



# Iron deficiency triggered transcriptome changes in bread wheat

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## ABSTRACT

A series of complex transport, storage and regulation mechanisms control iron metabolism and thereby maintain iron homeostasis in plants. Despite several studies on iron deficiency responses in different plant species, these mechanisms remain unclear in the allohexaploid wheat, which is the most widely cultivated commercial crop. We used RNA sequencing to reveal transcriptomic changes in the wheat flag leaves and roots, when subjected to iron limited conditions. We identified 5969 and 2591 differentially expressed genes (DEGs) in the flag leaves and roots, respectively. Genes involved in the synthesis of iron ligands i.e., nicotianamine (NA) and deoxymugineic acid (DMA) were significantly up-regulated during iron deficiency. In total, 337 and 635 genes encoding transporters exhibited altered expression in roots and flag leaves, respectively. Several genes related to MAJOR FACILITATOR SUPERFAMILY (MFS), ATP-BINDING CASSETTE (ABC) transporter superfamily, NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (NRAMP) family and OLIGOPEPTIDE TRANSPORTER (OPT) family were regulated, indicating their important roles in combating iron deficiency stress. Among the regulatory factors, the genes encoding for transcription factors of BASIC HELIX-LOOP-HELIX (bHLH) family were highly up-regulated in both roots and the flag leaves. The jasmonate biosynthesis pathway was significantly altered but with notable expression differences between roots and flag leaves. Homoeologs expression and induction bias analysis revealed subgenome specific differential expression. Our findings provide an integrated overview on regulated molecular processes in response to iron deficiency stress in wheat. This information could potentially serve as a guideline for breeding iron deficiency stress tolerant crops as well as for designing appropriate wheat iron biofortification strategies.

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## 1. Introduction

Iron (Fe) plays a pivotal role in several metabolic and biosynthetic pathways essential for plant growth. It not only functions

as a cofactor to some key enzymes such as cytochromes and hydrogenase, but is also involved in the electron transport chain of photosynthesis and respiration [1]. Fe deficiency in plants severely affects the overall crop yield. Several transcriptomic studies, using

**Abbreviations:** ABC, ATP-BINDING CASSETTE; AEC, AUXIN EFFLUX CARRIER; ACC, 1-aminocyclopropane-1-carboxylate; AOC, ALLENE OXIDE CYCLASE; AOS, ALLENE OXIDE SYNTHASE; AQP, AQUAPORIN; AVA, avenic acid; bHLH, BASIC HELIX-LOOP-HELIX; bZIP, BASIC LEUCINE ZIPPER; DEGs, differentially expressed genes; DMA, deoxymugineic acid; DMAS, DEOXYMUGINEIC ACID SYNTHASE; DPA, days post anthesis; ERF, ETHYLENE-RESPONSIVE FACTOR; epiHMA, 3-epihydroxymugineic acid; epiHDMA, 3-epihydroxy-2'-deoxymugineic acid; FAD, FATTY ACID DESATURASE; FDR, false discovery rate; FIT, FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR; FRO, FERRIC REDUCTASE OXIDASE; GCN, gene co-expression network; GO, Gene ontology; GSH, GLUTATHIONE; HC, high confidence; HMA, HEAVY METAL-ASSOCIATED; 3-HMA, 3-hydroxymugineic acid; IDE, iron deficiency-responsive *cis*-acting element; IDEF, IDE BINDING FACTOR; IHW, independent hypothesis weighting; ILR3, IAA-LEUCINE RESISTANT3; IREG/FPN, IRON REGULATED PROTEIN/FERROPORTIN; Iron, Fe; IRT1, IRON-REGULATED TRANSPORTER; JAs, jasmonates; JMT, JASMONATE O-METHYLTRANSFERASE; KAT, 3-KETOACYL-COA THIOLASE; LOX, LIPOXYGENASE; MA, mugineic acid; MATE, MULTI ANTIMICROBIAL EXTRUSION PROTEIN; MFS, MAJOR FACILITATOR SUPERFAMILY; MRP, MULTIDRUG RESISTANCE PROTEIN; MT, METALLOTHIONEIN; NA, nicotianamine; NAAT, NICOTIANAMINE AMINOTRANSFERASE; NAC, NO APICAL MERISTEM (NAM)/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR (ATAF)/CUP-SHAPED COTYLEDON (CUC); NAS, NICOTIANAMINE SYNTHASE; NRAMP, NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN; NRT1/PTR, NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER; OPCL, 4-COUMARATE COA LIGASE; OPR, 12-OXOPHYTYLDIENOATE REDUCTASE; OPT, OLIGOPEPTIDE TRANSPORTER; PDR, PLEIOTROPIC DRUG RESISTANCE; PLA, PHOSPHOLIPASE A1; PRI, POSITIVE REGULATOR OF IRON DEFICIENCY RESPONSE; P<sub>S</sub>s, phyto siderophores; PT, peptide transport; PYE, POPEYE; SAM, S-adenosyl-L-methionine; SAMS, S-ADENOSYL-L-METHIONINE SYNTHETASE; SLC40A1, SOLUTE CARRIER FAMILY 40 MEMBER 1; SWEET, SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS; TOM, TRANSPORTER OF MUGINEIC ACID; VIT, VACUOLAR IRON TRANSPORTER; YSL, YELLOW STRIPE LIKE; ZIFL, ZINC INDUCED FACILITATOR-LIKE; ZIP, ZINC/IRON PERMEASE.

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either qRT-PCR based expression analysis, microarray hybridization or RNA sequencing, have been conducted to characterize the mechanisms underlying iron deficiency induced response in different plant species, for example, in *Arabidopsis* [2–5], soybean [6–8], tomato [9], rice [10], barley [11,12], maize [13], and wheat [14–16]. It became evident with such studies that the uptake, transport and storage of iron in plants involve a systematic cooperation between tissues and cell organelles, as well as a fine coordination of iron chelators, transporters and several regulatory factors. Genome wide expression profiling of iron deficiency responses has not been systematically studied in bread wheat - one of the world's most important staple crop. In particular, the few studies reported so far analyzed the expression of some pre-selected genes [14] or transcriptome analysis in a single tissue from the plants at the seedling stage [15,16]. Comprehensive analysis of iron deficiency response in fully grown wheat plants, for example at the reproductive stage, is currently lacking. Such analyses are important because the plants' response to Fe deficiency at the commencement of grain filling would be crucial for overall yield.

Nicotianamine (NA) and deoxymugineic acid (DMA) are among the well-known iron chelators responsible for iron acquisition and translocation. Nicotianamine functions in long-distance as well as cellular iron transport universally in higher plants [17]. Nicotianamine also serves as an intermediate for the production of DMA, which is one of the types of phytosiderophores (PSs), produced specifically in graminaceous species for iron uptake and transport. Deoxymugineic acid is synthesized from S-adenosyl-L-methionine (SAM) via a conserved pathway of sequential enzymatic reactions mediated by NICOTIANAMINE SYNTHASE (NAS), NICOTIANAMINE AMINOTRANSFERASE (NAAT), and DEOXYMUGINEIC ACID SYNTHASE (DMAS) [18]. Deoxymugineic acid is either directly involved in iron transport and acquisition, or is further converted to other PSs such as mugineic acid (MA), 3-hydroxymugineic acid (3-HMA), avenic acid (AVA), 3-epihydroxy-2'-deoxymugineic acid (epiHDMA) and 3-epihydroxymugineic acid (epiHMA), depending on plant species [18,19]. In bread wheat, an increase in expression for most of the identified NAS gene copies was observed when plants were exposed to iron deficiency [20]. In addition, *TaNAAT1*, *TaNAAT2*, and *TaDMAS1* with three homologs each were identified, and their expression was up-regulated in wheat roots under iron deficiency [21].

Deoxymugineic acid is released into the rhizosphere by the efflux transporter TRANSPORTER OF MUGINEIC ACID 1 (TOM1), a member of the MAJOR FACILITATOR SUPERFAMILY (MFS) [22]. The Fe(III)-MA complex is transported back to roots by the members of YELLOW STRIPE LIKE (YSL) transporter family [23]. The YSL proteins belong to OLIGOPEPTIDE TRANSPORTER (OPT) family and have been widely studied in rice. The OsYSL15 is a plasma membrane-localized transporter which functions to transport Fe(III)-DMA from the rhizosphere [23,24]. The other YSL proteins including OsYSL2, OsYSL16 and OsYSL18 play a role in translocation of Fe(III)-DMA and/or Fe(II)-NA complexes [25–28]. The peptide transport (PT) clade, second subfamily of the OPT family, also transports Fe(II)-NA as well as Fe(III)-NA in rice [29].

Intracellular iron translocation is relatively less understood; however a few vacuolar and mitochondrial transporters have been identified in graminaceous species as well as in *Arabidopsis*. The NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (NRAMP) transporters export iron from the vacuoles in *Arabidopsis* [30] whereas the VACUOLAR IRON TRANSPORTERS (VITs) perform the opposite function in cereal species [31,32]. FERRITIN, mainly found in the plastids, encodes the main iron storage protein, and its expression level is down-regulated when *Arabidopsis* suffers from iron deficiency [2]. Among the regulatory factors, BASIC HELIX-LOOP-HELIX (bHLH) is the prominent transcription factor family reported to regulate iron deficiency responsive genes and

iron homeostasis in general [33]. Rice FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (OsFIT/OsbHLLH156) [34,35] interacts with OsIRO2 [36,37] to positively regulate iron deficiency responses. Rice OsbHLLH133 negatively regulates root to shoot iron translocation [38]. In rice, OsIRO3 serves as a negative regulator of iron deficiency response [39], and it is also regulated by POSITIVE REGULATOR OF IRON DEFICIENCY RESPONSE 1, 2 and 3 (OsPRI1/OsbHLLH060, OsPRI2/OsbHLLH058, and OsPRI3/OsbHLLH059) [40–42]. Additionally, the NO APICAL MERISTEM (NAM)/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR (ATAF)/CUP-SHAPED COTYLEDON (CUC) (NAC) and WRKY transcription factor families have also been identified as iron deficiency responsive [43,44].

In order to gain a comprehensive insight into molecular responses of bread wheat when exposed to iron deficiency, we studied transcriptomic changes in the roots and flag leaves of wheat plants subjected to iron-deficient and iron-sufficient conditions during early grain filling. A number of iron deficiency responsive genes and key regulatory factors were identified. Homoeolog expression and induction bias analysis between three subgenomes (A, B and D) of bread wheat was carried out. In addition, gene co-expression network was constructed to reveal specific gene expression associations. Our results serve as an important genetic resource for future investigations aimed at deciphering roles of individual genes as well as molecular mechanisms involved in iron homeostasis regulation in wheat and for designing optimal breeding strategies to protect yield losses to iron deficiency stress.

## 2. Materials and methods

### 2.1. Plant material and iron deficiency stress treatment

*Triticum aestivum* cv. Bobwhite S26 seeds were germinated on wet filter paper for 7 days and then transferred to the hydroponic system in the greenhouse conditions (22C/18C with 16-hour-light/8-hour-dark cycle, 60% relative humidity). All the seedlings were first maintained in iron-sufficient hydroponic solution for 7 days and then half of the plants were transferred to iron-deficient hydroponic solutions. Hydroponic solutions (pH 6.0) were prepared as described previously [14], using 0.88 mM K<sub>2</sub>SO<sub>4</sub>, 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.0 mM MgSO<sub>4</sub>, 0.1 mM KCl, 1.0 μM H<sub>3</sub>BO<sub>3</sub>, 1.0 μM MnSO<sub>4</sub>, 0.2 μM CuSO<sub>4</sub>, 0.02 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, and 1.0 μM ZnSO<sub>4</sub> with different Fe(III)-EDTA concentrations (iron-sufficient condition: 100.0 μM Fe; iron-deficient condition: 10.0 μM Fe). The hydroponic solutions were refreshed every week. Air was continuously pumped into the hydroponics for efficient circulation. After around 90 days growth on hydroponic solution, plants reached the anthesis stage. Roots and flag leaves were collected at 8–10 days post anthesis (DPA), i.e., around the commencement of grain filling. Three biological replicates were collected for each sample.

### 2.2. Measurement of chlorophyll and carotenoids content

Around 25 mg of flag leaves samples were grounded followed by incubating in 15 mL of 95% ethanol solution in falcon tubes for 24 h in dark at room temperature. The mixture was inverted several times and the supernatant ethanol extract was used for measuring absorbance at 664 nm, 649 nm, and 470 nm wavelength, which are the absorption maxima of chlorophyll *a*, chlorophyll *b* and carotenoids in 95% ethanol solution, respectively [45]. Two technical replicates were measured from each of the three biological replicates with a Ultrospec 3000 UV/Visible spectrometer measurements (Pharmacia Biotech, Sweden). The quantification of chlorophyll *a*, chlorophyll *b* and carotenoids content per

gram of flag leaf fresh weight (FW) were calculated according to the Lichtenthaler and Buschmann's protocol [45].

2.3. Total RNA extraction, library preparation and Illumina sequencing

Isolation of total RNA from the roots and flag leaves was carried out using the Isol-RNA Lysis Reagent (5 PRIME, USA). The total RNA was digested with RNase-Free DNase (Qiagen, Germany) to remove the genomic DNA followed by RNA cleaning with RNeasy Plant Mini Kit (Qiagen, Germany). The quality of the isolated RNA was determined with a Qubit® (1.0) Fluorometer (Life Technologies, USA) and a Bioanalyzer 2100 (Agilent, Germany).

Strand-specific cDNA libraries (dUTP method) were prepared using TruSeq Stranded mRNA Sample Prep Kit (Illumina, USA). Total RNA samples (1 µg) were poly-A enriched and then reverse-transcribed into double-stranded cDNA with Actinomycin added during first-strand synthesis. The cDNA samples were fragmented, and end-repaired before ligation of TruSeq adapters containing the index for multiplexing fragments. After ligation, these TruSeq adapters were selectively enriched by PCR amplification on both ends. The quality and quantity of the enriched libraries were validated using Qubit® (1.0) Fluorometer and the Caliper GX LabChip® GX (Caliper Life Sciences, USA). The product is a smear with an average fragment size of approximately 250 bp. The libraries were normalized to 10 nM in 10 mM Tris-Cl, pH8.5 with 0.1% Tween 20. TruSeq PE Cluster Kit v3-cBot-HS (Illumina, USA) was used for cluster generation using 8 pM of pooled normalized libraries on the cBOT. Sequencing was performed on the Illumina HiSeq 2000 paired end at 2 × 101 bp (Illumina, USA).

2.4. RNA sequencing data analysis

RNA sequencing reads were quality-checked with fastqc [46]. The reads were trimmed with Trimmomatic-0.36 by cutting Illumina TruSeq adapters from the reads, and read length below 36 bp was dropped. Reads were aligned to high confidence (HC) genes of IWGSC wheat genome assembly RefSeq v1.0 [47] with STAR (2.7.3a) allowing maximum 5 bp mismatches [48]. Reads specificity to A, B, or D subgenome was identified by HomeoRq [49] and gene expression was quantified by featureCounts (v1.6.4) [50]. Differential expression analysis was performed using DESeq2 (1.26.0) [51] with 0.05 nominal false discovery rate (FDR) control level by using independent hypothesis weighting (IHW) algorithm (1.14.0) [52]. The genes which passed the threshold of FDR < 0.05 and a fold change of ≥ 2 were considered as differentially expressed genes (DEGs). Gene ontology (GO) enrichment analysis was performed with R package 'TopGO (v2.34.0)' [53]. WeightFisher algorithm was used to calculate significance of GO terms [53]. Homoeologs information was downloaded from EnsemblPlants database, and 25,978 triplets, 14,817 duplets and 15,312 monoplets were extracted (cases for 1-to-many, many-to-many were included) [47]. As the 1-to-many and many-to-many cases exist, one gene might appear in several triplets. Triplet DEGs were analyzed further and 'homoeolog induction bias' was assigned if one or two homoeologs in a triplet were differentially expressed between normal and iron deficient condition [54]. Data statistics and figure plotting was performed in RStudio (Version 1.1.456) with R (Version 3.5.0) and various R plotting packages (ggplot2, ggnetwork, sna, VennDiagram and Pheatmap) and Excel [55–59]. Expression pattern of few selected genes were validated using qRT-PCR (Supplementary Fig. 1).

2.5. Wheat gene co-expression network (GCN) construction

Publicly available independent wheat transcriptome datasets were download from URGI (<https://urgi.versailles.inra.fr/down->

[load/iwgc/IWGSC\\_RefSeq\\_Annotations/v1.1/](https://urgi.versailles.inra.fr/down-load/iwgc/IWGSC_RefSeq_Annotations/v1.1/)). In total, 938 wheat transcriptome datasets including various tissues such as roots, leaves/shoots, grains, spikes, etc. from seedling, vegetative, and reproductive developmental stages were used to construct the gene co-expression network based on Markov Cluster (MCL) Algorithm. Genes were clustered to identify function modules by MCL Algorithm with inflation value set as 2.5. Results were visualized by t-distributed Stochastic Neighbor Embedding (t-SNE). Hypergeometric test was carried out and WeightFisher algorithm was used to calculate significance of the DEGs enrichment in each clustered module.

3. Results

3.1. DEGs responding to iron deficiency stress

High quality reads, ranging from 12.92 to 25.09 million reads, were generated among different samples. Among them, 50.69 to 70.34% of the reads aligned to HC genes of IWGSC wheat genome assembly RefSeq v1.0 (Supplementary Table 1). 5969 genes were identified as differentially expressed (FDR < 0.05, fold change ≥ 2) in flag leaves, among which 4226 genes were up-regulated and 1743 genes were down-regulated (Fig. 1) under iron deficiency condition. The roots samples had less number of DEGs (2591 genes) as compared to the flag leaves, with 1186 up-regulated and 1405 down-regulated genes (Fig. 1). The flag leaves and roots share 684 common DEGs, with 472 genes exhibiting a similar expression pattern between the two tissues. Out of these 472 genes, 343 genes were up-regulated while 129 genes were down-regulated in both of the tissues. The remaining 212 genes had the opposite regulation patterns between flag leaves and roots (Fig. 1).

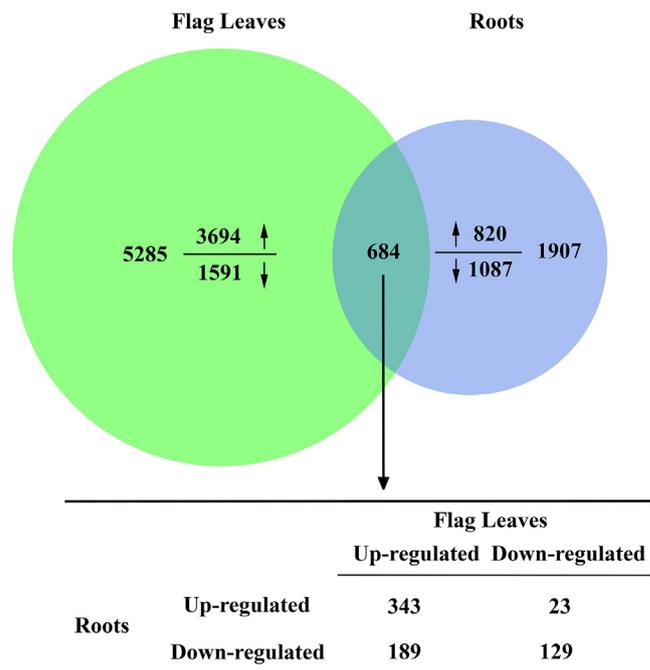


Fig. 1. DEGs in the roots and flag leaves samples subjected to iron-deficient condition. Venn diagram shows total DEGs (FDR < 0.05, fold change ≥ 2) in roots and flag leaves. Green color represents total DEGs in flag leaves; blue color represents total DEGs in roots. Upward arrow indicates up-regulation of gene expression; downward arrow indicates down-regulation of gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Furthermore, the chlorophyll *a* and chlorophyll *b* decreased nearly 70% as well as carotenoids decreased over 50% in the wheat plants subjected to iron deficiency, reconfirming the implementation of iron deficiency stress treatment (Supplementary Fig. 2).

### 3.2. GO analysis of the DEGs

To acquire functional information on the DEGs, GO enrichment analysis was carried out and DEGs were assigned into three categories i.e., biological process (BP), molecular function (MF) and cellular component (CC). The GO terms with *p*-value < 0.001 were considered as significantly enriched. GO analysis of roots DEGs revealed 19 BP GO terms to be significantly enriched (Fig. 2A; Supplementary Table 2). More than half of these GO terms were related to plant metabolic and biosynthetic processes, e.g. 'nicotianamine biosynthetic process' (GO:0030418), 'lignin catabolic process' (GO:0046274), 'malate metabolic process' (GO:0006108),

'glutamine metabolic process' (GO:0006541), 'cellulose biosynthetic process' (GO:0030244), 'tryptophan catabolic process' (GO:0006569), 'oxaloacetate metabolic process' (GO:0006107), 'regulation of jasmonic acid biosynthetic process' (GO:0080141), 'methionine metabolic process' (GO:0006555), 'cytokinin biosynthetic process' (GO:0009691), and more (Fig. 2A). Transport or ion homeostasis related GO terms i.e., 'transmembrane transport' (GO:0055085), 'metal ion transport' (GO:0030001), 'cellular iron ion homeostasis' (GO:0006879) were also enriched, suggesting an eminent role of transporters during iron deficiency. The root specific GO term 'lateral root formation' (GO:0010311) implied that the root development also responds to iron deficiency (Fig. 2A). The overrepresented MF GO terms 'nicotianamine synthase activity' (GO:0030410), 'iron ion binding' (GO:0005506), 'malate dehydrogenase (NADP<sup>+</sup>) activity' (GO:0046554), 'ATPase activity, coupled to transmembrane movement of substances' (GO:0042626), 'metal ion transmembrane transporter activity' (GO:0046873), 'citrate

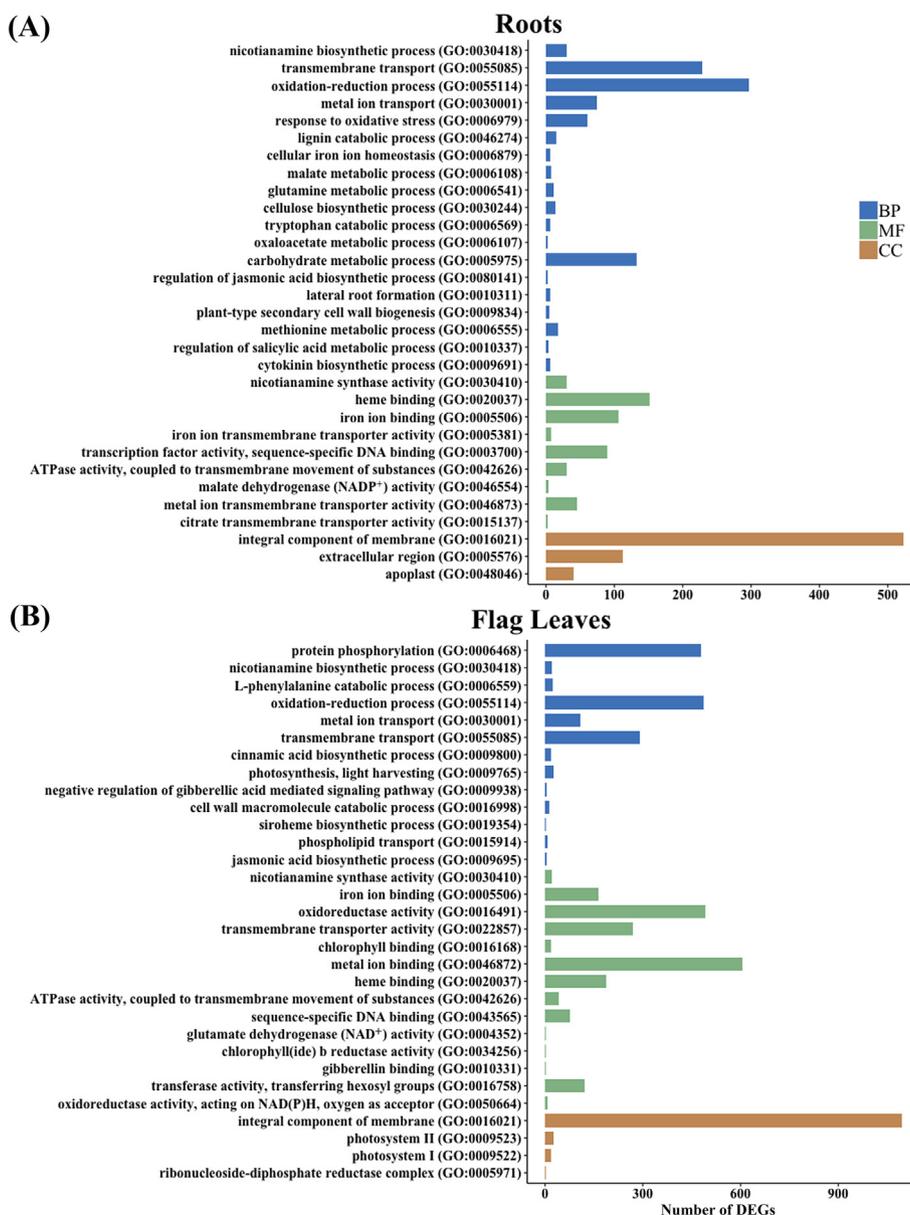


Fig. 2. GO analysis of the DEGs in iron-deficient wheat roots and flag leaves. DEGs are distributed to three major GO categories: i.e., BP (presented in blue); MF (presented in green); CC (presented in dark orange). X-axis represents the number of DEGs in each GO term. (A) roots and (B) flag leaves. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transmembrane transporter activity' (GO:0015137) indicated that synthesis of iron chelators and iron transport was regulated to maintain iron homeostasis (Fig. 2A). Among the CC GO terms, 'integral component of membrane' (GO:0016021), 'extracellular region' (GO:0005576), 'apoplast' (GO:0048046) were enriched in roots DEGs (Fig. 2A). In general, the overrepresentation of flag leaves DEGs GO terms were similar as that in case of roots, with several GO terms as exception (Fig. 2B; Supplementary Table 2). For example, the BP GO term 'photosynthesis, light harvesting' (GO:0009765), MF GO terms 'chlorophyll binding' (GO:0016168), 'chlorophyll(ide) b reductase activity' (GO:0034256), CC GO terms 'photosystem I' (GO:0009522) and 'photosystem II' (GO:0009523) all indicates the photosynthesis was significantly affected in wheat plants subjected to iron deficiency, which correlates well with the reduced chlorophyll content in the flag leaves (Supplementary Fig. 2). These results highlight the significance of membrane and/or membrane localized metal ion transporters as well as of regulatory and metabolic proteins during iron deficiency stress.

### 3.3. Genes encoding iron chelators and iron storage proteins are modulated under iron limited conditions

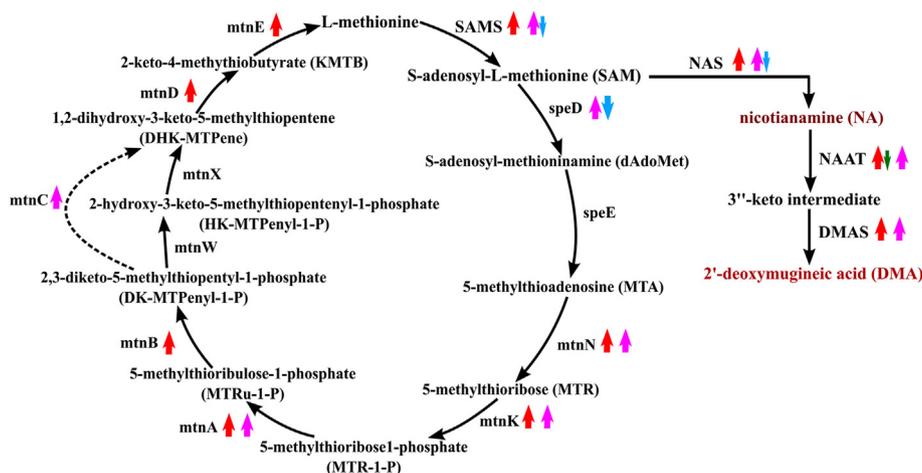
Genes involved in synthesis of NA and DMA were highly up-regulated in both roots and flag leaves. Several of the *NAS* gene homologs were up-regulated, with fold changes as high as 298.3-fold (TraesCS6D02G382900) in roots and over 1800-fold (TraesCS5A02G552400) in flag leaves (Fig. 3; Supplementary Tables 3&4). Likewise, *NAAT* and *DMAS* gene homologs were highly up-regulated in both roots and flag leaves (Fig. 3; Supplementary Table 4). PS synthesis links to methionine salvage pathway because SAM, a key precursor for the synthesis of MAs, is produced from methionine. In roots and flag leaves both, the methionine salvage was also accelerated as indicated by the increased expression of genes mediating sequential reactions in this pathway (Fig. 3; Supplementary Table 4). An increase in the expression levels of above discussed genes indicate plants' tendency to increase the production of NA and DMA in response to iron deficiency stress.

The expression levels of the three *FERRITIN* homologs (TraesCS5A02G152400, TraesCS5B02G151000, TraesCS5D02G157600) significantly declined by upto 6 and 10-fold during iron deficiency condition in roots and flag leaves, respectively (Supplementary Tables 3&5). However, one of the *FERRITIN* (TraesCS4B02G137200)

genes was up-regulated 2.7-fold in flag leaves. The reduced expression of *FERRITIN* indicates plants' preference to distribute the available iron to different plant parts in order to combat iron deficiency rather than storage. The expression levels of several genes annotated as *METALLOTHIONEIN* (*MT*), encoding for metal binding protein involved in ion storage and detoxification, were also altered in both roots and flag leaves. Most of the *MT* gene homologs exhibited increased expression with changes as high as 679-fold (TraesCS1B02G239400) and 3.3-fold (TraesCS3A02G531300) in the iron-deficient roots and flag leaves, respectively. On the contrary, two other *MT* gene homologs (TraesCS1B02G042200 and TraesCS3A02G074600) were down-regulated 4.2-fold and 3.1-fold in iron-deficient roots, respectively. Similarly, expression of two *MT* gene homologs (TraesCS3D02G074400 and TraesCS3A02G074600) showed 5.3-fold and 4.1-fold down-regulation in flag leaves, respectively (Supplementary Tables 3&5). The regulated *MTs* appear to play a significant role in maintaining metal ion homeostasis.

### 3.4. Several transporters coordinate effective iron translocation during iron deficiency stress

GO enrichment and transcript abundance analysis demonstrated that transport related genes were significantly regulated under iron deficiency conditions in both roots and flag leaves (Fig. 2; Supplementary Table 6). In case of roots, 337 genes encoding for transporters and transmembrane channels were identified as differentially expressed and were further assigned to different protein families based on the InterPro IDs (Supplementary Table 6). The *MFS* comprised the largest set among the DEGs encoding for transporters in wheat roots with 78 genes belonging to this category (Supplementary Table 6). The different gene families belonging to the *MFS* exhibited variable expression patterns. The genes encoding *SOLUTE CARRIER FAMILY 40 MEMBER 1* (*SLC40A1*, *IRON REGULATED PROTEIN/FERROPORTIN*, *IREG/FPN*), *ZINC INDUCED FACILITATOR-LIKE 1* (*ZIFL*), and a high proportion of the DEGs encoding *NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER* (*NRT1/PTR*) family were significantly up-regulated during the iron deficiency condition, whereas some genes encoding for *NITRATE TRANSPORTER*, and *SUGAR TRANSPORTER* were down-regulated (Supplementary Table 6). With 37 members differentially expressed, *ATP-BINDING CASSETTE* (*ABC*) superfamily contained



**Fig. 3.** Gene expression regulation in the methionine salvage and phytosiderophore (PS) synthesis pathway in roots and flag leaves. Red and pink arrows represent up-regulated gene expression in roots and flag leaves, respectively. Green and light blue arrows represent down-regulated gene expression in roots and flag leaves, respectively. Smaller size of blue and light blue arrows stands for less number of down-regulated genes than up-regulated genes. SAMS, S-ADENOSYL-L-METHIONINE SYNTHETASE; NAS, NICOTIANAMINE SYNTHASE; NAAT, NICOTIANAMINE AMINOTRANSFERASE; DMAS, DEOXYMUGINEIC ACID SYNTHASE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

up-regulated genes from *ATP-BINDING CASSETTE TRANSPORTER SUBFAMILY A (ABCA)* family, *MULTIDRUG RESISTANCE PROTEIN (MRP)*, members of ABC transporter C family), and *PLEIOTROPIC DRUG RESISTANCE (PDR)* family. Most of the down-regulated genes encoding ABC transporters were from ABCG family (Supplementary Tables 6). The *AQUAPORIN-LIKE (AQP)* superfamily was the third most regulated group with 28 down-regulated and 2 up-regulated DEGs. *ZINC/IRON PERMEASE (ZIP)* family, *OPT* superfamily, *VIT* family, *MULTI ANTIMICROBIAL EXTRUSION PROTEIN (MATE)* family, *NRAMP* family, and *AUXIN EFFLUX CARRIER (AEC)* family were mostly up-regulated. Around half of the *HEAVY METAL-ASSOCIATED (HMA)* domain containing genes, such as *COPPER TRANSPORT PROTEIN* family were up-regulated, whereas *HEAVY METAL TRANSPORT/DETOXIFICATION* superfamily protein encoding genes were mostly down-regulated (Supplementary Table 6).

Flag leaves showed a higher number of DEGs encoding for transporters (635 genes) as compared to the roots (Supplementary Table 6). Genes encoding ABC transporter and MFS families contributed nearly 30% of the DEGs encoding for transporters in the flag leaves. Among the 115 MFS genes, the *SLC40A1* family, *ZIFL* family, *SUGAR TRANSPORTER* family, *NRT1/PTR* family 5.5 and 2.2 were significantly up-regulated. Among 85 DEGs encoding ABC transporters, the ABCB family, ABCC (including MRP) family, ABCCG family, and PDR family were significantly up-regulated. The other gene families, such as the MATE family, HMA family, OPT superfamily, the NRAMP family, and the POTASSIUM TRANSPORTER were also significantly up-regulated in flag leaves. For example, 75% and 91% genes from MATE and OPT family were up-regulated, respectively. In contrast, over 90% of the ZIP family, 77% of SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS (SWEET) proteins, and 68% of amino acid transporter family genes were down-regulated in flag leaves (Supplementary Table 6).

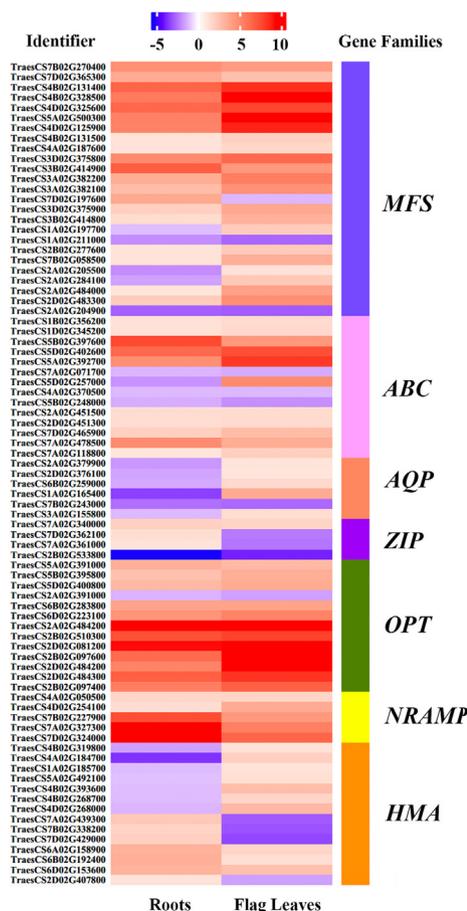
Apart from the differences observed for roots and flag leaves, 115 transport related DEGs were shared among roots and flag leaves (Supplementary Table 6). MFS, ABC transporter superfamily, HMA, OPT, AQP, NRAMP, and ZIP ranked as the top seven regulated gene (super) families (Fig. 4; Supplementary Table 6). Over 20% of the genes (25 genes) were classified as MFS. Among the 25 MFS genes, 19 were expressed more than 2-fold higher, both in roots and flag leaves (Supplementary Table 6).

Seven members of ZIFL gene family were up-regulated, with expression changes between 2.2- and 74.2-fold in roots and between 3- and 971.4-fold in flag leaves. Two of the SLC40A1 genes (TraesCS7B02G270400 and TraesCS7D02G365300), had higher expression levels in both roots and flag leaves (Fig. 4; Supplementary Tables 3&6). In case of NRT1/PTR FAMILY 5.5 gene homologs, the expression levels increased as high as 89.8-fold in roots and 21.8-fold in flag leaves. Four genes (TraesCS7D02G197600, TraesCS1A02G197700, TraesCS2A02G205500, and TraesCS2A02G284100) had opposite expression in roots and flag leaves (Fig. 4; Supplementary Tables 3&6). The remaining two genes (TraesCS1A02G211000 and TraesCS2A02G204900) were down-regulated in both the tissues (Fig. 4; Supplementary Tables 3&6). Among the genes encoding ABC transporters, three ABCB gene homologs (TraesCS5B02G397600, TraesCS5D02G402600, and TraesCS5A02G392700) exhibited increased expression under iron-deficient conditions, ranging from 23.8 to 145.5-fold in roots and 17.8 to 211-fold in flag leaves. Similarly, ABCA, MRP, and PDR gene homologs also showed increased expression. However, the ABCC gene homologs were mostly down-regulated in both of the tissues, except for one gene TraesCS5D02G257000, which showed 26.4-fold up-regulated expression in flag leaves (Fig. 4; Supplementary Tables 3&6). In total, thirteen gene homologs belonging to the OPT superfamily were regulated. An increased expression of as high as 441.5-fold in roots and 1230.9-fold in flag leaves were observed for 12 genes belonging to the OPT superfamily. However,

only one gene (TraesCS2A02G391000) exhibited decreased expression level in both the tissues (Fig. 4; Supplementary Tables 3&6). Similarly, five of the NRAMP gene homologs showed increased expression in both the tissues, when subjected to iron-deficient conditions. In case of gene homologs in AQP, ZIP, and HMA families, the expression patterns were variable, many of which showed reversed expression trend between roots and flag leaves (Fig. 4; Supplementary Table 6). These results further illustrate that iron uptake and translocation mechanisms are mediated by diverse range of transporters and ion channels. These transporters seem to coordinate iron distribution during iron deficiency condition and at the same time maintain iron homeostasis in wheat.

### 3.5. Regulatory factors controlling iron deficiency responses in wheat

In total, nearly 60% (108/179) of the DEGs encoding for transcription factors were down-regulated in roots. On the contrary, over 63% (231/366) of them showed significant up-regulation in flag leaves (Supplementary Table 7). Genes encoding regulatory factors from bHLH, NAC, MYB, BASIC LEUCINE ZIPPER (bZIP), WRKY, and ETHYLENE-RESPONSIVE FACTOR (ERF) families responded to iron deficiency stress in wheat and accounted for over 73% of the



**Fig. 4.** Seven most differentially expressed transporter encoding gene families shared among roots and flag leaves. Heatmaps show seven most differentially expressed transporter encoding gene families shared among roots and flag leaves, respectively. White to red color indicates up-regulation of gene expression; white to blue color indicates down-regulation. Also refer to Supplementary Table 6 for gene IDs and for actual fold change information. MFS: MAJOR FACILITATOR SUPERFAMILY; ABC: ATP-BINDING CASSETTE; AQP: AQUAPORIN; ZIP: ZINC/IRON PERMEASE; OPT: OLIGOPEPTIDE TRANSPORTER; NRAMP: NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN; HMA: HEAVY METAL-ASSOCIATED. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

corresponding DEGs in roots and flag leaves. A large fraction of the DEGs encoding bHLH transcription factors (78% of the total bHLH family homologs) were up-regulated in roots, ranging from 2.1-fold to 1291-fold change increase (Supplementary Table 7). The *bHLH* transcription factor gene family was also highly regulated in the flag leaves, with 67% of the differentially expressed *bHLH* genes up-regulated (Supplementary Table 7). In total, 26 *bHLH* DEGs were shared between roots and flag leaves (Table 1). The expression of *PYE* (*bHLH47*) homolog genes (TraesCS2A02G081300, TraesCS2B02G095900, TraesCS2D02G079100, TraesCS2A02G157800, and TraesCS2D02G163900) increased by at least 2.5-fold and 3.99-fold in roots and flag leaves, respectively. The

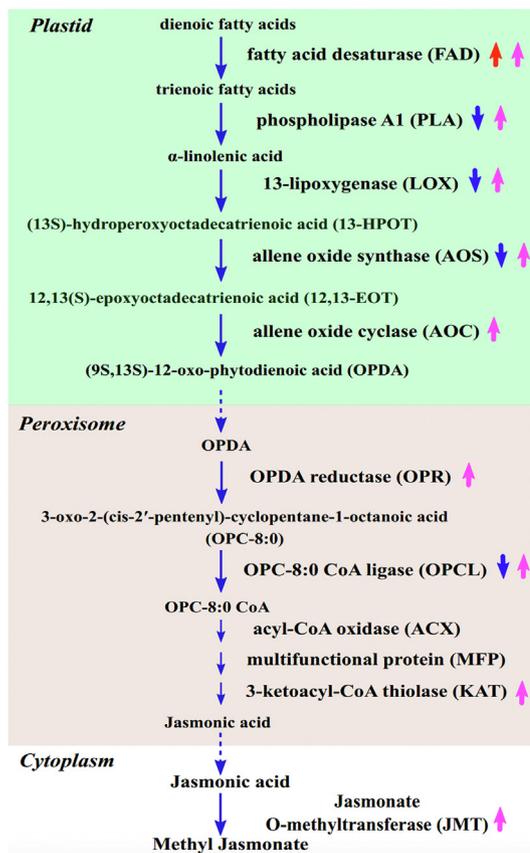
**Table 1**

Differentially expressed genes (DEGs) encoding transcription factors shared among roots and flag leaves. The table enlists the DEGs encoding for transcription factors (FDR < 0.05, fold change ≥ 2) and shared among roots and flag leaves under iron deficient condition. Positive number of fold change indicates up-regulation of gene expression. Negative number of fold change indicates down-regulation of gene expression.

| Identifier         | Blast Hit                                 | Fold change |             |
|--------------------|---|-------------|-------------|
|                    |   | Roots       | Flag Leaves |
| TraesCS2D02G425700 | <i>ERF4</i> -like                         | -3          | 2.47        |
| TraesCS4B02G299400 | <i>TaERFVII.4-4B</i>                      | -3.36       | -14.61      |
| TraesCS2A02G288000 | <i>ERF113</i> -like                       | -2.27       | 7.07        |
| TraesCS2A02G081300 | <i>PYE</i> -like ( <i>bHLH47</i> -like)   | 6.85        | 13.41       |
| TraesCS2B02G095900 | <i>PYE</i> -like ( <i>bHLH47</i> -like)   | 17.6        | 25.79       |
| TraesCS2D02G079100 | <i>PYE</i> -like ( <i>bHLH47</i> -like)   | 10.86       | 8           |
| TraesCS2A02G157800 | <i>PYE</i> -like ( <i>bHLH47</i> -like)   | 2.5         | 3.99        |
| TraesCS2D02G163900 | <i>PYE</i> -like ( <i>bHLH47</i> -like)   | 2.66        | 4.12        |
| TraesCS3A02G489600 | <i>ORG2</i> -like ( <i>bHLH38</i> -like)  | 433.99      | 2594.54     |
| TraesCS3B02G550000 | <i>ORG2</i> -like ( <i>bHLH38</i> -like)  | 188.05      | 121.61      |
| TraesCS3D02G495600 | <i>ORG2</i> -like ( <i>bHLH38</i> -like)  | 1291.76     | 413.83      |
| TraesCS3A02G489500 | <i>ORG2</i> -like ( <i>bHLH38</i> -like)  | 108.42      | 203.98      |
| TraesCS3B02G549800 | <i>ORG2</i> -like ( <i>bHLH38</i> -like)  | 1137.69     | 22.67       |
| TraesCS3D02G495400 | <i>ORG2</i> -like ( <i>bHLH38</i> -like)  | 61.39       | 36.01       |
| TraesCS2A02G515300 | <i>bHLH100</i> -like                      | 768.85      | 361.45      |
| TraesCS2B02G543700 | <i>bHLH100</i> -like                      | 271.83      | 234.78      |
| TraesCS2B02G543800 | <i>bHLH100</i> -like                      | 1106.44     | 165.75      |
| TraesCS2D02G517000 | <i>bHLH100</i> -like                      | 316.02      | 181.92      |
| TraesCS3A02G489700 | <i>bHLH101</i> -like                      | 19.18       | 94.19       |
| TraesCS3B02G550200 | <i>bHLH101</i> -like                      | 13.06       | 131.74      |
| TraesCS3D02G495700 | <i>bHLH101</i> -like                      | 8.18        | 488.49      |
| TraesCS2A02G281200 | <i>FIT</i> -like ( <i>bHLH29</i> -like)   | 10.71       | 22.23       |
| TraesCS2B02G298600 | <i>FIT</i> -like ( <i>bHLH29</i> -like)   | 5.47        | 20.73       |
| TraesCS2D02G280100 | <i>FIT</i> -like ( <i>bHLH29</i> -like)   | 14.65       | 8.8         |
| TraesCS1A02G281300 | <i>ILR3</i> -like ( <i>bHLH105</i> -like) | -2.66       | -6.99       |
| TraesCS1B02G290500 | <i>ILR3</i> -like ( <i>bHLH105</i> -like) | -3.27       | -18.47      |
| TraesCS1D02G280600 | <i>ILR3</i> -like ( <i>bHLH105</i> -like) | -2.61       | -10.42      |
| TraesCS6B02G220300 | <i>bHLH121</i> -like                      | -2.18       | -13.93      |
| TraesCS2B02G289900 | <i>TabHLH27</i>                           | -7.51       | 11.69       |
| TraesCS2D02G437600 | <i>PHL11</i> -like                        | 2.48        | 2.24        |
| TraesCS6B02G201700 | <i>TaMYB1</i>                             | 3.08        | 7.1         |
| TraesCS3A02G367600 | <i>TaMYB41</i>                            | -2.17       | -3.33       |
| TraesCS2D02G104500 | <i>WRKY72</i> -like                       | 4.33        | 4.33        |
| TraesCS2D02G104600 | <i>WRKY34</i> -like                       | 11.13       | 31.34       |
| TraesCS2D02G489700 | <i>WRKY70</i> -like                       | 5.42        | 3.52        |
| TraesCS5B02G183700 | <i>WRKY62</i> -like                       | 3.98        | 8.29        |
| TraesCS7A02G420400 | <i>bZIP10</i>                             | -3.23       | -3.5        |
| TraesCS7B02G391800 | <i>bZIP9</i> -like                        | -2.41       | -2.47       |
| TraesCS7D02G475100 | <i>bZIP9</i> -like                        | -2.2        | -4.22       |
| TraesCS3B02G404800 | <i>LG2</i> -like                          | -2.86       | -2.22       |
| TraesCS2A02G467100 | <i>bZIP 27</i> -like                      | -3.21       | -2.7        |
| TraesCS2D02G324700 | <i>NAC 92</i> -like                       | -2.45       | 5.69        |
| TraesCS5A02G143200 | <i>TaNAC69-3</i>                          | -2.2        | 39.99       |
| TraesCS4A02G131000 | <i>NAM-B1</i> -like ( <i>NAC</i> family)  | -2.24       | 3.77        |
| TraesCS2D02G563000 | <i>DOF1.7</i> -like                       | -6.11       | 7.14        |
| TraesCS2B02G592700 | <i>DOF1.7</i> -like                       | -6.02       | 17.98       |
| TraesCS3A02G360300 | <i>SCL9</i> -like                         | 3.74        | 3.64        |
| TraesCS3D02G354100 | <i>SCL9</i> -like                         | 3.17        | 4.59        |
| TraesCS6A02G240400 | <i>HOX24</i> -like                        | -8.04       | -5.55       |
| TraesCS6D02G222600 | <i>HOX24</i> -like                        | -5.16       | -4.03       |
| TraesCS3A02G103500 | <i>TaDOG11</i>                            | -2.4        | 2.22        |
| TraesCS7A02G429300 | Uncharacterized protein                   | -3.21       | -2.26       |

*ORG2* (*bHLH38*) homolog genes (TraesCS3A02G489600, TraesCS3B02G550000, TraesCS3D02G495600, TraesCS3A02G489500, TraesCS3B02G549800, and TraesCS3D02G495400) showed up-regulated expression in the range of 61 to 1291-fold for roots and 22 to 2594-fold for flag leaves. Four *bHLH100* homolog genes i.e., TraesCS2A02G515300, TraesCS2B02G543700, TraesCS2B02G543800, and TraesCS2D02G517000 were up-regulated over 271-fold and 181-fold in roots and flag leaves, respectively. The *bHLH101* homolog genes, exhibited at least 8.1-fold and 94.2-fold elevated expression in roots and flag leaves under iron deficiency, respectively. Expression of *FIT* (*bHLH29*) homolog genes (TraesCS2A02G281200, TraesCS2B02G298600, and TraesCS2D02G280100) increased over 5.5-fold and 8.8-fold in roots and flag leaves, respectively. The *IAA-LEUCINE RESISTANT3* (*ILR3/bHLH105*) homolog genes (TraesCS1A02G281300, TraesCS1B02G290500 and TraesCS1D02G280600) and *bHLH121* homolog (TraesCS6B02G220300) were down-regulated by over 2.2-fold in roots and up to 18-fold in the flag leaves. However, *TabHLH27* had opposite expression pattern in roots and flag leaves.

Unlike the *bHLH* gene family, other large transcription factor families were mostly down-regulated in roots. For example, 97% of *ERF* specific DEGs, 78% of *MYB* DEGs, 89% of *bZIP* DEGs, and 67% of the *NAC* genes were down-regulated in roots (Supplementary Table 7). On the contrary, 75% of the *WRKY* genes showed up-regulation in roots. In flag leaves, 96% and 100% of the genes encoding *NAC* and *WRKY* transcription factors, respectively, were up-regulated. The *MYB* and *ERF* genes have almost equal proportion of up- and down-regulated DEGs in flag leaves. Similar as roots, expression of 85% of *bZIP* genes was suppressed in flag leaves



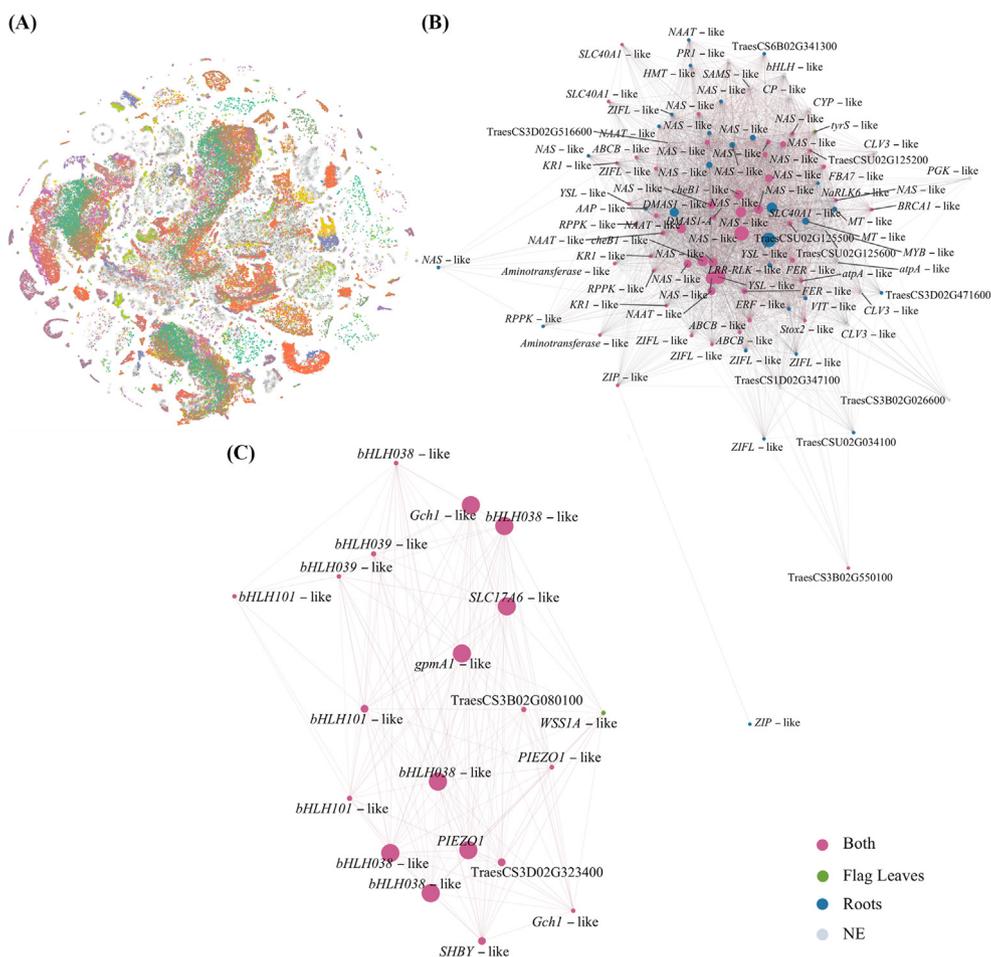
**Fig. 5.** Gene expression modulation in the jasmonate biosynthesis pathway in roots and flag leaves. Red and pink arrows represent up-regulated gene expression in roots and flag leaves, respectively. Blue arrows represent down-regulated gene expression in roots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Supplementary Table 7). In total, 34 and 24 *ERF* genes were differentially expressed in roots and flag leaves, respectively (Supplementary Table 7). However, only three of these *ERF* genes were shared among the roots and flag leaves (Table 1). The TraesCS4B02G299400 was suppressed 3.3-fold and 14.6-fold in roots and flag leaves, respectively. The TraesCS2D02G425700 and TraesCS2A02G288000 had a reduced expression in roots but was up-regulated in the flag leaves (Table 1). Similar to the *ERF* genes, only four *WRKY* transcription factor genes were shared between both the tissues. All four of these shared *WRKY* DEGs (TraesCS2D02G104500, TraesCS2D02G104600, TraesCS2D02G489700 and TraesCS5B02G183700) were up-regulated, with *WRKY34*-like up-regulated by 11-fold and 31-fold in roots and flag leaves, respectively (Table 1). Five *bZIP* genes were shared between roots and flag leaves and were down-regulated by up to 4.2-fold. Roots and flag leaves shared three *NAC* DEGs but with at least 2.2-fold down-regulation in roots and a 39-fold up-regulation of *TaNAC69-3*-like gene in the flag leaves. Given the high expression changes observed during iron deficiency stress, *bHLH* appeared to be the most regulated gene family as compared to the *NAC*, *WRKY*, *bZIP*, *ERF*, and *MYB* (Table 1; Supplementary Table 7). The observed differential and high expression changes in above transcription factors signify their regulatory role in controlling iron deficiency

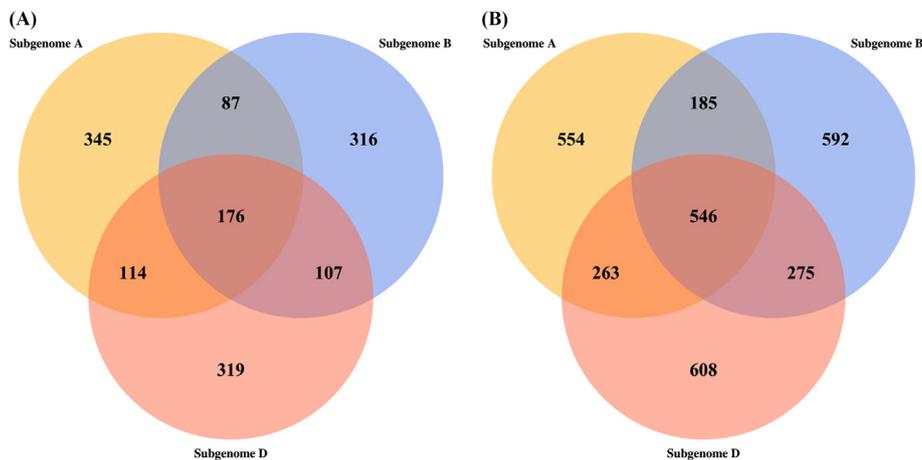
responses in bread wheat and indicate a complex regulatory network. Particularly, the transcription factors that are shared between the roots and flag leaves indicate a more systemic response to iron deficiency stress.

### 3.6. Jasmonate biosynthesis is altered in response to iron deficiency

Jasmonates (JAs) are important signal molecules for bridging the molecular responses and physiological reactions together. In our study, iron deficiency stress significantly affected the biosynthesis of JAs (Fig. 5). Generally, the biosynthesis pathway of JAs was up-regulated in flag leaves, where the expression of genes encoding FATTY ACID DESATURASE (FAD) increased as high as 85-fold (TraesCS6D02G260300) (Supplementary Tables 3&8). Four out of six *PHOSPHOLIPASE A1* (*PLA*) genes exhibited increased expression, ranging from 2.2- to 58-fold in flag leaves. All the differentially expressed *LIPOXYGENASE* (*LOX*), *ALLENE OXIDE SYNTHASE* (*AOS*), *ALLENE OXIDE CYCLASE* (*AOC*) genes showed up-regulated expression in flag leaves. The expression of *LOX* gene homologs increased as high as 513-fold (TraesCS5D02G013400) in flag leaves. The *AOS* gene homologs were up-regulated as high as 54-fold (TraesCS4B02G237600) in flag leaves. In addition, more than 2-fold increased expression of *12-OXOPHYTODIENOATE*



**Fig. 6.** Gene co-expression network in bread wheat. (A) Overview of gene co-expression network in bread wheat. Gene co-expression network in bread wheat was clustered to modules. Top 50 modules (ranked with gene number in each module) are labeled with colors. Hypergeometric test across all modules demonstrated that Module 90 (B) and Module 300 (C) are the most DEGs enriched modules in roots and flag leaves, respectively. Pink nodes (Both) represent differential expression in roots and flag leaves. Green nodes (Flag leaves) represent differential expression in flag leaves only. Blue nodes (Roots) represent differential expression in roots only. Gray nodes (NE) represent no differential expression detected in either tissue. The size of the node indicates interaction of one gene to other genes in this module. The larger the size, higher is the interaction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 7.** Homoeolog induction bias in response to iron deficiency condition. (A) Homoeolog induction bias in roots under iron deficiency condition. (B) Homoeolog induction bias in flag leaves under iron deficiency condition. Yellow, blue, and pink circles show differentially expressed triplet genes in subgenome A, B, and D, respectively. Number in the Venn diagrams indicate triplets with only one differentially regulated homoeolog copy (nonoverlapping regions); triplets with two genes as differentially regulated (overlapping region between two colors); or triplets with all three genes as differentially regulated (overlapping region among three colors). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

REDUCTASE (*OPR*), 4-COUMARATE COA LIGASE (*OPCL*), 3-KETOACYL-COA THIOLASE (*KAT*) and JASMONATE O-METHYLTRANSFERASE (*JMT*) was also observed in iron-deficient wheat flag leaves. Interestingly, the jasmonate biosynthesis related genes, such as *PLA*, *LOX*, and *OPCL*, were significantly down-regulated in roots of bread wheat under iron deficiency (Fig. 5). The expression of three out of four AOS genes also showed down-regulation in roots. However, all the *FAD* genes, except one (TraesCS5A02G186200), were up-regulated in roots (Supplementary Tables 3&8). These results indicate that JAs are important signal molecules contributing to iron homeostasis maintenance in bread wheat.

### 3.7. Co-expression analysis of the DEGs

Co-expression network was built to reveal gene associations by correlating and clustering bread wheat genes. Only the modules with at least 20 genes were considered. In total, 303 modules containing over 82,600 genes were generated, representing 76.5% of total wheat HC genes (Fig. 6A). Enrichment of DEGs in flag leaves and roots in each module was evaluated by hypergeometric test. Module 90 and Module 300 ranked as most DEGs enriched modules in roots and flag leaves, respectively (Supplementary Table 9). Module 90 contains 94 genes, 78 of these genes were differentially expressed in roots and 50 of these genes were differentially expressed in flag leaves. GO analysis demonstrated the Module 90 is related to nicotianamine synthase activity (GO:0030410). It contains 23 *NAS*-like genes, 7 *ZIFL*-like genes, 5 *NAAT*-like genes, 3 *YSL*-like genes, 3 *SLC40A1*-like genes, 2 *DMAS1*-like, 2 *ZIP*-like genes, 2 *FER*-like genes, which play an important role in iron acquisition, translocation, or storage (Fig. 6B, Supplementary Table 9). All DEGs in flag leaves belonging to this module were up-regulated, and 77 out of 78 DEGs of roots in Module 90 were observed as up-regulated under iron deficiency. The increased expression of genes belonging to this module suggested a strong coordinated response to these genes to combat iron deficiency stress. Module 300 is a DNA binding (GO:0003677) related module, which only contains 20 genes. However, 19 of these genes were differentially expressed in roots and 20 of these genes were differentially expressed in flag leaves. Ten *bHLH*-like genes are included in this module, with 5 *bHLH038*-like genes, 3 *bHLH101*-like genes, and 2 *bHLH039*-like genes. Genes in Module 300 were all significantly up-regulated, as high as 1291-fold (TraesCS3D02G495600) up-regulation in roots and 2594-fold (TraesCS3A02G489600) up-

regulation in flag leaves (Fig. 6C, Supplementary Table 9). These results further signify the role of *bHLH* family of transcription factors and of genes within module 90 and 300 in maintaining iron homeostasis in wheat.

### 3.8. Homoeologs induction bias analysis

The DEGs were assigned to three wheat subgenomes, which shared a similar number of DEGs with an order of A genome (869) > D genome (850) > B genome (836) > unknown (36) in roots; D genome (2072) > B genome (1920) > A genome (1886) > unknown (91) in flag leaves. To understand if homoeologs expression or induction bias exists when bread wheat is experiencing iron deficiency stress, DEGs were distributed to triplet genes (if three homoeologous copies exist), duplet genes (if two homoeologous copies exist), or monoplex (if only one copy of a homoeolog was detected). Among the DEGs, 1957 genes distributed in 1464 triplets in roots, whereas 4506 genes distributed in 3023 triplets in flag leaves (Fig. 7). Only 176 triplets were observed with all three homoeologs differentially expressed in roots. Whereas 87, 114, 107 triplets have two copies of homoeologs differentially expressed in AB, AD, and BD subgenome groups, respectively. In addition, 345, 316, and 319 triplets have only one copy of homoeolog differentially expressed in subgenome A, B, and D, respectively (Fig. 7A). Similar as roots, 546 triplets were observed differentially expressed in flag leaves with all three copies of homoeologs. Homoeolog genes differentially expressed in AB, AD, and BD subgenome groups with two copies corresponded to 185, 263, 275 triplets, respectively. Whereas 554, 592, and 608 triplets have one copy of homoeolog differentially expressed in subgenome A, B, and D in flag leaves, respectively (Fig. 7B). In summary, around 88% of the triplets in roots and 82% of the triplets in flag leaves have a biased induction. In addition, among the triplets where all three homoeologs are differentially expressed, most of the DEGs appeared to be regulated in a very similar manner between the three subgenomes (Supplementary Fig. 3).

## 4. Discussion

Although the soils are abundant in iron, conditions like high pH and presence of sodium carbonate in the soil adversely affect iron uptake and absorption by plants, thereby negatively affecting the

agricultural produce. From human nutrition point of view as well, it is equally necessary to study the mechanisms controlling iron uptake and translocation, so as to design effective biofortification strategies to tackle iron deficiency anemia. Plant responses to iron deficiency have been extensively studied in non-graminaceous plant species like *Arabidopsis* [60] and graminaceous plants including rice [61]. To date, several studies have been carried out to characterize the iron homeostasis related genes in wheat [14,20,21,62–65]. However, there is very limited information available from the transcriptomics studies of bread wheat suffering from iron deficiency, especially during the grain filling developmental stage [16].

#### 4.1. Enhanced production of iron chelators during iron deficiency

Iron homeostasis in plants is tightly controlled by a concerted involvement of various iron ligands, transporters and regulatory factors. Generally, iron combines with ligands for transportation or storage to reduce the risk of toxic ROS production by the free iron [66]. Therefore, the production of iron ligands often depends on the amount of iron available for uptake and/or translocation. In rice roots, *OsNAS1*, *OsNAS2*, *OsNAS3*, *OsNAAT1* and *OsDMAS1* are up-regulated upon iron deficiency, suggesting important roles in iron acquisition and translocation of NA and DMA [62,67,68]. In addition to functioning as a key intermediate for biosynthesis of DMA, NA is also an iron chelator which forms stable complexes with ferrous and ferric iron for symplastic and long-distance iron translocation [69–71]. DMA is also a multifunctional iron ligand which is also employed in long-distance transport of iron in xylem, as well as phloem [72,73]. Our data also suggests increased production of NA and DMA. During iron deficiency stress, the key genes involved in PSs synthesis i.e., *NAS*, *NAAT*, *DMAS* were up-regulated in wheat roots and flag leaves, which is consistent with the previous findings [16,20,21] in wheat and rice [62,67,68]. The results also correspond well to observations made *via* qRT-PCR based analysis of 19 preselected Fe homeostasis related genes [14]. We observed up-regulated expression of *S-ADENOSYL-L-METHIONINE SYNTHETASE (SAMS)* in wheat roots during iron deficiency, which is similar as *OsSAMS1* and *OsSAMS2* in rice [10]. In contrast, no change in *SAMS* expression was observed in barley when comparing iron deficient and control growth conditions [74]. A proteomic study in cucumber roots even showed a reduction of *SAMS* protein [75].

#### 4.2. Concerted action of a range of transporters is required for iron translocation in bread wheat

A variety of transporters play an important role in intercellular and intracellular plant iron translocation and the cooperation of these proteins is vital to plant growth and development. Under iron-deficient conditions, many transporter gene families were significantly differentially regulated in bread wheat flag leaves and roots, such as *MFS*, *ABC* transporter family, *MATE*, *OPT* superfamily and *NRAMP* family. The *MFS* together with *ABC* transporters are the two universal transmembrane superfamilies across the living kingdom [76]. As the largest secondary transport carrier superfamily, *MFS* facilitates the transport of various substances such as amino acids, peptides, drugs, nucleotides, as well as iron chelates, etc. [77]. A *MFS* family member, *TOM1*, exports DMA to the soil and is involved in internal iron transportation of DMA to the phloem and xylem in rice plants [22]. The zinc-induced facilitators *OsZIFL4*, *OsZIFL5*, *OsZIFL7* and *OsZIFL12* were up-regulated in rice roots under iron deficiency which suggested their role in iron transport [78]. The expression of *ZIFLs* was not altered in rice leaves though [78]. In case of wheat, we observed increased expression for most of the *ZIFL* including *TOM* genes in both the flag leaves and roots.

However, two of the *ZIFL* genes (*TraesCS5B02G137400*, *TraesCS4A02G218000*) were suppressed in flag leaves when suffering from iron deficiency, which can be confirmed by another study in wheat [79]. We also detected an increased expression of the *SLC40A1* genes under iron-deficient conditions in roots and flag leaves, which is similar to the response observed in *Arabidopsis* [80]. These results demonstrate that *MFS* genes play an important role for iron transport in bread wheat and various subfamily members are involved in a coordinated manner, likely serving different functions to maintain overall iron homeostasis.

The *ABC* superfamily is one of the largest primary active transporter family, which transports mineral ions, lipids, and peptides, and hence is very crucial for import and export of metabolites [81]. Several of the genes belonging to *ABC* transporter family were significantly regulated in wheat roots and flag leaves during iron deficiency. The expression of *NtPDR3* has been reported to be increased in *Nicotiana tabacum* suspension cells under iron-deficient conditions [82]. Likewise, the *OsABCB14* gene belonging to B family of *ABC* transporters has been reported as an auxin transporter influencing iron homeostasis in rice plants [83]. In plants, the *MRP* genes were reported to transport phytic acid into vacuoles [84]. In mammalian cells, *MRP1* was identified as *GLUTATHIONE (GSH) conjugate transporter* and was associated to NO-mediated iron efflux from cells [85,86]. Similarly, our data identified several wheat homologs of genes encoding *ABC* transporters that were differentially regulated in wheat roots and flag leaves under iron-deficient conditions and suggests their direct and/or indirect involvement in iron transport.

The plant *MATE* family, localized in vacuoles and the plasma membrane, is known to mainly facilitate transportation of secondary metabolites and xenobiotics [87]. *MATE* transporters have been reported to mediate the efflux of citrate into the root vasculature in rice, soybean and *Arabidopsis* and therefore could improve plant iron transport efficiency [88–91]. We also observed increased expression of several *MATE* genes, both in roots and flag leaves. These differentially expressing *MATE* transporters appear to be important for Fe(III)-citrate transport in wheat.

Transporter families such as *OPT*, *NRAMP* and *ZIP* families have been studied in *Arabidopsis*, rice and maize. As one of the well characterized metal transporter family, the *OPT* family member *YSL* transporters have been shown to be involved in iron acquisition from the soil, long-distance transport through phloem, and iron translocation in developing seeds [23–26,92]. The *NRAMP* family is also considered as an important metal transporter family with several of these genes participating in iron transport through vacuoles and plasma membrane [30,93]. Similarly, the *ZIP* family is involved in transport of divalent metals such as iron, zinc and manganese [94]. The expression of genes from *OPT*, *NRAMP* and *ZIP* families were significantly regulated in bread wheat during the iron deficiency conditions, suggesting a similar role in long-distance and/or intracellular iron translocation in wheat.

#### 4.3. Complex regulatory and signaling network controls iron homeostasis

Among the regulatory factors, the bHLH transcription factor family is one of the most widely reported to be involved in regulation of plant iron homeostasis related genes. In case of rice, the *IRO2* regulates the expression of genes responsible for DMA synthesis and iron transport such as *OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1*, *OsYSL15* and *TOM1* [36,37,95]. In this study, expression of rice *OsIRO2* homologs was up-regulated in roots and flag leaves, suggesting a similar regulation role in bread wheat. In *Arabidopsis*, *bHLH38*, together with the other three Ib subgroup bHLH transcription factors *bHLH39*, *bHLH100* and *bHLH101* were up-regulated in leaves as well as roots, under iron-deficient conditions [96]. The

bHLH38, bHLH39, bHLH100, and bHLH101 have been reported to form heterodimers with FER-like iron deficiency-induced transcription factor (FIT/bHLH29), in order to regulate the genes encoding *FERRIC CHELATE REDUCTASE (FRO2)* and *IRON-REGULATED TRANSPORTER (IRT1)* in Arabidopsis [97,98]. In addition to FIT, another important bHLH transcription factor POPEYE (PYE), which is OsIRO3 orthologue, positively regulates plant growth and development and acts as a transcriptional repressor to certain iron homeostasis related genes such as *NAS4*, *ZIFL1*, and *FERRIC REDUCTASE OXIDASE3 (FRO3)* [99]. In our study, several bHLH members including homologs of bHLH38, bHLH100, bHLH101, FIT, and PYE were found to be up-regulated under iron deficiency, suggesting a similar regulation mechanism of iron deficient response in bread wheat and Arabidopsis. ILR3 and bHLH121 are the upstream transcription factors which could regulate many other iron homeostasis related transcription factors [100]. The ILR3 could form homo- and heterodimers with its IVC bHLH homologs, namely bHLH34, bHLH104, and bHLH115 to induce the expression of Ib bHLH transcription factors [101–104]. Similarly, the PYE homolog, bHLH121, activates the expression of Ib bHLH transcription factor and PYE encoding genes by interacting with ILR3 [100]. The down-regulation of the *ILR3* and *bHLH121* homologs in roots and flag leaves suggests a complex regulation network in bread wheat (Table 1). Further experimentation is necessary to give a deep insight into the iron homeostasis regulation mechanism.

In addition, the bHLH regulation network connects to the signaling pathways. One such is the jasmonate signaling. Jasmonates are lipid-derived plant hormones which originate from oxidative metabolism of polyunsaturated fatty acids [105]. They function as signal molecules and participate in various physiological responses to regulate developmental processes or to respond to environmental stimuli [105]. The biosynthesis of jasmonic acid in soybean was altered in iron limiting growth condition [7]. Jasmonates has been observed to suppress the expression level of iron deficiency-inducible genes functioning in iron acquisition or transport, such as *IRT1* and *FRO2* in roots of Arabidopsis, and *OsNAS1*, *OsDMAS1*, and *TOM1* in rice roots [106–108]. However, JA signaling was reported to be activated in rice roots during very early iron deficiency responses but was weakened or vanished after long term iron deficiency treatment [107]. Jasmonates suppress iron deficiency response by repressing the expression of *FIT*, *bHLH38*, *bHLH39*, *bHLH100* and *bHLH101* genes [106]. The down-regulation of JA biosynthesis related genes in wheat roots under Fe deficiency condition was observed in this study. In addition, the endogenous JA level is elevated in senescent Arabidopsis leaves, accompanied with up-regulation of JA biosynthesis related genes such as *LOX1*, *LOX3*, *LOX4*, *AOC1* [109]. Similarly, the expression of JA biosynthesis related genes in flag leaves was elevated, suggesting JAs might promote leaf senescence and may accelerate iron translocation between leaves and grains in bread wheat when suffering from iron deficient stress. However, the expression pattern of genes encoding key enzymes regulating ethylene production i.e., 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase did not indicate increased production of ethylene, a known promoter of leaf senescence and fruit ripening in plants (data not shown) [110,111]. To further understand the role of JAs on wheat iron deficiency responses, more physiological and biochemical studies for instance, measuring the endogenous JAs concentration in different wheat organs, and/or evaluating iron deficiency responses by applying exogenous JAs could be beneficial. In addition, the interaction between JA and other hormonal signaling network such as auxin and ethylene under Fe deficiency would be an interesting scientific question.

Both NAC and WRKY transcription factor families have been widely reported to be involved in regulating abiotic stress such as cold, drought, salinity stress etc. [112,113]. Some of the factors

in the NAC family have also been reported to regulate iron transport in both wheat and rice [43,63]. The NAC family member, IRON DEFICIENCY-RESPONSIVE CIS-ACTING ELEMENT 2 (IDE2) BINDING FACTOR (OsIDEF2) binds to OsYSL2 promoter region and regulates the expression of OsYSL2 [43]. Among the WRKY family, OsWRKY80 was associated with gene regulation during iron excess [114]. Increased gene expression of OsWRKY17 was observed in iron deficient rice roots [44]. In addition, expression of OsWRKY45 was reported as up-regulated in rice shoots [36]. We also detected elevated expression levels for many of the NAC and WRKY family members especially in leaves, suggesting their regulatory role in maintaining wheat iron homeostasis.

## 5. Conclusions

The RNA sequencing data revealed the genes modulated during iron deficiency stress in wheat and reflected on the role of several iron ligands, transporters as well as regulatory mechanisms involved in iron homeostasis. Methionine salvage and PS synthesis were observed as accelerated, perhaps due to enhanced demand of iron chelators such as NA and DMA during iron deficiency conditions. Several gene families including the *MFS*, *ABC* transporters, *OPT*, *NRAMP* families were significantly regulated, thereby facilitating effective transport of iron. Transcription factors, especially the bHLH family actively reacted to the iron deficiency in roots as well as flag leaves, and might facilitate a link between JA signaling and iron deficiency response. Induction bias was detected among homoeolog triplet DEGs. Further, gene co-expression network elucidated the gene relationships involved in iron homeostasis in bread wheat. These observations lay an important foundation for further research on iron deficiency stress responses in wheat and as well for future biofortification programs.

## 6. Availability of data and materials

The data analyzed in this work has been provided as part of figures and tables as well as [Supplementary data](#) sheets.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Author contributions

N.K.B. conceived and designed the experiments. M.W. carried out the experiments. M.W., J.Z.G., and N.K.B. analyzed the data. M.W. and N.K.B. wrote the manuscript. M.W., J.Z.G., and N.K.B. edited the manuscript. All authors have read the manuscript and agree with its content.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.csbj.2020.09.009>.

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