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Molecular Analysis of Iron Deficiency Response in Hexaploid Wheat

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Iron deficiency leads to severe chlorosis in crop plants, including wheat, thereby reducing total yield and quality. Furthermore, grains of most bread wheat varieties are poor source of iron, which is vital for human nutrition. Despite the significance, iron uptake and translocation mechanisms in bread wheat have not been studied in detail, particularly under iron limited growth conditions. In this study, bread wheat plants were grown under iron deficiency stress until maturity. Data were collected at three distinct developmental time points during grain-filling. The plants experiencing low iron availability exhibited significantly lower chlorophyll content as well as low iron concentration in leaves and grains. The expression levels of bread wheat genes homologous to iron deficiency responsive genes of rice, barley, and Arabidopsis were significantly changed under iron deficiency stress. The wheat homologs of genes involved in phytosiderophore (PS) synthesis and transport were significantly up-regulated in the iron-deficient roots through all development stages, confirming an important role of deoxymugineic acid (DMA) in iron acquisition. The up-regulation of *NICOTIANAMINE SYNTHASE* (*NAS*) and *DEOXYMUGINEIC ACID SYNTHASE* (*DMAS*) in flag leaves and grains suggested the involvement of nicotianamine (NA) and DMA in iron chelation and translocation in wheat, particularly at the commencement of grain-filling. In line with this, the homolog of gene encoding TRANSPORTER OF MUGINEIC ACID (TOM) was up-regulated in the wheat roots under iron deficiency. Additionally, genes encoding long-distance iron transporter YELLOW STRIPE-LIKE (YSL), the vacuolar transporter NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (NRAMP), and the transcription factor BASIC HELIX-LOOP-HELIX (bHLH), were also up-regulated upon iron starvation. A tissue specific and growth stage specific gene expression differences in response to iron deficiency stress were observed, providing new insights into iron translocation, storage and regulation in bread wheat.

Keywords: iron deficiency, gene expression, wheat, phytosiderophore synthesis, transcription factors, iron transporters

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INTRODUCTION

Iron is an essential micronutrient for all living organisms. In plants, iron functions as redox-active metal in many important reactions of metabolic processes such as photosynthesis, mitochondrial respiration, nitrogen assimilation, hormone biosynthesis, and the production and scavenging of reactive oxygen species (Hansch and Mendel, 2009). Although iron ranks as the fourth most

abundant element in the earth's crust (Buckhout and Schmidt, 2013), plants often suffer from iron deficiency due to the low bioavailability of iron in aerobic, calcareous and/or high pH soils (Morrissey and Guerinot, 2009). Iron deficiency causes interveinal chlorosis because of insufficient chlorophyll production that is often identified as alternate yellow and green stripes in younger leaves of most cereals (Barker and Stratton, 2015). As one of the key agricultural problems, iron deficiency restrains plant growth, ultimately leading to yield losses. Additionally, the grain iron content in cereal crops affected by iron deficiency is further reduced, thereby impacting human nutrition. Several studies investigated plant responses to iron deficiency stress and its effect on iron uptake, translocation and utilization (Thimm et al., 2001; Kobayashi et al., 2005; Buckhout et al., 2009; Yang et al., 2010; Rodriguez-Celma et al., 2013) but such information is rather limited for wheat (Bonneau et al., 2016; Beasley et al., 2017; Connorton et al., 2017; Garnica et al., 2018; Kumar et al., 2018; Mathpal et al., 2018).

Since excess iron can also be reactive and toxic via the Fenton reaction, plants tightly control and maintain iron homeostasis. Non-graminaceous and graminaceous plants utilize different strategies for iron acquisition from soil. Non-graminaceous plants acquire iron through the reduction strategy (strategy I), in which protons are first released into the rhizosphere by H^+-ATPases to increase the solubility of iron (Santi and Schmidt, 2009). FERRIC CHELATE REDUCTASES (FRO) reduce ferric iron [Fe(III)] to ferrous iron [Fe(II)] (Robinson et al., 1999; Waters et al., 2002). IRON REGULATED TRANSPORTER 1 (IRT1), which is a divalent metal ion transporter localized on the plasma membrane, subsequently transports Fe(II) into the plant roots (Vert et al., 2002). Graminaceous plants use the chelation strategy (strategy II) for iron uptake from the soil. The production of iron chelators, i.e., the mugineic acid (MA) family phytosiderophores (PSs), is the key step. The PSs are synthesized from S-adenosyl-L-methionine, through a series of conserved reactions catalyzed by NICOTIANAMINE SYNTHASE (NAS), NICOTIANAMINE AMINOTRANSFERASE (NAAT) and DEOXYMUGINEIC ACID SYNTHASE (DMAS) (Kobayashi et al., 2010). In addition to being a precursor for DMA production, NA also chelates iron forming Fe(II)-NA and Fe(III)-NA complexes, and transports iron within the plants (von Wiren et al., 1999; Takahashi et al., 2003). The DMAs are either directly exported to the soil as chelators for iron acquisition, for example in rice, or they are further converted into other MAs as is the case in barley (Kobayashi et al., 2010). In wheat, 21 *NAS* genes were identified and classified into two distinct clades (Bonneau et al., 2016). Under iron deficiency condition, the clade I *NAS* genes were up-regulated in root tissues (Bonneau et al., 2016). In addition, six *TaNAAT* homeologs and three *TaDMAS1* homeologs have been identified, and these are most closely related to the barley *HvNAAT* and *HvDMAS1* (Beasley et al., 2017). The *TaNAAT1*, *TaNAAT2*, and *TaDMAS1* genes were also significantly upregulated in root tissues under Fe deficiency treatment (Beasley et al., 2017). In rice, the PSs are transported to the rhizosphere by the phytosiderophore efflux transporter TRANSPORTER OF MUGINEIC ACID 1 (TOM1) (Nozoye et al., 2011). The released PSs chelate Fe(III) and the resulting Fe(III)-PS complexes are transported back into roots cells by the YELLOW STRIPE-LIKE (YSL) family of transporters (Kobayashi and Nishizawa, 2012). *OsYSL15* in rice encodes a Fe(III)-DMA and Fe(II)-NA transporter and is induced in the root epidermis under iron deficiency (Inoue et al., 2008; Lee et al., 2009). The YSL family of transporters is also suggested to facilitate long-distance iron transport through the phloem (Inoue et al., 2009; Lee et al., 2009; Ishimaru et al., 2010; Kumar et al., 2018). The function of YSL transporters in iron translocation is well-established in several crop plants, including maize and barley (Curie et al., 2001; Koike et al., 2004; Murata et al., 2006; Aoyama et al., 2009; Ishimaru et al., 2010; Kakei et al., 2012). Recently, 26 wheat *YSL* genes have been identified and comprise the largest plant *YSL* gene family known so far. These wheat *YSL* genes are differently regulated in wheat roots and shoots in response to iron-deficient stress (Kumar et al., 2018).

Specific transporters control iron translocation between the subcellular compartments. Iron in plants is often stored in vacuoles and iron import and export between vacuoles and the cytoplasm is dynamically regulated by the plant iron status. Some NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (NRAMP) such as AtNRAMP3 and AtNRAMP4 function in transporting iron out of vacuoles, whereas VACUOLAR IRON TRANSPORTER (VIT) has the opposite function (Lanquar et al., 2005; Zhang et al., 2012). Most of the iron in plants is found in leaf chloroplasts (Buckhout and Schmidt, 2013). PERMEASE IN CHLOROPLASTS 1 (PIC1) is associated with iron transport across the inner chloroplast envelope in Arabidopsis and tobacco (Duy et al., 2007, 2011; Gong et al., 2015), and Arabidopsis AtYSL4 and AtYSL6 export iron out of chloroplasts (Divol et al., 2013). The MITOCHONDRIAL IRON TRANSPORTER (MIT) (Bashir et al., 2011) is involved in iron transport into mitochondria.

In addition to the storage in vacuoles, iron is also stored in plastids where it is bound to FERRITIN (Briat et al., 2010). The FERRITIN globular protein complex can harbor up to 4,500 iron atoms (Harrison and Arosio, 1996) and therefore is an important iron storage protein in plants. Because of this iron storage capacity, targeted expression of FERRITIN in the endosperm is often used in iron biofortification strategies (Wirth et al., 2009; Boonyaves et al., 2016, 2017; Trijatmiko et al., 2016; Wu et al., 2018).

Additionally, several transcription factor families such as the BASIC HELIX-LOOP-HELIX (bHLH), WRKY, and NO APICAL MERISTEM (NAM)/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR (ATAF)/CUP-SHAPED COTYLEDON (CUC) (NAC) families are known to regulate the iron deficiency stress response (Ogo et al., 2006; Uauy et al., 2006; Sperotto et al., 2008; Wang et al., 2013). Transcription factors from the bHLH family, such as Fe-DEFICIENCY-INDUCIBLE BASIC HELIX-LOOP-HELIX TRANSCRIPTION FACTOR 2 (OsIRO2) and OsbHLH133 in rice, are known to regulate iron homeostasis-related genes in many plants (Ogo et al., 2006; Wang et al., 2013). Studies have shown that bHLH proteins are also involved in the regulation of iron uptake in chrysanthemum (Zhao et al., 2014) and *Populus* plants (Huang and Dai, 2015).

Wheat is the most widely cultivated commercial crop in the world and feeds 40% of the population and provides 20% of the energy for human nutrition (Gupta et al., 2005). Although the knowledge pool concerning iron homeostasis in bread wheat is slowly advancing, the information is very limited when compared to model plants such as Arabidopsis and rice (Uauy et al., 2006; Waters et al., 2009; Bonneau et al., 2016; Beasley et al., 2017; Garnica et al., 2018; Kumar et al., 2018; Mathpal et al., 2018). In particular, the molecular response of bread wheat to iron deficiency stress is unknown but could help breeders to increase grain iron content and growers to optimize agricultural management. We investigated wheat plants grown on iron deficiency condition and observed their response. The chlorophyll content was significantly lower in plants experiencing iron deficiency condition. The expression of endogenous iron homeostasis-related genes in roots, flag leaves and grains of iron deficient wheat plants indicated tissue- and growth stage-specific roles for iron chelators, transporters and transcription factors.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Bread wheat (*Triticum aestivum* cv. Bobwhite S26) seeds were germinated on wet filter paper for 1 week. The seedlings were then transferred to hydroponic nutrient solutions (pH 6.0): 0.88 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM KCl, 1.0 µM H_3BO_3, 1.0 µM $MnSO_4$, 0.2 µM $CuSO_4$, 0.02 µM $(NH_4)_6Mo_7O_{24}$, 1.0 µM $ZnSO_4$, and 100.0 µM Fe(III)-EDTA for maintenance of growth (control condition) (Durmaz et al., 2011). After 1 week, half of seedlings were transferred to hydroponic nutrient solutions with 10.0 µM Fe(III)-EDTA (for iron deficiency stress treatment) under greenhouse conditions (22°C/18°C with 16-h light/8-h dark cycle, 60% humidity). Air was continuously pumped into the hydroponic system for better circulation. The hydroponic medium was changed once per week and the solution was neutralized to pH 6.0 twice per week. Roots, flag leaves and grains were harvested at three different development stages, i.e., 8–10 DPA - days post anthesis (stage 1), 25–28 DPA (stage 2), and 33–35 DPA (stage 3). The three sampling stages correspond to 96, 113, 121 days after transplantation to the hydroponics solution, respectively. Three biological replicates were collected for each sample.

Chlorophyll Content Measurement

Twenty-five (25) mg of ground flag leaves samples were incubated in 15 mL of 95% ethanol solution for 24 h in dark at room temperature. Extraction at room temperature for 24 h is suggested to have better efficiency than in the refrigerator or for shorter duration (Arvola, 1981). Three biological replicates and two technical replicates were analyzed. The ethanol extract was subjected to Ultrospec 2100 UV/Visible spectrometer measurements (Pharmacia Biotech, Sweden) at A_{649} and A_{664}. The quantification of chlorophyll a and chlorophyll b were calculated according to the protocol by Lichtenthaler and Buschmann (2001). The sample used for chlorophyll measurement was standardized at 25 mg fresh weight (FW) and chlorophyll content/g FW was calculated accordingly.

Metal Measurements

Roots, flag leaves and grain samples were ground and digested with 4 mL 32.5% HNO_3 in a single reaction chamber microwave system (turboWave, MLS GmbH, Switzerland). After the digestion, samples were volumed up to 10 mL with water, and this solution was further diluted 10 times to be analyzed in ICP-OES (ICPE 9820, Shimadzu, Japan). The wavelength used for iron, zinc, copper and manganese was 259.940, 202.548, 324.754, and 257.610, respectively.

Total RNA Extraction and cDNA Synthesis

Isolation of total RNA from the roots and flag leaves was carried out using the Isol- RNA Lysis Reagent (5 PRIME, USA). RNA extraction from wheat grains was carried out following the protocol described by Singh and colleagues (Singh et al., 2003). Total RNA used for real-time quantitative PCR was treated with DNase I (Thermo Fisher Scientific, USA). The RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, USA) was used for cDNA synthesis. All steps were carried out following the manufacturers' instructions.

Gene Expression and Statistical Analysis

Gene expression analysis was carried out using qRT-PCR performed with the LightCycler® 480 Instrument II Real Time PCR system (Roche, Switzerland) using Taqman hydrolysis probes (Roche, Switzerland). The total reaction volume of each sample was set as 10 µL, comprising 5 µL of mastermix (Applied Biosystems, USA), 3 µL of 10× diluted cDNA, 0.9 µL of forward primer, 0.9 µL of reverse primer, 0.1 µL of the probe and 0.1 µL of H_2O.

The primers were designed based on the Roche universal probe library assay design center website (https://lifescience.roche.com/shop/CategoryDisplay?catalogId=10001&tab=&identifier=Universal±Probe±Library&langId=-1&storeId=15006). The corresponding probes were also selected using the same webportal. The primer sequences and probe numbers are provided in **Supplementary Table S1**. Primer efficiency was determined using a standard curve calculated with the following formula: $E = 10^{\wedge(-1/\text{slope})}$ (**Supplementary Table S1**). Genes for the study were selected based on % sequence identity to known homologs in other plant species using NCBI UniGene database as well as on published reports on wheat specific genes. The 19 studied wheat genes included phytosiderophore biosynthesis-related genes as well as several genes encoding different iron transporters, transcription factors and the storage protein FERRITIN (**Table 1**). Amplicons were searched against IWGSC (International Wheat Genome Sequencing Consortium) chromosome-based wheat genome sequencing (RefSeq assembly v1.0) in Ensembl database (**Supplementary Table S2**). For the already characterized wheat

genes, same gene name as in the original publication are used in this study (Borg et al., 2012; Bonneau et al., 2016; Beasley et al., 2017; Connorton et al., 2017; Guo and Wang, 2017; Kumar et al., 2018). The qRT-PCR reaction was carried out using the following steps: incubation at 50°C for 2 min, initialization at 95°C for 10 min, followed by denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min (these two steps were repeated for 40 cycles).

The reference genes for qRT-PCR normalization were chosen from 16 candidate genes that were demonstrated as stably expressed genes in the Genevestigator™ database and the literature (Zimmermann et al., 2005; Paolacci et al., 2009). Among these 16 tested genes, Ta.55681 (casein kinase II subunit beta-like), Ta.40026 (BTB/POZ and MATH domain-containing protein 4-like) and Ta.22845 (26S proteasome regulatory subunit) were found to be the most stable reference genes across all of the tested samples (unpublished data). Ta.55681 was used for the normalization of qRT-PCR data on genes of interest. Data normalization was performed as described by Schefe and colleagues (Schefe et al., 2006). Statistical analysis (one-way ANOVA) was carried out using RStudio (Version 1.1.456) and Excel. Figures were prepared in RStudio using the R plotting packages (ggplot2, pheatmap).

RESULTS

Iron Deficiency Stressed Plants Have Lower Tissue Iron Concentration and Lower Chlorophyll Content in Flag Leaves

The chlorophyll content in flag leaves and metal concentration in roots, flag leaves and grains were measured in the iron deficiency stressed plants in comparison to the plants grown on normal condition. For the chlorophyll measurement, the samples collected at three development stages during grain filling, i.e., 8–10 days post anthesis (DPA; stage 1), 25–28 DPA (stage 2), and 33–35 DPA (stage 3) were analyzed. In growth stage 1, the content of chlorophyll a and chlorophyll b, together with the total chlorophyll content (chlorophyll a+b), decreased around 70% in the wheat plants experiencing iron deficiency (**Figure 1**). In stage 2, the total chlorophyll content and chlorophyll a content decreased nearly 50%. In stage 3, 50% decrease in chlorophyll a content could be observed in iron-deficient wheat flag leaves. For the metal concentration measurement, the samples collected at 25–28 DPA (stage 2) were analyzed. The iron concentration in flag leaves and grains was significantly lower in plants under iron deficiency stress (**Figure 1**). On the other hand, the concentration of copper and zinc in all tissues and the manganese concentration in roots and flag leaves was higher in plants treated with low iron (**Supplementary Figure S1**). Further, plant height and panicle number were significantly reduced under iron deficiency stress (**Supplementary Figure S2**). Together, these observations indicate strong iron stress in the plants (**Figure 1**). We explored the expression differences of iron homeostasis related genes under iron deficiency stress (results below).

Iron Deficiency Induces Expression of Genes Involved in the Phytosiderophore Synthesis and Secretion

The relative mRNA levels of the wheat homologous genes *SAMS* (Ta.69768), *TaNAS6-D* (Ta.37977), *NAS* (Ta.5549), *TaNAAT2-B* (Ta.4977), and *TaDMAS1-A* (Ta.5335) that are related to PS biosynthesis were significantly increased in response to iron-deficient conditions, particularly in the roots (**Table 1**, **Figure 2**). In the iron-sufficient growth condition, their expression was similar in all tissues and development stages (**Figure 2**). **Table 1** summarizes the fold change difference between iron sufficient and deficient condition, while relative gene expression level differences can be observed in **Figure 2**, **Supplementary Table S3**. During iron deficient growth, the expression of *SAMS* (Ta.69768) increased in roots by 8.2-fold at stage 1, and around 3-fold at stages 2 and 3 (**Table 1**). The *NAS* homologs were up-regulated in roots at least 11-fold at all three stages, but in stage 2 their mRNA levels increased more than 200-fold, indicating a very strong but transient up-regulation of the genes during the peak grain filling period (**Table 1**). Likewise, the expression of *TaNAAT2-B* (Ta.4977) increased in roots by 5-fold at stage 1 and 2, and by 3.6-fold at stage 3. The expression of *TaDMAS1-A* (Ta.5335) was highest at stage 1 (21.8- fold up-regulated in roots), followed by stage 2 (15.1-fold) and 3 (6.9-fold). In line with the increased expression of genes involved in phytosiderophore synthesis, the expression of the phytosiderophore efflux transporter *TOM* homolog Ta.5180 was also significantly up-regulated in iron-deficient roots at all the development stages, with highest expression at stage 2 (243.3-fold) (**Table 1**, **Figure 2**).

The expression of phytosiderophore biosynthesis related genes was also increased in the flag leaves in iron-deficient conditions, including *SAMS* (Ta.69768) at stage 1, *TaNAS6-D* (Ta.37977), *NAS* (Ta.5549) at stage 2 and *TaDMAS1-A* (Ta.5335) at stage 3, but generally the increase of their expression was lower than in roots. In addition to roots and the flag leaves, iron deficiency also increased gene expression in developing wheat grains. With a few exceptions, most of the expression changes in grains were restricted to growth stage 1 (**Table 1**, **Figure 2**). The increased expression of the phytosiderophore biosynthesis and iron acquisition-related genes indicate that NA and DMA production was enhanced in response to iron deficiency, especially in the roots. The stronger increase in expression in roots as compared with the flag leaves and grains is consistent with the required increase in phytosiderophore production by the roots and their export into the rhizosphere for iron chelation. Furthermore, the up-regulation of *NAS* and *DMAS* in the flag leaves and grains suggests the involvement of NA and DMA in long-distance iron translocation within the plants, and also indicates an active transport of iron into the grains at growth stage 1 (**Figure 2**).

Expression of Genes Encoding Long-Distance Iron Transporters

The transcript levels of *TaYS1A* (Ta.48303), *TaYSL19-2A* (Ta.5463), *TaYSL9* (Ta.29321) encoding the wheat homologs of

TABLE 1 | Gene expression fold change between iron deficient and sufficient condition in bread wheat.

Gene	Unigene ID	Roots			Flang leaves			Grains		
		Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
Phytosiderophore synthesis and secretion										
SAMS	Ta.69768	8.2*	3.1**	3.0*	2.8*	−1.2	−1.4	2.0**	1.5	2.0
TaNAS6-D	Ta.37977	35.3**	231.3**	11.7**	81.8	4.4*	3.1	1.6**	1.6*	1.5
NAS	Ta.5549	70.7**	852.6**	18.2**	612.1	5.6*	36.9	1.5	−9.8	2.8
TaNAAT2-B	Ta.4977	5.3**	5.6**	3.6*	1.9	−14.1	−1.5	1.7**	−1.3	1.1
TaDMAS1–A	Ta.5335	21.8*	15.1**	6.9*	2.9	1.2	1.6*	1.4**	1.7**	1.0
TOM	Ta.5180	38.2**	243.3**	11.3**	410.9	6.1	3.7	−2.3	−46.3	3.7
Long-distance iron transport										
TaYS1A	Ta.48303	14.8**	22.8**	9.8**	3.4	2.4*	1.4	−1.6	−1.6	3.4
TaYSL19-2A	Ta.5463	69.3	48.3**	49.3**	341.2*	79.5	16.1	–	–	–
TaYSL9	Ta.29321	9.8**	12.9**	6.2*	2.6	−1.6**	−1.2	1.2*	2.1	1.5
ZIP	Ta.31727	3.2**	2.8**	2.5*	1.8	1.3	1.1	1.4	1.0	1.2
ZIP	Ta.38190	−2.9	−10.4	1.1	−8.1*	−43.0*	−21.8	−1.2	−1.4	1.3
Intracellular iron transport and storage										
NRAMP	Ta.13247	2.2**	2.0**	2.3**	12.3**	2.5	2.1*	1.9**	1.1	1.2
IREG/FPN	Ta.72669	17.3**	20.3**	8.2*	20.8	1.2	2.7*	1.3**	−1.3*	1.4
VIT	Ta.34653	11.0**	7.0**	14.2**	–	–	–	−1.0	−14.4	2.6
TaVIT2-D	Ta.22759	1.6	3.9*	−1.0	−12.7	−13.1	−22.7*	−1.4	1.1	−1.4
FRO	Ta.10673	1.9**	1.6*	2.3	14.6*	12.2*	7.1*	4.2**	−2.3	5.7
TaFer1-D	Ta.5220	−6.6**	−1.5	−6.0	−7.9*	−9.1**	−5.3	−1.8*	−2.0**	−1.3
Transcriptional regulation										
bHLH	Ta.34545	3112.1**	8097.4**	288.8**	594.3**	1185.2	1115.9*	1.4	−3.9	1.9
wbHLH056	Ta.93472	8.8**	20.1**	4.8*	194.8**	3373.4	2599.2	10.3**	–	2.6

*The numbers indicate the fold change between iron deficient and iron sufficient growth conditions. Positive values and the yellow to red color indicates the up-regulation. Negative values and the light blue to blue color indicates the down-regulation of the gene expression. Significant differences are marked with * when $p \leq 0.05$ or with ** when $p \leq 0.01$. "-" indicates no detection of expression. SAMS, S-adenosyl-L-methionine Synthetase; NAS, nicotianamine synthase; NAAT, nicotianamine aminotransferase; DMAS, deoxymugineic acid synthase; TOM, transporter of mugineic acid; YSL, yellow stripe like; ZIP, zinc-regulated transporter (ZRT)/ iron-regulated transporter (IRT)-like; FRO, ferric chelate reductases; NRAMP, natural resistance associated macrophage protein; IREG/FPN, iron regulated/ferroportin; VIT, vacuolar iron transporter; bHLH, basic HELIX-LOOP-HELIX. Among the tested genes, NA/MA biosynthesis genes (SAMS, NAS, NAAT, DMAS), VITs and ZIPs may also contribute to Zn uptake and transport. Based on studies in rice, VIT (OsVITs1 & 2; Zhang et al., 2012), ZIPs (OsZIP1; Ramesh et al., 2003), OsZIP5 (Lee et al., 2010a), OsZIP8 (Yang et al., 2009; Lee et al., 2010b), OsZIP4 (Ishimaru et al., 2005), OsZIP3 (Sasaki et al., 2015) are putatively involved in Zn transport in rice. NA/MA biosynthesis genes (SAMS, NAS, NAAT, DMAS) also play a role in Zn homeostasis because NA/DMA may complex Zn in the phloem in rice (Suzuki et al., 2008; Nishiyama et al., 2012).*

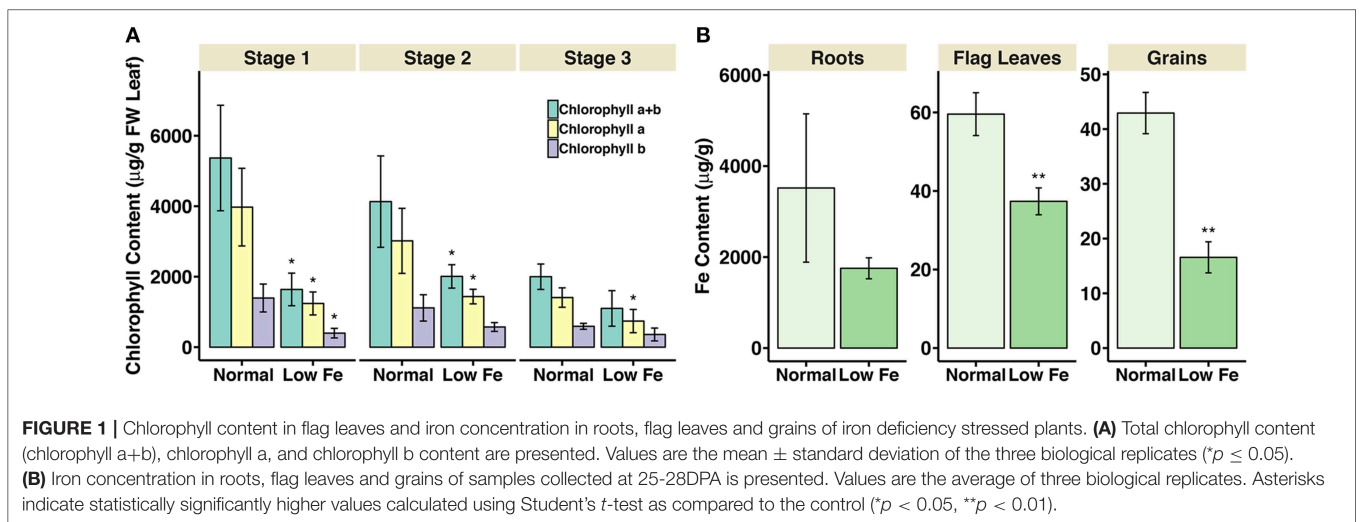

FIGURE 1 | Chlorophyll content in flag leaves and iron concentration in roots, flag leaves and grains of iron deficiency stressed plants. **(A)** Total chlorophyll content (chlorophyll a+b), chlorophyll a, and chlorophyll b content are presented. Values are the mean ± standard deviation of the three biological replicates (*$p \leq 0.05$). **(B)** Iron concentration in roots, flag leaves and grains of samples collected at 25-28DPA is presented. Values are the average of three biological replicates. Asterisks indicate statistically significantly higher values calculated using Student's *t*-test as compared to the control (*$p < 0.05$, **$p < 0.01$).

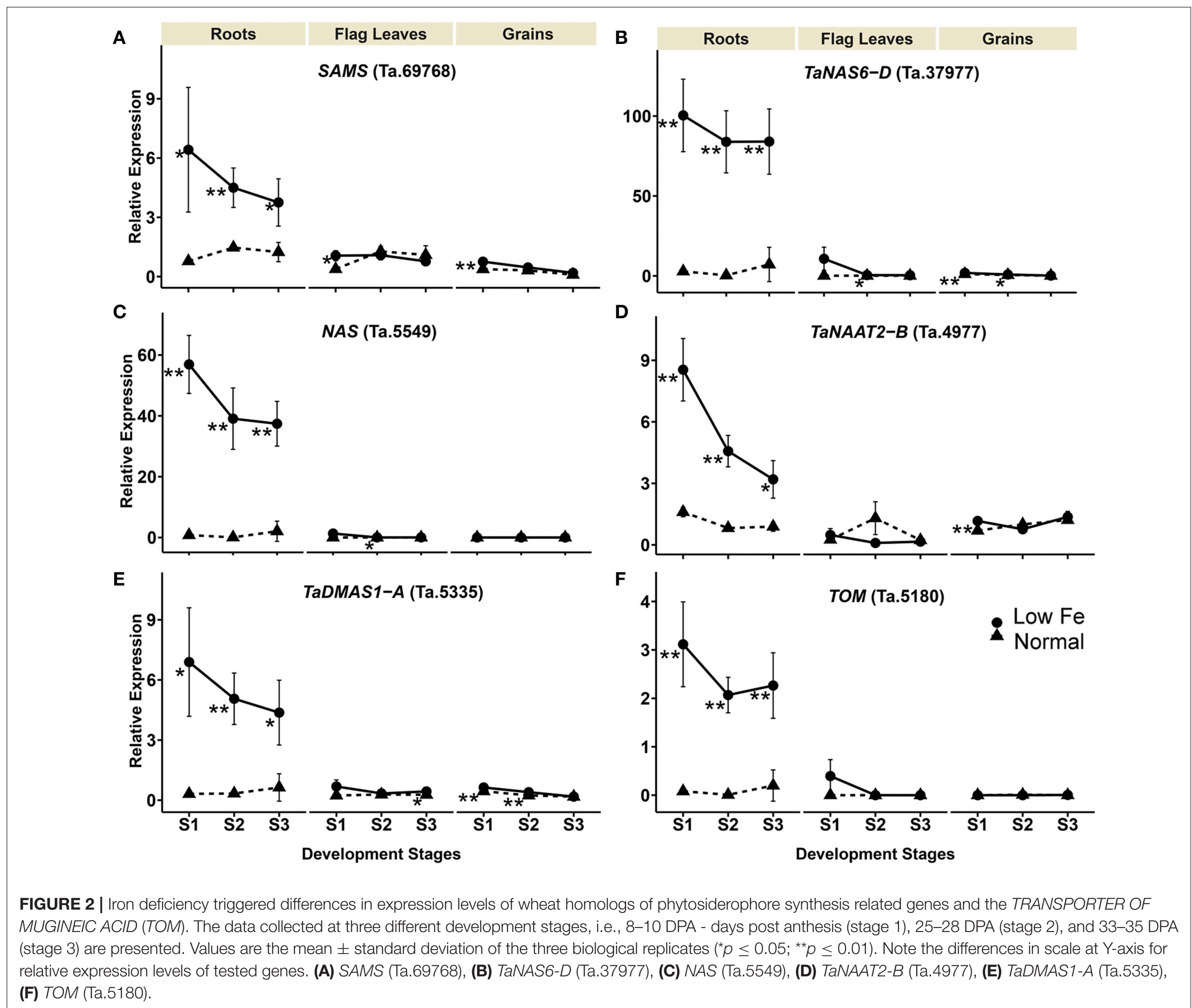

FIGURE 2 | Iron deficiency triggered differences in expression levels of wheat homologs of phytosiderophore synthesis related genes and the *TRANSPORTER OF MUGINEIC ACID* (*TOM*). The data collected at three different development stages, i.e., 8–10 DPA - days post anthesis (stage 1), 25–28 DPA (stage 2), and 33–35 DPA (stage 3) are presented. Values are the mean ± standard deviation of the three biological replicates (*$p \leq 0.05$; **$p \leq 0.01$). Note the differences in scale at Y-axis for relative expression levels of tested genes. **(A)** *SAMS* (Ta.69768), **(B)** *TaNAS6-D* (Ta.37977), **(C)** *NAS* (Ta.5549), **(D)** *TaNAAT2-B* (Ta.4977), **(E)** *TaDMAS1-A* (Ta.5335), **(F)** *TOM* (Ta.5180).

the metal-nicotianamine transporter YSL increased significantly in the iron deficient condition (**Table 1**). The expression of *TaYS1A* (Ta.48303) was up-regulated more than 9-fold in roots at all stages, and by 2.4-fold in flag leaves at stage 2 (**Table 1**, **Figure 3**). The expression of *TaYSL19-2A* (Ta.5463) in roots increased by 48.3- and 49.3-fold at stages 2 and 3, respectively, and in the flag leaves by 341.2-fold at stage 1. Expression of the *TaYSL9* (Ta.29321) was also significantly increased in roots at all the growth stages (**Table 1**, **Figure 3**). While a small increase in the expression of *TaYSL9* was detected in the grains at stage 1, the gene was down-regulated in the flag leaves collected at stage 2, suggesting that the wheat genes encoding the YSL homologs are differentially regulated.

The expression of two genes from the ZIP family (Ta.31727, Ta.38190) differed in the iron-deficient condition (**Table 1**, **Figure 3**). Expression of the *ZIP* homolog (Ta.31727) in roots was increased more than 2.5-fold at all development stages but was not altered in the flag leaves and grains. On the contrary, Ta.38190 showed a significant decrease in expression in the flag leaves at stages 1 and 2.

Differential Expression of Genes Encoding Intracellular Iron Transporters and Storage Proteins

Expression of the *NRAMP* homolog Ta.13247 increased in the flag leaves by 12- and 2- fold in stages 1 and 3, respectively. In the roots, the Ta.13247 expression increased approximately 2-fold in all of the development stages. In the grains, the gene was up-regulated in the stages 1 with 1.9-fold increase (**Table 1**, **Figure 4**). IRON REGULATED PROTEIN/ FERROPORTIN (IREG/FPN) homolog (Ta.72669) showed increased expression in the roots during all stages. In addition, the expression of Ta.72669 also increased by 2.7- and 1.3-fold in stage 3 of flag

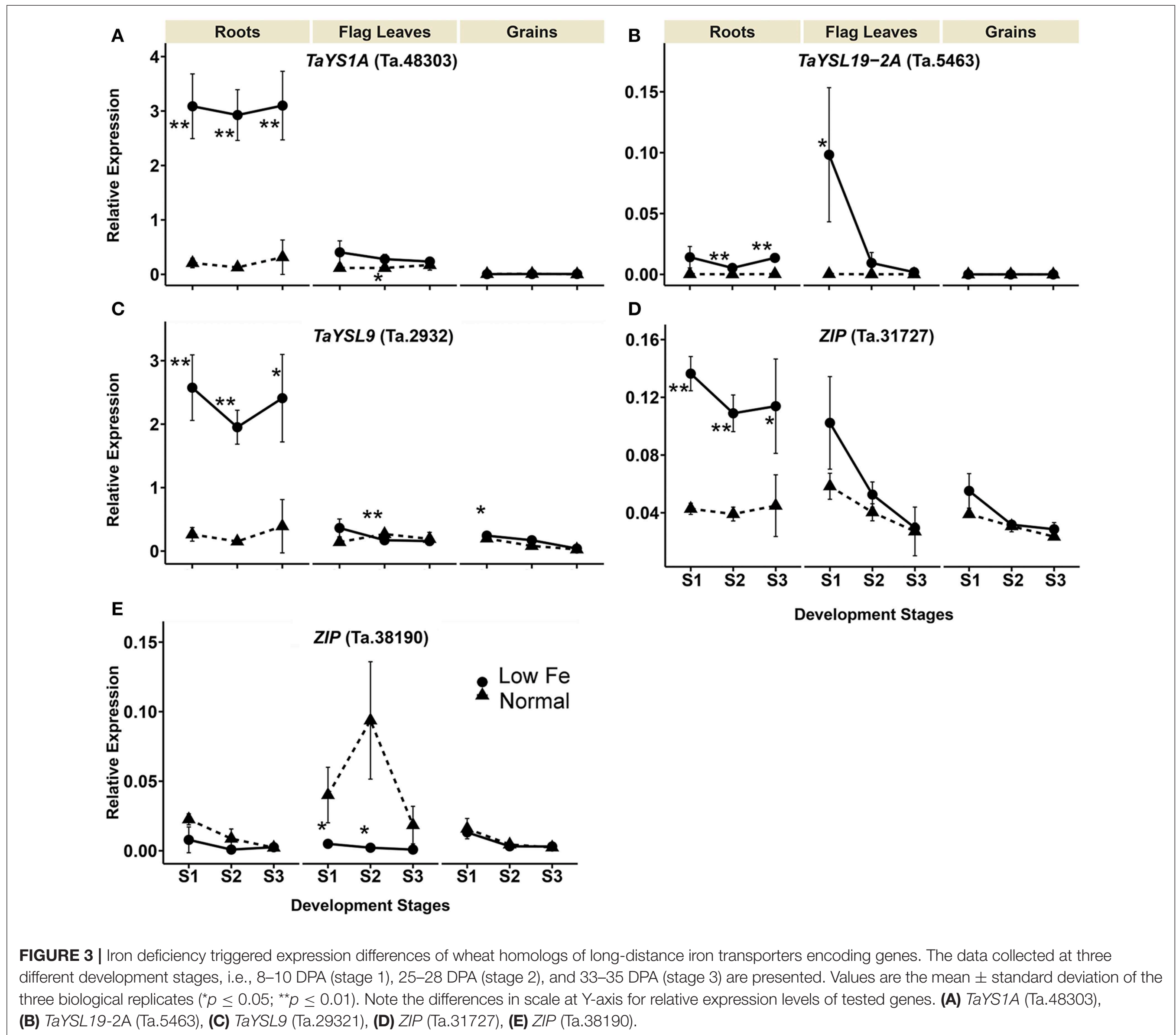

FIGURE 3 | Iron deficiency triggered expression differences of wheat homologs of long-distance iron transporters encoding genes. The data collected at three different development stages, i.e., 8–10 DPA (stage 1), 25–28 DPA (stage 2), and 33–35 DPA (stage 3) are presented. Values are the mean ± standard deviation of the three biological replicates (* $p \leq 0.05$; ** $p \leq 0.01$). Note the differences in scale at Y-axis for relative expression levels of tested genes. **(A)** *TaYS1A* (Ta.48303), **(B)** *TaYSL19*-2A (Ta.5463), **(C)** *TaYSL9* (Ta.29321), **(D)** *ZIP* (Ta.31727), **(E)** *ZIP* (Ta.38190).

leaves, and in stage 1 of the grains, respectively. However, a 1.3-fold decrease in expression of Ta.72669 was detected in the grains in stage 2 (**Table 1**, **Figure 4**). The expression of *VIT* homolog (Ta.34653) could not be detected in the flag leaves regardless of the growth conditions and stage of development. However, a significant increase in gene expression was observed in the roots, ranging from 7- to 14.2-fold changes (**Table 1**). The *TaVIT2-D* (Ta.22759) was only up-regulated in the roots in stage 2, but a significant decrease (22.7-fold) in gene expression was observed in the flag leaves in stage 3 (**Table 1**, **Figure 4**). The dynamic modulation of vacuolar iron transporters suggest that plant cells tightly maintain the balance between iron storage and usage, and actively re-distribute iron to the cell organs based on the iron status and demand within the plants. Iron deficiency also caused an increase in the expression of the *FRO* homolog Ta.10673 in roots at stages 1 and 2 (1.9- and 1.6-fold, respectively) (**Table 1**, **Figure 4**). *FRO* expression was also highly up-regulated in the flag leaves at all development stages (14.6-, 12.2-, and 7.1-fold) and also 4.2-fold in the grains during stage 1.

The expression of *FERRITIN* homolog (*TaFer1-D*, Ta.5220) was down-regulated in the roots, flag leaves and grains due to iron starvation. A more than 6-fold decrease in the expression of *TaFer1-D* was observed in the roots during stage 1. In the flag leaves and grains, the gene was down-regulated in stages 1 and 2 by more than 7.9- and 1.8-fold, respectively (**Table 1**, **Figure 4**). The results indicate that the plants reduce iron storage under low iron availability.

Expression Modulation of bHLH Transcription Factor Encoding Genes

Upon iron deficiency stress, the expression of *bHLH* homolog Ta.34545 was significantly up-regulated by 3112.1-, 8097.4-,

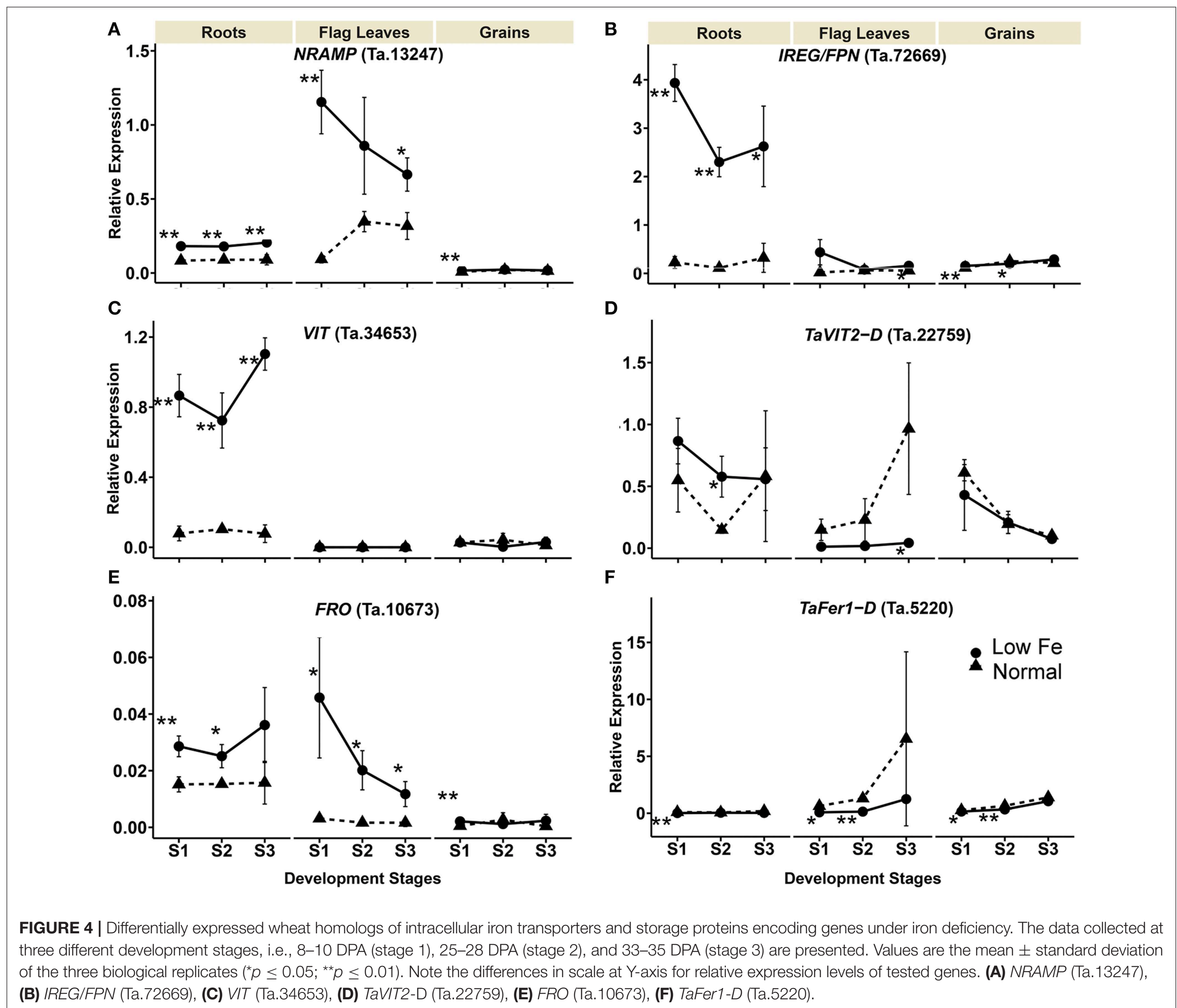

FIGURE 4 | Differentially expressed wheat homologs of intracellular iron transporters and storage proteins encoding genes under iron deficiency. The data collected at three different development stages, i.e., 8–10 DPA (stage 1), 25–28 DPA (stage 2), and 33–35 DPA (stage 3) are presented. Values are the mean ± standard deviation of the three biological replicates (* $p \leq 0.05$; ** $p \leq 0.01$). Note the differences in scale at Y-axis for relative expression levels of tested genes. **(A)** *NRAMP* (Ta.13247), **(B)** *IREG/FPN* (Ta.72669), **(C)** *VIT* (Ta.34653), **(D)** *TaVIT2*-D (Ta.22759), **(E)** *FRO* (Ta.10673), **(F)** *TaFer1-D* (Ta.5220).

and 288.8-fold in the roots in stage 1, 2, and 3, respectively. Similar increases were observed for the flag leaves, particularly in stage 1 and 3, with at least a 594.3-fold increase in gene expression. No significant difference in the expression was observed in the grains. Additionally, the expression of the *IRO2* homolog (*wbHLH056*, Ta.93472), which is also a *bHLH* member, was up-regulated by 4.8- to 20.1-fold in the roots when plants experienced iron-deficiency (**Table 1**, **Figure 5**). Increased expression of *wbHLH056* was observed in flag leaves (195-fold) and grains (10-fold), particularly during stage 1 (**Table 1**, **Figure 5**).

DISCUSSION

It has been suggested that PS production increases during iron deficiency in strategy II plants, including barley, rice, maize, sorghum, red fescue, and wheat (Kanazawa et al., 1994; Ma et al., 2003; Ishimaru et al., 2006; Tsednee et al., 2012; Garnica et al., 2018). Several reports have shown that iron deficiency in rice, barley and maize increases the expression of genes involved in NA and DMA synthesis (Negishi et al., 2002; Inoue et al., 2003, 2008; Kobayashi et al., 2005; Bashir et al., 2006; Zheng et al., 2009; Zhou et al., 2013). Similarly, an increased expression of *TaNAS* genes, *TaNAAT* genes, and *TaDMAS1* in wheat roots in response to iron-limited conditions during the vegetative growth stage have been reported (Bonneau et al., 2016; Beasley et al., 2017). In this study, we find that the expression of *SAMS*, *NAS*, *NAAT*, and *DMAS* homologs is increased in iron-deficient conditions, particularly in roots, suggesting increased production of NA and DMA for iron acquisition and translocation (**Figure 6**). NA chelates iron for long-distance and intracellular transport, and also is an intermediate in DMA production (Takahashi et al., 2003; Schuler et al., 2012). Recent evidence suggests that in addition to its well-known role in iron uptake from the

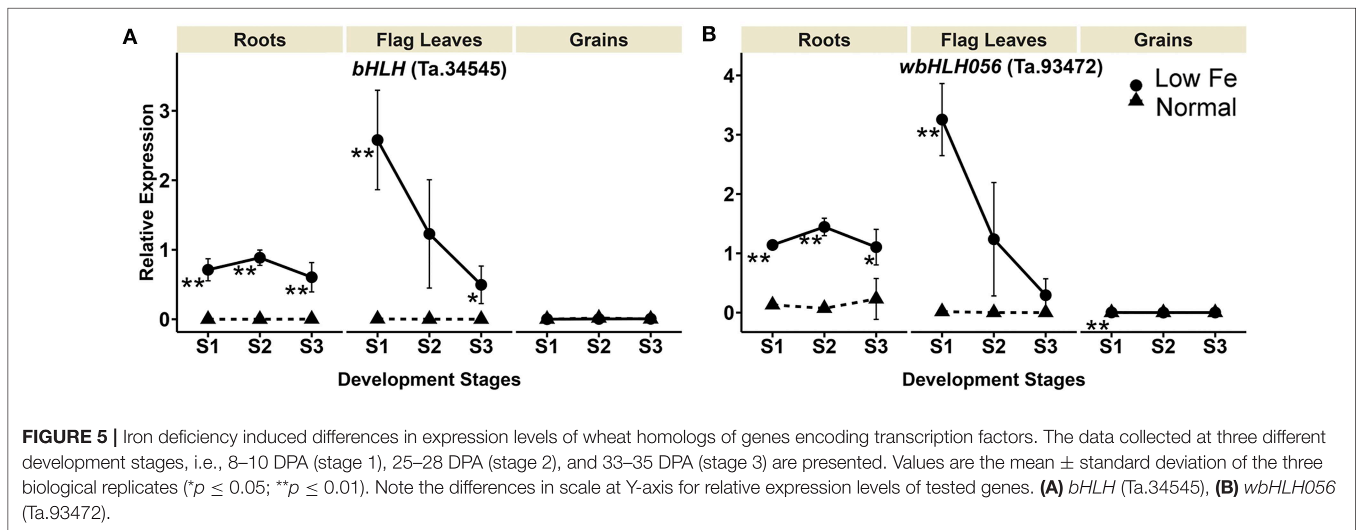

FIGURE 5 | Iron deficiency induced differences in expression levels of wheat homologs of genes encoding transcription factors. The data collected at three different development stages, i.e., 8–10 DPA (stage 1), 25–28 DPA (stage 2), and 33–35 DPA (stage 3) are presented. Values are the mean ± standard deviation of the three biological replicates (* $p \le 0.05$; ** $p \le 0.01$). Note the differences in scale at Y-axis for relative expression levels of tested genes. **(A)** *bHLH* (Ta.34545), **(B)** *wbHLH056* (Ta.93472).

rhizosphere, DMA also chelates iron in the xylem and phloem for long-distance transport in rice (Kakei et al., 2009; Nishiyama et al., 2012). The increased expression of *NAS* and *DMAS* genes in the flag leaves and grains suggest similar roles for DMA and NA in long-distance iron translocation in wheat. In rice, contrary to the other two *NAS* gens, *OsNAS3* is not induced under low iron stress in shoots and it is likely to be rather involved in mitigation of excessive iron stress (Inoue et al., 2003; Suzuki et al., 2008; Aung et al., 2018, 2019). Of the wheat *NAS* genes investigated in the present study, no *NAS* gene showed a similar expression pattern as *OsNAS3*, which is in line with findings by Bonneau et al. (2016).

Parallel to the increased PS production, the expression of genes encoding PS transporters is also positively modulated by iron deficiency to facilitate efficient iron translocation. TOM1 and TOM2 transporters facilitate DMA secretion into the rhizosphere and vascular loading, respectively (Nozoye et al., 2011, 2015). The wheat *TOM* homolog Ta.5180 has high DNA sequence similarity with the barley and rice *TOM1* genes and was significantly increased in wheat roots during iron deficiency, suggesting a potential role of the transporter in DMA efflux in wheat roots. The YSL transporters in non-grass plants such as Arabidopsis have been studied in detail for their function in iron transport and homeostasis. For example, YSL2 is proposed to facilitate lateral movement of metals in the vasculature (DiDonato et al., 2004), and YSL4 and YSL6 control iron release from chloroplasts (Divol et al., 2013). Detailed functions of the wheat YSL homologs in the iron deficiency response and maintenance of homeostasis remain unclear, but their increased expression in roots at all development stages suggest a primary role in iron uptake as well (Kumar et al., 2018; this study).

In rice, the transcription factor OsIRO2 regulates the expression of genes involved in phytosiderophore synthesis (*OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1)*, and genes encoding the PS transporter TOM1 as well as other iron transporters such as OsYSL15 (Ogo et al., 2006, 2007, 2011). Under iron deficiency, *OsIRO2* expression is positively regulated by the transcription factor IDE BINDING FACTOR (OsIDEF1) (Kobayashi and Nishizawa, 2012), which together with the NAC family transcription factor IDEF2 also regulates some of the iron deficiency-responsive genes in rice (Ogo et al., 2008). The Arabidopsis genes encoding the Ib subgroup bHLH transcription factors, including *bHLH38*, *bHLH39*, *bHLH100*, and *bHLH101*, have a closer phylogenetic relationship with the rice *OsIRO2* gene (Hindt and Guerinot, 2012). These bHLH transcription factors function in Arabidopsis iron homeostasis and are up-regulated under iron starvation (Wang et al., 2007). bHLH38 and bHLH39 can form heterodimers with the FER-Like iron deficiency-induced transcription factor (FIT) and they regulate the expression of *FRO2* and *IRT1* in Arabidopsis (Yuan et al., 2008). In contrast, the *bHLH100* and *bHLH101* function in a FIT-independent manner (Sivitz et al., 2012). The wheat homologs of *IRO2* were significantly up-regulated in roots and the flag leaves during iron deficiency, suggesting that they have a significant function in iron homeostasis and regulation of iron deficiency-responsive genes in wheat. Similar to reports from other plants (Ogo et al., 2006, 2007; Wang et al., 2007; Sivitz et al., 2012), the increased expression of PS-related genes and genes encoding iron transporters parallels the up-regulation of *IRO2* during iron deficiency in wheat.

The expression of iron homeostasis-related genes often depends on the external and/or internal iron status of plants. This is also relevant for the gene encoding important iron storage protein FERRITIN. Similar to the Arabidopsis *AtFERRITIN* genes (Buckhout et al., 2009; Rodriguez-Celma et al., 2013), the *TaFer1-D* gene in bread wheat was down-regulated under the iron-deficient conditions. Excess iron induces the *FERRITIN* transcript levels (Briat et al., 2010), supporting the storage function of FERRITIN in wheat. Further, we observed that the expression level of the *TaFer1-D* gene in the grains was also growth stage dependent, since a gradual increase in the expression of *TaFer1-D* was observed

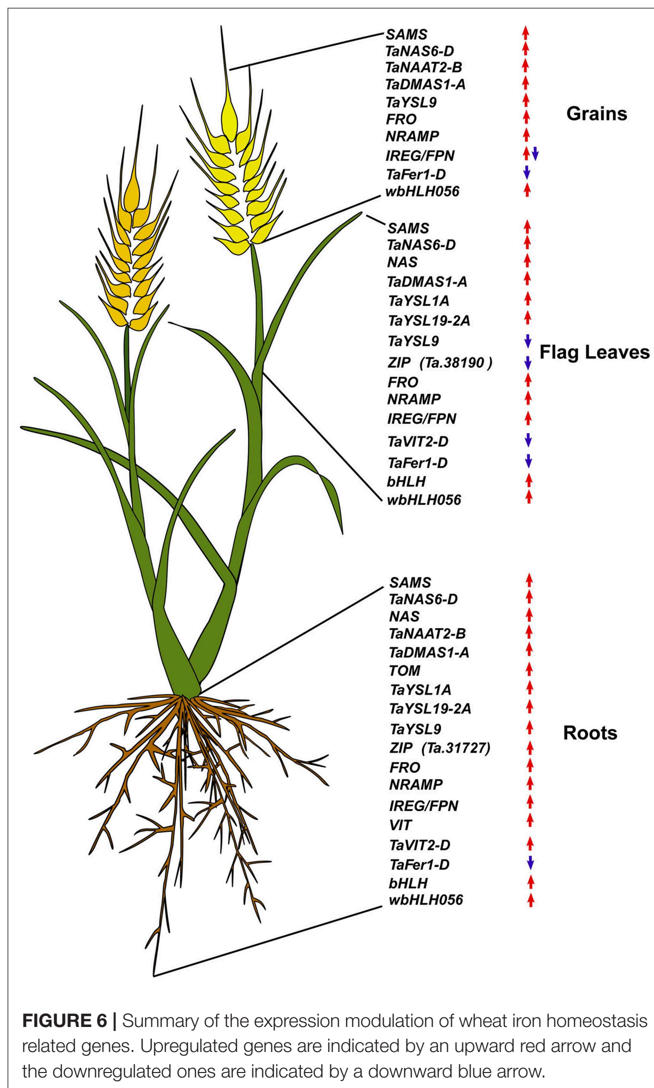

FIGURE 6 | Summary of the expression modulation of wheat iron homeostasis related genes. Upregulated genes are indicated by an upward red arrow and the downregulated ones are indicated by a downward blue arrow.

during the three development stages (**Supplementary Figure S3**, **Supplementary Table S3**).

Localizing on the vacuolar membrane, AtNRAMP3 and AtNRAMP4 mediate iron efflux from the vacuoles in Arabidopsis (Lanquar et al., 2005). The VIT and IRON REGULATED PROTEIN2/FERROPORTIN2 (IREG2/NPN2) perform the opposite function (Lanquar et al., 2005; Morrissey et al., 2009; Zhang et al., 2012). The expression of their homologs in wheat roots was significantly increased upon iron deficiency, which is in agreement with the expression modulation of *AtNRAMP3*, *AtNRAMP4* and *IREG2/FPN2* in Arabidopsis but conflict with the *OsVIT2* expression in rice (Lanquar et al., 2005; Morrissey et al., 2009; Zhang et al., 2012). This induction of *VITs* in roots can be due to the accumulation of metals other than iron, such as zinc and manganese, in the roots of plants under iron deficiency. It is known that VITs can transport not only iron but also zinc and manganese (Zhang et al., 2012; Connorton et al., 2017). In the roots of iron-deficient plants, we detected higher concentration of zinc and manganese, which can be toxic when present in excess (Marschner, 1995). The plants under low iron treatment potentially up-regulated the VIT genes in order to maintain the cytoplasmic zinc and manganese concentration low. In contrast to the roots, the *VIT* expression was either unaltered or reduced in the flag leaves and grains.

Localized on the root epidermal cells, FRO2 has been reported to function in iron reduction in strategy I plants (Robinson et al., 1999; Waters et al., 2002). Furthermore, leaf mesophyll cells (Bruggemann et al., 1993) and chloroplasts (Jeong et al., 2008) also rely on FROs for iron uptake. Recently, in rice, it has been suggested that *OsFRO1* localizes on tonoplast and play a role in making more iron available in the cytoplasm (Li et al., 2019). The induction of *FRO* expression in bread wheat roots, flag leaves and grains under iron limited conditions suggested their role being similar to their homolog in rice. However, whether FROs in wheat are involved in iron uptake from the soil into root cells needs to be verified by further experimentation.

In summary, iron homeostasis in bread wheat is maintained via a comprehensive modulation of various genes and gene families. Under iron-limited conditions, overall iron uptake and transport appears to be regulated by iron deficiency inducible transcription factors, such as those from the *bHLH* gene family (**Figure 6**). In order to cope with reduced iron supply during iron deficiency stress, the genes related to the synthesis of iron chelators (NA and DMA) as well as those encoding various iron translocation related transporters are up-regulated throughout the grain filling period. This suggests that iron uptake by the root and/or iron translocation from the roots to different plants parts lasted until the maturity in iron-deficient plants. Consistently, the root iron concentration was not significantly different between normal and iron-deficient plants. On the other hand, in the flag leaf and the grains, such genes involved in iron homeostasis were more highly expressed in iron-deficient plants than in normal plants at Stage 1, but from Stage 2 onwards, the gene expression difference between the iron treatments became insignificant or the expression level became even higher in normal plants than in iron-deficient plants. Given that YSLs play a role in iron remobilization in rice (Aoyama et al., 2009; Ishimaru et al., 2010), these results indicate that low external iron availability prompted both the onset and the end of iron remobilization from the flag leaf to the grains. The upregulation of genes involved in NA/PS synthesis in grains under iron deficiency implies a promotional role of NA/DMA in grain iron storage, which is in line with the study in rice (Lee et al., 2009). In rice, OsYSL9 is involved in iron export from the endosperm to embryo (Senoura et al., 2017). The up-regulation of *TaYSL9* in grains in iron-deficient plants may indicate that this gene plays a similar role as *OsYSL9*.

Overall, zinc, copper and manganese accumulated in tissues in an opposite way than iron. In other graminaceous species, it is known that YSLs can transport other metals than iron (Schaaf et al., 2004; Zheng et al., 2012; Zhang et al., 2018). Therefore, it is likely that at least some of the *YS/YSL* genes investigated in this study can transport both iron and other metals and that increased concentration of these metals is a consequence of the up-regulation of some *YS/YSL* genes.

A comprehensive study comprising of transcriptome profiling of the iron deficiency wheat plants could provide further

understanding of the coordinated roles of different transporters in combating iron deficiency stress. The insight into the wheat molecular iron homeostasis provided in this study may help researchers and breeders design well-tuned and more diversified biofortification studies (**Figure 6**). Experience from rice biofortification efforts suggest that the same biofortification strategies work differently in different genetic backgrounds (Masuda et al., 2012; Aung et al., 2013; Trijatmiko et al., 2016), and therefore it is necessary to have a wide range of biofortification strategies available. To date, however, there are only a limited number of wheat iron biofortification strategies reported (Borg et al., 2012; Connorton et al., 2017; Singh et al., 2017). In the present study, we found some endogenous transporters and transcription factors putatively involved in wheat iron homeostasis, whose use has not yet been explored in biofortification efforts. In particular, combining boost in NA/PS synthesis and iron remobilization from leaves to grains by tissue-specific expression of *YSL* genes could be an effective approach. Future biofortification studies may capitalize on this information to design and test novel biofortification strategies.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

NB conceived the experiments. MW and YK carried out the experiments. MW and NB interpreted the data and wrote the manuscript. All authors revised the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2019.00067/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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