIAE_CS42_7AL_TGACv1_558250_AA1791610 | -2.32 | Similar to Pleiotropic drug resistance protein 3. |
| TRIAE_CS42_2AL_TGACv1_097246_AA0323500 | -1.72 | Similar to Peroxidase (EC 1.11.1.7). |
| TRIAE_CS42_7AL_TGACv1_556210_AA1758330 | -2.21 | Similar to Kaurene synthase A (Fragment). |
| TRIAE_CS42_1AL_TGACv1_000555_AA0014640 | -1.84 | No apical meristem (NAM) protein domain containing protein. |
| TRIAE_CS42_6AL_TGACv1_471077_AA15524260 | -1.79 | Similar to OSIGBa0145M07.8 protein. |
| TRIAE_CS42_7AL_TGACv1_559906_AA1801190 | -1.68 | Similar to H0801D08.12 protein. |
| TRIAE_CS42_7AL_TGACv1_558101_AA1790150 | -1.76 | Similar to Acyl-ACP thioesterase (Fragment). |
| TRIAE_CS42_6AL_TGACv1_472321_AA1520860 | -2.06 | Similar to Subtilisin-like protease (Fragment). |

**B Genome**

| Genomic Location | LogLik | Gene Name and Description |
|------------------|--------|---------------------------|
| TRIAE_CS42_5BS_TGACv1_423346_AA1374840 | 2.19 | Similar to Calmodulin NtCaM13. |
| TRIAE_CS42_3B_TGACv1_224030_AA0791180 | 2.2 | Similar to IN2-2 protein. |
| TRIAE_CS42_2BL_TGACv1_129296_AA0323500 | 2.46 | Similar to OSIGBa0127A14.7 protein. |
| TRIAE_CS42_6BL_TGACv1_499646_AA1588130 | 2.32 | TGF-beta receptor, type I/II extracellular region family protein. |
| TRIAE_CS42_7BS_TGACv1_592587_AA1940840 | 2.25 | Similar to RING-H2 finger protein ATL1R (RING-H2 finger protein ATL8). |
| TRIAE_CS42_2BS_TGACv1_146290_AA0461590 | 2.64 | Similar to Senescence-associated protein SAG102. |
| TRIAE_CS42_5BS_TGACv1_404610_AA1306090 | 1.84 | Similar to Senescence-associated protein SAG102. |
| TRIAE_CS42_5BL_TGACv1_405319_AA1324840 | 2.77 | Similar to Transporter associated with antigen processing-like protein. |
| TRIAE_CS42_7BL_TGACv1_577614_AA1879540 | 2.69 | Peptidase A1 domain containing protein. |
| TRIAE_CS42_4BS_TGACv1_329166_AA1098520 | 2.32 | Similar to Alcohol dehydrogenase. |
| TRIAE_CS42_4BL_TGACv1_321683_AA1064050 | 2.56 | Protein of unknown function DUF1262 family protein. |
| TRIAE_CS42_7BL_TGACv1_591489_AA1920550 | NA | Similar to zinc transporter 4. |
| TRIAE_CS42_7BL_TGACv1_577301_AA1871590 | 2.41 | Delta-tonoplast intrinsic protein. |
| TRIAE_CS42_4BS_TGACv1_329309_AA1100040 | NA | Similar to Major facilitator superfamily antiporter. |
| TRIAE_CS42_2BL_TGACv1_129348_AA0379680 | 2. | Hypothetical conserved gene. |
| TRIAE_CS42_2BS_TGACv1_148847_AA0495340 | 4.34 | Helix-loop-helix DNA-binding domain containing protein. |
| TRIAE_CS42_2BL_TGACv1_130820_AA0418390 | 1.88 | |
| TRIAE_CS42_1BL_TGACv1_031794_AA0120680 | -2.79 | Divalent ion symporter domain containing protein. |
| TRIAE_CS42_5BL_TGACv1_406838_AA1305360 | -2.29 | Similar to anther-specific proline-rich protein APG. |
Table 3: Percentage of homoeolog triads categorised into ideal genome bias categories in control and Fe starved conditions.

|                | Control  | -Fe       |
|----------------|----------|-----------|
| Balanced       | 77.07%   | 77.89%    |
| A-suppressed   | 6.90%    | 6.90%     |
| B-suppressed   | 7.09%    | 6.82%     |
| D-suppressed   | 5.15%    | 4.93%     |
| A-dominant     | 1.12%    | 0.94%     |
| B-dominant     | 1.24%    | 1.18%     |
| D-dominant     | 1.43%    | 1.32%     |

Legends for Figures:

**Fig. 1.** Effect of Fe-starvation (-Fe) on the growth parameters of wheat seedlings post 20 days after starvation. (A) Phenotype of wheat seedlings exposed to Fe starvation. (B) Total biomass of roots and shoots of wheat seedlings after 20 DAS. 12-15 seedlings were collected for calculating the fresh tissue weight (in grams). (C) Number of first order lateral roots in roots subjected to –Fe condition and control plants (+Fe). (D) Primary root length of wheat roots. 10-12 seedlings were used for measuring the total primary root length of wheat seedlings under –Fe and +Fe condition. # indicate significant difference at p<0.05.

**Fig. 2.** Volcano plots, Principal component and analysis of differentially expressed genes modulated by Fe starvation in wheat roots. (A) Principal component analysis of samples from control and –Fe conditions. (B) Volcano plot of DEGs, the x-axis shows the fold change difference in the expression of genes in iron starved condition w.r.t. control, and the y-axis
indicates the adjusted P-values for the differences in expression. Genes without significant differences are indicated by grey dots. The up-regulated genes are represented by red dots, and the down-regulated genes are represented by green dots in the scatter plots. (C) Number of DEGs (-1 < logFC > 1) in –Fe;

**Fig. 3.** Genomics distribution and homoeolog bias studies during Fe starvation. (A) Chromosomal inclination of DEGs. (B) Pie charts showing (left panel) genome bias in triads where one of the homoeologs was up/down-regulated. A, B and D depict the sub-genome to which the DE homoeolog belongs; (right Panel) distribution of triads for which two of the homoeologs were differentially expressed. AB refers to the triads for which up/down regulation was observed in the homoeologs belonging to A and B subgenomes, while the D genome homoeolog behaved normal w.r.t control. Up/down regulation was observed in the homoeologs belonging to A and B sub-genomes, while the D genome homoeolog behaved normal with respect to control. (C) Sankey diagram depicting the homoeolog expression bias in Control and Fe starvation. Homoeolog triads were classified into seven categories based on relative normalised expression within each triad. Nodes flowing from Control to -Fe (Fe starvation) represent the triads with same as well as changed expression patterns across both conditions. Distinct colors represent the flow of triads belonging to the seven categories into same category under -Fe or transition into a different category.

**Fig. 4.** Gene ontology (GO) categories of the differentially expressed unigenes and its analysis. (A) WEGO plot describing GO annotation and classification of DEGs, with bars showing the number of genes belonging to respective terms (grey bars for down-regulated genes and red bars for up-regulated genes). Percentage and number of genes were calculated for the three broad main categories; (B) Enriched GO terms in DEGs under –Fe condition, y-axis depicting the significance of GO term enrichment. (C) Table showing top 20 enriched KEGG pathways in the up-regulated genes under iron starvation condition. Y-axis showing names of the pathways, x-axis showing number of genes enriched in respective pathways.

**Fig. 5.** Co-expression/hub genes and function enrichment network for identified DEG’s. Function enrichment network for DEG’s associated under Iron starvation in wheat roots with high significance (FDR ≤ 0.05) for (A) Up-regulated and (B) Down-regulated genes. Enriched GO functional categories are clustered with correlated DEG’s and represented by node circles.

**Fig. 6.** Top up-regulated and down-regulated genes in –Fe condition, annotated via KOBAS using rice RAP-DB/RefSeq annotations as reference. The heat map shows top 50 genes those are highly up-regulated (red-left panel) and down-regulated (green-right panel) genes
identified in wheat roots under –Fe condition with respect to control roots. For expression analysis, FPKM values were obtained using Cufflinks, and CuffDiff was used to identify DEGs by calculating significant changes in transcript expression between the stressed and normal samples (FDR≤0.05).

**Fig. 7.** Transcriptional factors (TFs) significantly associated with Fe starvation (FDR ≤ 0.05) in wheat roots. (A) List of TFs in response to Fe starvation stress. Blue lines represent up-regulated DEG’s and Red lines represent down-regulated DEG’s. (B) Co-expression/hub genes and network analysis using Fe responsive TFs (FDR ≤ 0.05) in wheat roots. Circles indicate the process associated with it (green for down-regulated genes; Blue for up-regulated genes). Enriched GO functional categories are clustered with correlated DEG’s and represented by node circles.

**Fig. 8.** Measurement of GST activity and metabolite profiling of wheat roots subjected to Fe starvation. (A) Glutathione-S-transferase activity of wheat roots under –Fe and +Fe condition. (B) Metabolite profiling of amino acids, sugars, polyols, organic acids and related compounds. Change in abundance of significant (P < 0.05) metabolites identified by GC-MS in Fe-starved roots. Abundance variation of each metabolite is represented in Log2 fold values of response ratio (-Fe/+Fe) of metabolite concentrations. Values are means of three biological replicates with bar representing Log ratio of standard error. * indicate significant difference at p<0.01; # indicate significant difference at p<0.05.

**Fig. 9.** Schematic representation of describing the core components involved in Fe starvation. The red font indicates the genes/metabolite those were highly up-regulated/high-accumulation during our study; whereas the green font indicates down-regulated/low accumulation. Methionine salvage pathway Bins indicate the level of the gene expression levels for the transcript indicated next to it. (Abbreviation used: FBP: fructose-1,6-bisphosphatase I, PFP: diphosphate-dependent phosphofructokinase, ALDO: fructose-bisphosphate aldolase, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, PGAM: 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase, PDC: pyruvate decarboxylase, GLYK: D-glycerate 3-kinase, GlyA: glycine hydroxymethyltransferase, ltaE: threonine aldolase, BMT2: homocysteine S-methyltransferase, metE: homocysteine methyltransferase, metK: S-adenosylmethionine synthetase, SamDC: S-adenosylmethionine decarboxylase, SRM: spermidine synthase, MTN: 5′-methylthioadenosine nucleosidase, mtnK: 5-methylthioribose kinase, mtnA: methylthioribose-1-phosphate isomerase, DEP1: enolase-phosphatase E1, mtnC: enolase-phosphatase E1, mtnD: 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase, TyrAT: tyrosine aminotransferase, NAS: nicotiamine synthase, NAAT: nicotiamine aminotransferase, DMAS: 3''-deamino-3''-oxonicotiamine reductase, YSL: Yellow Stripe Like, maiA: maleylacetoacetate isomerase, AAT: aspartate aminotransferase, TAT: tyrosine aminotransferase, HPD: 4-hydroxyphenylpyruvate dioxygenase, GGT: gamma-
glutamyltranspeptidase, AOS3: hydroperoxide dehydratase, OPR: 12-oxophytodienoic acid reductase, ACX: acyl-CoA oxidase, MFP2: enoyl-CoA hydratase, ACAT: acetyl-CoA acyltransferase 1)

Supporting information

**Supplementary Fig. S1:** MapMan visualization depicting the differentially expressed transcription factors families, with red and blue colored bins for up and down-regulated transcripts, respectively. Numbers in the scale represent fold changes in expression levels expressed as Log2.

**Supplementary Fig. S2:** Overview of genes modulated by the Fe starvation in wheat roots. MapMan overview demonstrating differentially expressed transcripts under iron starvation in general metabolic pathways. Log2 fold change values of DEGs were imported into MapMan. Red and blue bins represent up-regulation and down-regulation, respectively, in terms of log2 fold change, as shown by the scale.

**Supplementary Fig. S3:** qRT-PCR validation of selected genes form the DEG’s during Fe-deficient roots after 20 days of starvation. A total of 2 μg of RNA (DNA free) was used for cDNA synthesis and qRT-PCR was performed using gene specific primers (Supplementary Table S1). C\textsubscript{T} values were normalized against wheat ARF\textsubscript{1} as an internal control.

**Supplementary Fig. S4:** Estimation of Nitrate levels under Fe-starvation using salicylic acid method. Three Biological replicate root samples of 10, 15 and 20 days after starvation (DAS) were completely dried for the extraction. Yellow coloration (in test tubes: lower panel) represents level of nitrate in the sample. Potassium Nitrate was used as standard in 0-70 μg concentrations.

**Supplementary Fig. S5:** Characterization of wheat ABCB25 transporter. (A) Phylogeny analysis of TaABCB25 along with its closest orthologs from rice, arabidopsis and yeast. (B) Expression analysis of TaABCC25 in roots of wheat seedlings subjected to Fe starvation. Wheat seedlings (5-7 days old) were subjected to Fe stress and samples were harvested after 12 hour, 3, 6, 9 and 15 days(d) post starvation. The relative qRT-PCR was performed using wheat ADP-ribosylation factor (ARF) as an internal control gene. Fold accumulation was calculated with respect to the control roots. (C) Schematic comparision of different domains of AtABCB27, OsABCB25 and TaABC25. The number indicates the predicted amino acid position of the TM domains.

**Supplementary Table S1:** List of primers used in the current study. *wheat genes named according to rice RAP-DB/RefSeq based on KOBAS annotation.
Supplementary Table S2: Summary of filtered and mapped reads for each sample. Obtained RNA-seq reads were quality filtered using Trimmomatic v0.35. TopHat was used to map the obtained reads to the wheat genome (TGACv1).

Supplementary Table S3: DEGs in response to Fe starvation in wheat roots List of up-regulated genes, downregulated genes (sheet 2), genes exclusively expressed in response to Fe starvation (sheet 3). Table enlists Control and Fe starvation expression values, logFC for Fe starvation wrt Control samples. Each DEG is annotated with information like rice ortholog, gene definition, KEGG Orthology, Pathways and Pfam domains, which were obtained through KOBAS 3.0 stand-alone tool, using Oryza sativa RAP-DB and RefSeq as reference.

Supplementary Table S4: List of 8473 homoeolog triads that were used for homoeolog induction and expression biasness analysis.

Supplementary Table S5: Expression profiles of genes/gene of different transcription factors those are differentially Up- and down regulated. MapMan was used to identify TFs and categorize them into TF families. Table gives logFC value for starvation vs control for each TF showing significantly altered expression. A gradient of red and green is used for up-regulated and down-regulated TFs respectively.

Supplementary Table S6: Gene Ontology analysis of up- and down-regulated genes in response to Fe starvation. WEGO tool was used to categorize DEGs into GO categories and identify significant GO terms. Table lists the number and percentage of up- and down-regulated genes as well as p-values for each GO term.

Supplementary Table S7: Expression profiles of genes/gene families involved in strategy-II mode of Fe uptake. Strategy-II components of Fe uptake were identified and classified based on screening wheat genes annotated by KOBAS for respective KO IDs for NAS, NAAT, YSL and DMAS genes.

Supplementary Table S8: Expression profiles of genes/gene families involved in strategy-I mode of Fe uptake. Strategy-I components of Fe uptake were identified and classified based on screening wheat genes annotated by KOBAS for respective KO IDs for AHA, IRT, FRO, PEZ genes.

Supplementary Table S9: Expression profiles of genes involved during the process of glutathione mediated detoxification process. DEGs mapped to glutathione metabolism were identified using KOBAS. Table lists expression values and annotation for genes. Red color denotes up-regulated genes and green color represent down-regulation.
Supplementary Table S10: GC-MS analysis of wheat roots subjected to 20 days of Fe starvation.
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 9