Gene expression pattern of vacuolar-iron transporter-like (VTL) genes in hexaploid wheat during metal stress

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Iron is one of the important micronutrients that is not just essential for the human body, but also required for crop productivity and yield-related traits. To address the Fe homeostasis in crop plants, multiple transporters belonging to the category of Major facilitator superfamily are being explored. In this direction, Vacuolar iron transporters (VIT) are being reported and have been characterized functionally as an important candidate to address biofortification in cereal crops. In the present study, the identification and characterization of new members of Vacuolar iron transporters-like proteins (VTL) was performed. Phylogenetic analyses demonstrated distinct clustering of all the VTL genes from the previously known VIT genes. Our analysis identifies multiple VTL genes from hexaploid wheat with the highest number of this gene family localized on chromosome 2. Quantitative expression analysis suggests that most of the VTL genes are induced only during the Fe surplus condition, thereby reinforcing their role metal homeostasis. Interestingly, most of the wheat VTL genes were significantly up-regulated in a tissue-specific manner under Zn, Mn and Cu deficiency conditions. Although, no significant changes in expression of wheat VTL genes were observed in roots under heavy metals, but TaVTL2, TaVTL3 and TaVTL5 were upregulated in the presence of cobalt stress. Overall, this work deals with the characterization of wheat VTL genes that could provide an important genetic resource for addressing metal homeostasis in bread wheat.

Keywords: micronutrient uptake, Triticum aestivum L., Zinc transport, biofortification, Iron deficiency.
1. Introduction

Successful micronutrient biofortification of crops through biotechnology requires detailed knowledge of complex homeostatic mechanisms that tightly regulate the micronutrient concentrations in plants. Iron (Fe) is one of the important micronutrients that is involved in multiple important cellular and physiological processes in plants [1–3]. Some of the important functions include its importance in photosynthesis, nitrogen fixation and respiration [4,5]. Although Fe may be present in the soil, yet due to alkaline rhizospheric conditions or unfavorable circumstances it is not been efficiently taken up by plants [6–9]. Moreover, the Fe is mobilized inside the plant tissue with an important goal to load in the filial tissue of grains that basically involves a multistep process encompassing many bottlenecks [10–13]. Researchers worldwide is generating information for the means to enrich Fe rich grains and their storage with enhanced bioavailability. To improve Fe content in cereal grains, multiple transporters and chelators have been targeted including molecular approaches [14–16]. In addition to these, a number of genes are still unaddressed that are potential candidates for micronutrient biofortification, including transporters belong to the inventory of the Major facilitator superfamily (MFS) gene family [17]. Also, a very few reports are available that deals with the identification and molecular characterization of wheat genes or gene families involved in Fe and Zn homeostasis. Recent reports are emerging for the identification of few functional gene families, for example belonging to, yellow stripe like transporters [18], nicotianamine synthase (NAS), deoxymugineic Acid Synthase (DMAS) [19], yet there are still many genes families that remained to be characterized in hexaploid wheat. Similarly, genes encoding for Zinc–Induced Facilitator-Like family (ZIFL) of transporters have been described for their role during Fe homeostasis beside been regulated by a few heavy metals [20].

Fe storage in seeds gets compartmentalized in two major subcellular stores that include chloroplasts and vacuoles. For example, 95% of the iron is stored in vacuoles in the Arabidopsis seeds [21]. Vacuoles are an important site for Fe mobilization wherein, they are bound to various chelators like phytic acid, nicotianamine and other organic acids etc. Therefore, uptake of Fe into vacuoles could be an alternate strategy to enhance total
micronutrient content with a minimized tradeoff for its toxicity in the tissue. To design such strategy, the role of vacuolar transporters needs to be addressed and exploited [15,22].

Previously, one such vacuolar iron transporters (VIT) were shown to be playing an important role to maintain Fe in the optimal physiological range and prevent cellular toxicity. VIT genes from multiple plant species have been characterized and assessed for their ability to enhance Fe content in cereal crops [15]. These VIT genes show high homology with a small family of nodulin like protein containing a CCC-1 (Ca²⁺-Sensitive Cross Complementer) like domain with yeast Cccp1 [23]. CCC-1 like the domain was initially discovered in yeast encoded for the vacuolar iron transporter in yeast. Furthermore, mutant cccI cells show increased sensitivity to external iron [21,24] AtVIT1 is one of the early characterized genes showing the presence of CCC-1 like domain and transport of iron to vacuoles [21]. Utilizing the bioinformatics resources, subsequent studies led to the identification of many vacuolar iron transporters-like (VTL) proteins from different plant species. Model species, Arabidopsis genome encodes five VTL proteins and overexpression of the few genes have shown increased Fe content in seeds. AtVIT1 protein can transport iron into the vacuoles to counter the toxicity and support the seedling development under enhanced iron conditions [23,25]. This suggested that these genes likely to have a function in regulation in Fe homeostasis.

Therefore, the characterization of vacuolar transporters in an important crop such as wheat becomes a prerequisite to address the global issue of biofortification. Wheat is an important crop that is consumed in many developing countries, including India and is therefore being targeted for trait improvement for nutritional quality. In the current work genome-wide identification of wheat VTL genes was performed. Further, expression studies during different regimes of Fe, Zn and heavy metals was done that provide an insight into the regulation of wheat VTL genes in a tissue-specific manner.

### 2. Results

#### 2.1 Identification, phylogenetic analysis and genomic distribution of wheat VTL genes
Thirty-one wheat VIT family sequences were identified based on Ensembl Pfam search and bidirectional blast analysis (Table S1). Subsequently, to study the phylogenetic relationship among VIT family protein sequences from wheat, *Brachypodium*, maize, rice, *Arabidopsis* and *S. cerevisiae*, an unrooted neighbour-joining tree was constructed. This analysis separated the sequences into two distinct clades for VTL and VIT proteins that corresponds to the previously stated distribution of the VIT family. This also led to the classification of the wheat VIT family into 8 VIT and 23 VTL sequences (Figure 1, Table S2). Due to the occurrence of homoeologs, the 23 VTL sequences representing only 4 *VTL* genes, named as *TaVTL1*, *TaVTL2*, *TaVTL4* and *TaVTL5* based on their corresponding rice orthologs followed by the chromosome on which they are present. None of the orthologs in wheat showed high confidence similarity with rice vacuolar iron transporter homolog 3. *TaVTL1* and *4* were found to have three homoeologs, while *TaVTL2* has 4. In contrast, the phylogenetic analysis grouped 13 highly similar sequences together with rice vacuolar iron transporter homolog 5, these were named as *TaVTL5* (Figure S1).

*TaVIT1* and *TaVIT2* have already been reported earlier [15]. Interestingly, another new wheat *VIT* with two homoeologs at chromosome 7 (sub-genomes A and D) was identified and referred as *TaVIT3*. *VIT* genes are located on chromosome groups 2, 5 and 7 while *VTL* genes on chromosome groups 2, 4, and 6 with maximum contribution from chromosome 2. Nine *VTL* genes are present on B sub-genome, while seven each on A and D sub-genomes. Maximum number of *VTL* sequences are located on chromosome 2B (Figure 2A).

### 2.2 Gene, protein structure and subcellular localization

*VIT* genes in wheat have three and four intronic and exonic regions respectively, while *VTL* genes have a single exon each with the absence of any introns (Figure 2B), clearly dividing the VIT family into two sub-families based on gene structure also. CDS length was found to be varying from 657 to 747 nucleotides for wheat *VIT* genes. The CDS length for *VTL* genes was ranging from 549 to 810 nucleotides except for *TaVTL5-2A_3* that was 378 nucleotides long. The short length of one *VTL* gene is due to the missing sequence information at the stop site. The length of *TaVIT* peptides ranged from 218 to 256 while
TaVTL protein length varied from 125 to 269 amino acids. The division of VIT and VTL proteins was also evident from the sub-cellular localization (Table S2); while TaVIT proteins were predicted to be predominantly localized on the plasma membrane and chloroplast thylakoid membrane, maximum TaVTL proteins were predicted to be present on the vacuolar membrane (87%). TaVTL4-4A was predicted to be localized on plasma membrane. VIT proteins had 3-4 predicted TM domains. TaVTL1, 2 and 4 have 5 TM domains majorly, except for TaVTL4-4B which was predicted to have 6 TM domains. Only TaVTL5-2D_3 had 5 TM domains; other paralogs/homoeologs of TaVTL5 had lesser number of TM domains probably due to gene duplication events or missing information. To summarize, TaVTLs have 5 TM domains predominantly, which are depicted in Table S2.

Eucalyptus VIT1 (EgVIT1) crystal structure was deciphered recently [26] that was used to confirm the VIT family protein topology prediction using Phobius [27]. EgVIT1 was predicted to have only three TM domains while the crystal structure stated the presence of five TM domains. Therefore, VIT, as well as VTL protein sequences from wheat, were aligned to EgVIT1 to see the possible TM domains in addition to those predicted by Phobius (Figure S2).

2.3 Conserved domain and motif analysis

All the VIT and VTL genes were found to have the typical CCC1-like superfamily domains of yeast, which were demonstrated earlier for the iron and manganese transport from the cytosol to vacuole. Motif analysis using MEME webserver suggested that motifs 6, 9, and 10 are VIT specific with exceptions for motif 10 been absent in TaVIT3 and motif 6 absent in VIT3-7A. Similarly, motifs 5, 7, 8, 11 to 14 are VTL specific with the exceptions for motif 5 been absent in TaVTL5-2B_3 and TaVTL5-2B_5, where motif 7 was specific for TaVTL5 sequences except in TaVTL5-2A_3, TaVTL5-2B_6, TaVTL5-2D_3. Motif 8 was specific for TaVTL1, 2 and 4. Motif 11 for TaVTL1 and 2. Motif 12 and 13 were unique for TaVTL2, whereas Motif 14 was present only in TaVTL1 (Figure 3, Table S3).
2.4 Expression of wheat VTL genes under Fe deficiency and surplus condition

To check the regulation of VTL genes at the transcriptional level, the promoter for the wheat VTL genes were scanned for the cis-elements responsive for Fe and heavy metals. The analysis revealed the presence of multiple such sequences, including iron-deficiency-responsive element 1 (IDE1), metal response element (MRE), heavy metal responsive element (HMRE) and iron-related bHLH transcription factor 2 (IRO2) binding site (Table S4). In the most abundant category, iron-deficiency-responsive element 1 (IDE1) was predominant. Interestingly, the IRO2 binding site was present only in the regulatory region of TaVTL2B/D. These observations suggest that VTL expression could be regulated by the presence-absence of specific metals including micronutrients such as Fe and Zn.

Previously, VTL genes were reported to have differential expression patterns under the changing regimes of Fe and Zn [25]. Therefore, we tested if wheat VTL genes could respond at the transcript level when subjected to changing Fe concentration. The expression in roots and shoots of wheat seedlings was measured after subjecting them for 3 and 6 days of starvation. Our expression analysis suggests that in roots all the VTL genes (TaVTL1, TaVTL2, TaVTL4 and TaVTL5) were downregulated at both the days, whereas, only TaVTL5 was upregulated at 6 days of starvation (Figure 4A).

Similarly, in shoots also all the expression of wheat VTL genes was suppressed except for TaVTL2 that was upregulated only on 6 days post starvation (Figure 4B). These expression data demonstrate that under Fe deprivation VTL gene expression are negatively regulated in wheat seedling. Transcriptomic sequencing data from wheat seedlings after 20 days of Fe starvation (SRP189420) was also used to check expression response upon Fe starvation. Categorically, TaVTL5 group genes were seen to be upregulated upto 12-fold, with TaVTL5-2B_6 showing upregulation of ~60 fold, although the expression was not very high (Figure S3).

Next, we performed the gene expression analysis under the excess Fe regime. This was done to test if wheat VTL genes could be potentially involved in detoxification of excess Fe. Interestingly, we observed a significant up-regulation of all the TaVTL genes in roots at both the time points. Out of all, TaVTL4 showed the highest fold gene expression
(~100 fold) when compared to its control (Figure 5A). TaVTL2 show very early and high expression response, whereas both TaVTL1 and TaVTL5 were highly expressed at 6 days of treatment. At this time their gene expression level was more than ~14 fold compared to control. In contrast, in shoots most of the wheat VTL genes were expressed at the 3 days of treatment with TaVTL1 and TaVTL2 showing the transcript accumulation of 8-14 folds with respect to their control (Figure 5B).

2.5 Manganese, Zinc and Copper deficiency causes differential changes in VTL expression

Wheat VTL genes showed high similarity to previously known VIT genes. In addition to Fe, VIT genes are known to be affected by the perturbed concentration of Zn and Mn [15]. Since many of these cation transporters are known for their reduced substrate specificity, therefore, expression of wheat VTL genes during Zn and Mn deprivation was also studied. In general, during the changing regimes of Zn and Mn, wheat VTL genes showed specific expression in a tissue-specific manner (Figure 6). TaVTL2 was the only gene showing enhanced accumulation of its transcript under Zn deficiency in both root and shoot tissue, whereas TaVTL1 and TaVTL5 showed high expression in roots only under Zn deficiency. No significant changes in the expression of TaVTL4 were observed for the studied time point under the changing Zn regime. In contrast, no induction of wheat VTL genes was observed in roots under Mn deficiency with respect to its control, whereas, in shoots, TaVTL2 and TaVTL4 showed high transcript accumulation. Under Cu deficiency, all VTL genes showed an induced expression in shoots while only two of the genes, including TaVTL1 and TaVTL2 were upregulated in roots.

2.6 Heavy metal (Ni, Cd and Co) mediated expression of VTL genes

To check the effect of the heavy metal on the gene expression pattern, wheat seedlings were subjected to treatment with Ni, Cd and Co and; expression of VTL genes was performed. In the treated plants, decreased growth of the shoot and root length was observed, suggesting that heavy metals could affect the plant performance (data not shown). Interestingly, none of the wheat VTL genes showed enhanced expression in roots after 15 days of heavy metal
exposure. In shoots, only under the metal Co-treatment TaVTL2, TaVTL4 and TaVTL5 show significantly higher expression as compared to control shoot samples (Figure 7). This data suggested the metal-specific expression of wheat VTL genes in a tissue-specific manner. The previously reported wheat VIT genes showed grain specific expression data. Surprisingly, VTL genes showed very low or no expression in grains or their tissue parts, suggesting their probable roles in the organs of the plants (Figure S4).

3. Discussion

Fluctuation in the nutrient condition in the soil results in drastic adaptions by the plants. In general, plants rely on different physiological and molecular processes to minimize nutrient stress [28]. In this regard, MFS gene family plays an important role to provide the tolerance as well as mobilization of important minerals, including micronutrient to the foliar parts including seeds [17]. In this study, the characterization of the new gene family VTL was done. Our data reinforce the importance of this gene family for their roles not limited during metal homeostasis but also a good potential for biofortification in cereal crops such as wheat and rice.

MFS family has been widely explored for its role as metal transporters and providing the necessary support to provide multiple functions in plants [29]. Previously, five VTL genes were reported in Arabidopsis and five genes were reported in rice for this sub-class. Our study in wheat resulted in the identification of a maximum number of VTL genes from any crop plants. The high number of genes is due to the presence of multiple homoeologues and occurrence of the duplication of many wheat VTL genes. Interestingly, chromosome 2 has been linked with multiple quantitative trait loci (QTL) for the high grain Fe and Zn [30]. Further dissection is required in this direction to identify if any of the wheat VTL could be linked with the loading of micronutrient in grains. Based on our expression analysis and the support from the previous studies, it could be suggested that VTL genes could also be involved in providing the tolerance to high levels of Fe and Zn in the soils [25]. In fact, the predicted localization data indicate that VTL could be localized at either the plasma membrane or the vacuolar membrane. AtVTL1 was reported to be localized in
the vacuolar membrane and others also be associated with the plasma membrane [25]. Our

PSORT analysis suggests that most of the wheat VTL are localized in the vacuolar

membrane, thus making them a suitable candidate for sequestering micronutrients such as

Fe and Zn.

The substrate specificity of the metal transporters is a major bottleneck to achieve high

Fe and Zn rich grains. Manipulating the specificity of these metal transporters to enrich the

Fe and Zn remains the major challenge [20]. Therefore, studying the expression pattern of

VTL genes in the presence of heavy metals could provide preliminary clues to employ such

strategies. Consequently, the study was undertaken to see the influence of other metals like

Ni, Cd and Co. The expression of wheat VTL genes in roots and shoots suggested an

interesting phenomenon, where no significant changes in the expression of their transcript

was observed when exposed to either Ni and Cd. In contrast, only Co was able to induce

the expression of TaVTL1, TaVTL2, TaVTL4 and TaVTL5 in shoots only. This data suggests

the controlled expression of wheat VTL genes in specific tissues. Additionally, besides Fe

homeostasis, the vacuolar transporters are also linked with the impaired activity of Zn and

Mn transport [31]. In our study only, TaVTL2 was significantly induced by the Mn

deficiency in shoots. No such effects were observed in roots wherein, all the quantified

wheat VTL showed downregulation under Mn deficiency. Interestingly, TaVTL1 and

TaVTL2 showed upregulation in roots under Zn deficiency. The tissue dependent

equation patterns of wheat VTL genes under the changing regimes of the metal exposure

suggest was observed. It has been observed that VTL genes from Arabidopsis showed

transcriptional changes in response to Fe, Zn and Mn [25]. Based on the previous work and

our results it could be suggested that regulation of the VTL genes at the transcript level

could be conserved. Our works gene expression data also correlates with the presence of

multiple cis-elements in the promoter of wheat VTL genes.

Herein, a detailed inventory, structure and expression characterization of wheat VTL

genes was performed. The work presented here provides the preliminary clue for the

equation characterization of wheat VTL genes that further remains to be characterized for

their in-planta functional activity using forward and reverse genetics approaches.
4. Conclusions

The present work lead to the identification of high number of VTL genes from hexaploid wheat. Because of polyploidization, a very high number of genes from this sub-family was identified. The presence of high number of VTL been restricted to only chromosome 2, 4 and 6 of the wheat genomes. The expression of these gene under metal stress including changes in the presence of Fe and Zn concentrations and exposure to heavy metals reinforce the importance of this gene-family during metal homeostasis. Therefore, VTL class of gene family in wheat requires further characterization for their localization patterns and for their functional activity.

5. Materials and Methods

5.1 Identification of VIT family and classification of VTL genes in wheat

For the identification of wheat VTL genes, the Ensembl database was used to extract VIT family genes (Pfam ID: PF01988) for wheat. The identification was confirmed by bidirectional blast analysis. VIT family sequences from Arabidopsis, rice, maize, Brachypodium were also extracted using Pfam search. The identity of VIT family genes was further validated by confirming the presence of CCC1-like superfamily domain using NCBI-CDD domain search. CCC1 sequence for S. cerevisiae was also retrieved from its genome database. To separate out VTL genes from VIT genes and for further phylogenetic analysis, all the proteins were aligned through MUSCLE alignment and an unrooted neighbor-joining phylogenetic tree with 1000 bootstrap replicates was constructed with all the retrieved sequences. The tree was constructed through MEGA-7 [32]. Rice vacuolar iron transporter homolog 1-5 from UniProt were used for the nomenclature of the twenty-three TaVTL sequences based on the closest orthologs. The naming of the genes indicates the chromosome number and the sub-genome on which they are present.

5.2 Conserved domains and motif detection, analysis of gene, promoter and protein structure

Wheat VIT family genes were searched for conserved domains using NCBI-CDD database[33]. MEME suite v5.1.0 was used for further analysis to identify the common
conserved motifs for both VIT and VTL proteins. The maximum number of motifs was set
to 15 for MEME analysis. Gene structure for VITs and VTLs were studied using (GSDS)
(http://gsds.cbi.pku.edu.cn/) [34] using genomic and CDS sequences. Sub-cellular
localization and TM domains were predicted using web-based prediction programs Wolf
PSORT and Phobius respectively[35]. For promoter analysis, ~2Kb promoter elements of
the corresponding wheat VTL genes were surveyed for the presence of the respective cis-
elements. The promoter sequence was obtained for the respective genes suing the IWGSC

5.3 Plant materials and growth conditions

For stress experiments, hexaploid wheat *Triticum aestivum* cv. C-306 (received from
Punjab Agriculture University, Ludhiana) was used. Briefly, seeds were surface sterilized
using 1.2% sodium hypochlorite prepared in 10% ethanol and then rinsed twice with
autoclaved MQ. The seeds were kept on moist filter paper inside a Petri dish and stratified
for 1 day at 4 °C in dark condition. Stratified seeds were further kept for germination for 6
days at room temperature. The remaining seed/endosperm was excised from seedlings at
one leaf stage and was shifted to phytaboxes (10-12 seedling / phytabox) containing the
Hoagland nutrient media for respective treatments. The standard composition of nutrient
media for control includes 6 mM KNO₃, 1 mM MgSO₄, 2 mM Ca(NO₃)₂, 2mM
NH₄H₂PO₄, 20 μM Fe-EDTA, 25μM H3BO3, 2 μM MnSO₄, 0.5 μM CuSO₄, 2 μM
ZnSO₄, 50 μM KCl and 0.5 μM Na₂MoO₄. The variable concentrations used for
treatments were excess Fe (+Fe; 200μm), Fe starvation (-Fe;2μm), Zn deficiency (-ZnSO₄;
0μm), Mn deficiency (-MnSO₄; 0μm), Cu deficiency (-CuSO₄; 0μm), Cadmium stress
(+Cd; 50 μm), Cobalt stress (+Co; 50 μm) and Nickel stress (+Ni; 50 μm). The aerobic
condition was provided in hydroponics and the media was replaced every alternate day to
avoid any contamination and drastic nutrient depletion. The respective roots and shoots
samples belonging to iron deficient and sufficient plant groups were collected at 3 and 6
days after stress (D). For the rest of the treatments, root and shoot, samples were collected
on the 15th Day of treatments. All the experiments were performed in a growth chamber
under controlled environmental conditions at 22-24 °C temperature, 65-70% humidity, at a photoperiod of 16 hours day and 8 hours night and 300 nm of light.

5.4 RNA isolation and cDNA preparation

The collected root and shoot samples were ground separately in liquid nitrogen. Total RNA from respective samples was extracted by TRIZOL based method. The extraction was followed by the DNase treatment using Turbo DNAfree kit (Invitrogen, USA) to remove any genomic DNA contamination in the RNA samples. Subsequently, RNA purity was checked and quantified for the preparation of the cDNA. 2 μg of total RNA was used for cDNA synthesis using superScript III First-Strand Synthesis System (Invitrogen, USA). The cDNA quality was ascertained by using internal control and was further diluted 20X and used for gene expression studies.

5.6 Quantitative-real time PCR (qRT-PCR) expression analysis

To perform quantitative real time-PCR (qRT-PCR), forward and reverse primers of *TaVTL* genes were designed and used as listed in Table S5. The primers were designed from the conserved region of the all homoeologs of each gene. For *TaVTL5* the primers were designed from the conserved region of 9 sequences, the significant conserved region was not found for remaining 4 homoeologs (TraesCS2B02G454900, TraesCS2B02G610400, TraesCS2D02G431900, TraesCS2D02G588000). qRT-PCR was performed in 7500 Real-Time PCR System (Applied Biosystems, USA) using 1/20 times dilution of the respective cDNAs. All qRT-PCR reactions were performed using SYBR Green I (QuantiFast® SYBR® Green PCR Kit, Qiagen, Germany) chemistry and ARF (ADP-Ribosylation Factor: *TaARF1*—AB050957.1) as an internal control [36]. The efficiency of the qRT-PCR was checked and melt curve analysis was performed for each of the PCR reactions as per the guidelines. Gene expression analyses was carried out with three biological replicates and two-three technical replicates. Relative fold expression of genes was determined based on delta-delta CT-method (2^-ΔΔCT) [37].
5.7 RNA-seq expression analysis for VIT family genes

To get the transcript expression levels for VIT family genes under Fe stress, RNAseq data from SRA project ID SRP189420 was utilized to extract transcript expression values (as FPKM) from control as well as Fe starved wheat root samples using the cufflinks pipeline. Subsequently, for expression analysis of VTL and VIT genes in wheat grain tissue developmental time course [38], expression values as Transcripts Per Kilobase Million (TPM) were retrieved from expVIP database. Expression values from both studies were then used to plot heatmaps using MeV software.

5.8 Statistical Analysis

Excel was used for data analysis. The mean values were calculated form the standard deviation including three technical replicates form at least three biological replicates. Student t-tests were used to observe the significant differences between the mean values of treatment and control plants. The significance threshold used was \#P < 0.05.

Acknowledgments

All the authors thank Executive Director, NABI for facilities and support. This research was funded by the NABI-CORE grant to AKP. Support from International Wheat Genome Sequencing Consortium for providing the high-quality wheat genome resources is highly appreciated.

Conflicts of Interest: The authors declare no conflict of interest.

Author Contributions: Conceptualization, SS., AKP; methodology, SS, AK, VM and AKP; formal analysis, SS, AKP, GK and HR; investigation, SS, VM; writing—original draft preparation, AKP., AK., JK; writing—review and editing, AKP., AK., GK., and HR; visualization, AKP; funding acquisition, AKP.
References

1. Rout, G.R.; Sahoo, S. Role of Iron in Plant Growth and Metabolism. *Rev. Agric. Sci.* 2015, 3, 1–24.

2. Briat, J.F.; Curie, C.; Gaymard, F. Iron utilization and metabolism in plants. *Curr. Opin. Plant Biol.* 2007, 10, 276–282.

3. Morrissey, J.; Guerinot, M.L. Iron Uptake and Transport in Plants: The Good, the Bad, and the Ionome. *Chem. Rev.* 2009, 109, 4553–4567.

4. Miller, G.W.; Huang, I.J.; Welkie, G.W.; Pushnik, J.C. Function of iron in plants with special emphasis on chloroplasts and photosynthetic activity. In *Iron Nutrition in Soils and Plants*; Springer Netherlands, 1995; pp. 19–28.

5. Tang, C.; Robson, A.D.; Dilworth, M.J. A split-root experiment shows that iron is required for nodule initiation in Lupinus angustifolius L. *New Phytol.* 1990, 115, 61–67.

6. Marschner, P. *Marschner’s Mineral Nutrition of Higher Plants: Third Edition*; Elsevier Inc., 2011; ISBN 9780123849052.

7. Krohling, C.A.; Eutrópio, F.J.; Bertolazi, A.A.; Dobbss, L.B.; Campostrini, E.; Dias, T.; Ramos, A.C. Ecophysiology of iron homeostasis in plants. *Soil Sci. Plant Nutr.* 2016, 62, 39–47.

8. Jeong, J.; Guerinot, M.L. Homing in on iron homeostasis in plants. *Trends Plant Sci.* 2009, 14, 280–285.

9. Colangelo, E.P.; Guerinot, M.L. Put the metal to the petal: metal uptake and transport throughout plants. *Curr. Opin. Plant Biol.* 2006, 9, 322–330.

10. Kim, S.A.; Guerinot, M.L. Mining iron: Iron uptake and transport in plants. *FEBS Lett.* 2007, 581, 2273–2280.

11. DiDonato, R.J.; Roberts, L.A.; Sanderson, T.; Eisley, R.B.; Walker, E.L. Arabidopsis Yellow Stripe-Like2 (YSL2): A metal-regulated gene encoding a plasma membrane transporter of nicotianamine-metal complexes. *Plant J.* 2004, 39, 403–414.

12. Bashir, K.; Inoue, H.; Nagasaka, S.; Takahashi, M.; Nakanishi, H.; Mori, S.; Nishizawa, N.K. Cloning and characterization of deoxymugineic acid synthase genes...
from graminaceous plants. *J. Biol. Chem.* **2006**, *281*, 32395–32402.

13. Kobayashi, T.; Nishizawa, N.K. Iron Uptake, Translocation, and Regulation in Higher Plants. *Annu. Rev. Plant Biol.* **2012**, *63*, 131–152.

14. Aggarwal, S.; Kumar, A.; Bhati, K.K.; Kaur, G.; Shukla, V.; Tiwari, S.; Pandey, A.K. RNAi-Mediated Downregulation of Inositol Pentakisphosphate Kinase (IPK1) in Wheat Grains Decreases Phytic Acid Levels and Increases Fe and Zn Accumulation. *Front. Plant Sci.* **2018**, *9*, 1–12.

15. Connorton, J.M.; Jones, E.R.; Rodríguez-Ramiro, I.; Fairweather-Tait, S.; Uauy, C.; Balk, J. Wheat Vacuolar Iron Transporter TaVIT2 Transports Fe and Mn and Is Effective for Biofortification. *Plant Physiol.* **2017**, *174*, 2434–2444.

16. Masuda, H.; Ishimaru, Y.; Aung, M.S.; Kobayashi, T.; Kakei, Y.; Takahashi, M.; Higuchi, K.; Nakaniishi, H.; Nishizawa, N.K. Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Sci. Rep.* **2012**, *2*.

17. Ricachenevsky, F.K.; Sperotto, R.A.; Menguer, P.K.; Sperb, E.R.; Lopes, K.L.; Fett, J.P. ZINC-INDUCED FACILITATOR-LIKE family in plants: lineage-specific expansion in monocotyledons and conserved genomic and expression features among rice (Oryza sativa) paralogs. *BMC Plant Biol.* **2011**, *11*, 20.

18. Kumar, A.; Kaur, G.; Goel, P.; Bhati, K.K.; Kaur, M.; Shukla, V.; Pandey, A.K. Genome-wide analysis of oligopeptide transporters and detailed characterization of yellow stripe transporter genes in hexaploid wheat. *Funct. Integr. Genomics* **2019**, *19*, 75–90.

19. Beasley, J.T.; Bonneau, J.P.; Johnson, A.A.T. Characterisation of the nicotianamine aminotransferase and deoxymugineic acid synthase genes essential to Strategy II iron uptake in bread wheat (Triticum aestivum L.). *PLoS One* **2017**, *12*, e0177061.

20. Sharma, S.; Kaur, G.; Kumar, A.; Meena, V.; Kaur, J.; Pandey, A.K. Overlapping transcriptional expression response of wheat zinc-induced facilitator-like transporters emphasize important role during Fe and Zn stress. *BMC Mol. Biol.* **2019**, *20*, 1–17.

21. Kim, S.A.; Punshon, T.; Lanzirotti, A.; Li, L.; Alonso, J.M.; Ecker, J.R.; Kaplan, J.; Guerinot, M.L. Localization of Iron in Arabidopsis Seed Requires the Vacuolar
Membrane Transporter VIT1. Science (80-.). 2006, 314, 1295–1298.

22. Martinoia, E. Vacuolar transporters -Companions on a longtime journey. Plant Physiol. 2018, 176, 1384–1407.

23. Gollhofer, J.; Schläwicke, C.; Jungnick, N.; Schmidt, W.; Buckhout, T.J. Members of a small family of nodulin-like genes are regulated under iron deficiency in roots of Arabidopsis thaliana. Plant Physiol. Biochem. 2011, 49, 557–564.

24. Li, L.; Chen, O.S.; Ward, D.M.V.; Kaplan, J. CCC1 Is a Transporter That Mediates Vacuolar Iron Storage in Yeast. J. Biol. Chem. 2001, 276, 29515–29519.

25. Gollhofer, J.; Timofeev, R.; Lan, P.; Schmidt, W.; Buckhout, T.J. Vacuolar-iron-transporter1-like proteins mediate iron homeostasis in arabidopsis. PLoS One 2014, 9, 1–8.

26. Kato, T.; Kumazaki, K.; Wada, M.; Taniguchi, R.; Nakane, T.; Yamashita, K.; Hirata, K.; Ishitani, R.; Ito, K.; Nishizawa, T.; et al. Crystal structure of plant vacuolar iron transporter VIT1. Nat. plants 2019, 5, 308–315.

27. Käll, L.; Krogh, A.; Sonnhammer, E.L.L. A combined transmembrane topology and signal peptide prediction method. J. Mol. Biol. 2004, 338, 1027–1036.

28. Morgan, J.B.; Connolly, E.L. Plant-Soil Interactions: Nutrient Uptake. Nat. Educ. Knowl. 2013, 4, 2.

29. Haydon, M.J.; Cobbett, C.S. A Novel Major Facilitator Superfamily Protein at the Tonoplast Influences Zinc Tolerance and Accumulation. Plant Physiol. 2007, 143, 1705–1719.

30. Krishnappa, G.; Singh, A.M.; Chaudhary, S.; Ahlawat, A.K.; Singh, S.K.; Shukla, R.B.; Jaiswal, J.P.; Singh, G.P.; Solanki, I.S. Molecular mapping of the grain iron and zinc concentration, protein content and thousand kernel weight in wheat (Triticum aestivum L.). PLoS One 2017, 12.

31. Sinclair, S.A.; Krämer, U. The zinc homeostasis network of land plants. Biochim. Biophys. Acta - Mol. Cell Res. 2012, 1823, 1553–1567.

32. Sudhir Kumar, Masatoshi Nei, Joel Dudley, and K.T. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Br. Bioinform.
Marchler-bauer, A.; Lu, S.; Anderson, J.B.; Chitsaz, F.; Derbyshire, M.K.; Deweese-scott, C.; Fong, J.H.; Geer, L.Y.; Geer, R.C.; Gonzales, N.R.; et al. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* **2011**, *39*, 225–229.

Hu, B.; Jin, J.; Guo, A.; Zhang, H.; Luo, J. Genome analysis GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics* **2015**, *31*, 1296–1297.

Horton, P.; Park, K.; Obayashi, T.; Fujita, N.; Harada, H.; Nakai, K. WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* **2007**, *35*, 585–587.

Bhati, K.K.; Alok, A.; Kumar, A.; Kaur, J.; Tiwari, S.; Pandey, A.K. Silencing of *ABCC13* transporter in wheat reveals its involvement in grain development, phytic acid accumulation and lateral root formation. *J. Exp. Bot.* **2016**, *67*, 4379–4389.

Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2^(-ΔΔC_T) Method. *Methods* **2001**, *408*, 402–408.

Pfeifer, M.; Kugler, K.G.; Sandve, S.R.; Zhan, B.; Rudi, H.; Hvidsten, T.R.; Mayer, K.F.X.; Olsen, O.A.; Rogers, J.; Doležel, J.; et al. Genome interplay in the grain transcriptome of hexaploid bread wheat. *Science (80-)*. **2014**, *345*. 


Legends for the Figures

**Figure 1:** Phylogenetic analysis showing separation of VIT family genes in Arabidopsis, Brachypodium, Oryza sativa, Zea mays and Triticum aestivum into two distinct clades; VTL clade and VIT clade. The Neighbour-joining phylogenetic tree was generated using MEGA. The numbers represent bootstrap values from 1000 replicates.

**Figure 2:** Genomic distribution and exon-intron arrangements of VTL genes. (A) Wheat VTL genes were present on chromosome groups 2, 4 and 6 with maximum VTL genes on chromosome group 2, which was selected to show the VTL gene distribution on 2A, 2B and 2D chromosomes. (B) Genomic structure for wheat VTL and VIT genes. The intron-exon arrangement was identified using Gene Structure Display Server (GSDS). Exons and introns are represented using pink boxes and cyan lines, respectively. The scale determines the size of the genomic regions.

**Figure 3:** Conserved motifs identified for TaVIT and TaVTL proteins using MEME suite 5.1.0. The colored rectangles on each sequence represent specific conserved motifs numbered 1 through 14, as depicted by the color codes in the box.

**Figure 4:** Tissue-specific qRT-PCR expression analysis of wheat VTL genes during Fe deficiency (-Fe) and in the control (C) conditions. Wheat seedlings were subjected to Fe deficiency for 3 and 6 days represented as -Fe(3D) and -Fe(6D). The controls for the respective time points are represented as C(3D) and C(6D). (A) Fold expression analysis was performed in roots and (B) in shoots. 2 µg of total RNA was used for the cDNA preparation. Relative fold expression levels were calculated relative to C(3D). Ct values were normalized using wheat ARF1 as an internal control. Vertical bars represent the standard deviation. # represents the significant difference at p < 0.05 with respect to their respective control treatments.

**Figure 5:** Tissue-specific qRT-PCR expression analysis of wheat VTL genes during Fe surplus (+Fe) and in the control (C) conditions. Wheat seedlings were subjected to Fe surplus for 3 and 6 days represented as +Fe(3D) and +Fe(6D). The controls for the respective time points are represented as C(3D) and C(6D). (A) Fold expression analysis
was performed in roots and (B) in shoots. 2 µg of total RNA was used for the cDNA
preparation and relative fold expression levels were calculated relative to C(3D). Ct values
were normalized using wheat ARF1 as an internal control. Vertical bars represent the
standard deviation. # represents the significant difference at p < 0.05 with respect to their
respective control treatments.

Figure 6: Tissue-specific qRT-PCR expression analysis of wheat VTL genes during Mn (-Mn), Zn (-Zn) and Cu (-Cu) deficiency with rest to the control (C) conditions. (A) Fold
expression analysis was performed in roots and (B) in shoots. 2 µg of total RNA was used
for the cDNA preparation and relative fold expression levels were calculated relative to
control tissue (C). The Ct values were normalized using wheat ARF1 as an internal control.
Vertical bars represent the standard deviation. # represents the significant difference at p <
0.05 with respect to Control tissue.

Figure 7: Tissue-specific qRT-PCR expression analysis of wheat VTL genes upon heavy
metal treatments. Wheat seedlings were exposed to Ni (+Ni, 50 µm), Cd (+Cd, 50 µm) and
Co (+Co, 50 µm). Control seedlings (C) without any exposure to heavy metals were
compared with the treated ones. (A) Fold expression analysis was performed in roots and
(B) in shoots. 2 µg of total RNA was used for the cDNA preparation and relative fold
expression levels were calculated relative to control samples. Ct values were normalized
using wheat ARF1 as an internal control. Vertical bars represent the standard deviation. #
represents the significant difference at p < 0.05 with respect to their respective control
treatments.
Figure 2
Figure 3
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