Short title: Function of vacuolar iron transporters in wheat

Corresponding author: Janneke Balk, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK; Phone: + 44 1603 450621; E-mail: janneke.balk@jic.ac.uk

Title: Vacuolar Iron Transporter TaVIT2 transports Fe and Mn and is effective for biofortification

James M. Connorton[1,2], Eleanor R. Jones[1], Ildefonso Rodríguez-Ramiro[3], Susan Fairweather-Tait[3], Cristobal Uauy[1] and Janneke Balk[1,2]

[1] Department of Biological Chemistry, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK
[2] School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK
[3] Norwich Medical School, University of East Anglia, Norwich, NR4 7UQ, UK

One sentence summary: Altering expression of a vacuolar iron transporter doubles iron content in white wheat flour

List of author contributions: C.U. and J.B. conceived and designed the project; J.M.C. and E.R.J. designed and performed experiments; I.R.R. carried out the bioavailability assays; All authors analysed and interpreted data; J.M.C. and J.B. co-wrote the paper with contributions from the other authors.

Key words: Biofortification, Micronutrient, Iron deficiency anaemia, Wheat, Barley, Endosperm, Metal transport

Abstract

Increasing the intrinsic nutritional quality of crops, known as biofortification, is viewed as a sustainable approach to alleviate micronutrient deficiencies. In particular iron deficiency anaemia is a major global health issue, but the iron content of staple crops such as wheat is difficult to change because of genetic complexity and homeostasis mechanisms. To identify target genes for biofortification of wheat (*Triticum aestivum*), we functionally characterized homologs of the *Vacuolar Iron Transporter* (*VIT*). The wheat genome contains two *VIT* paralogs, *TaVIT1* and *TaVIT2*, which have different expression patterns, but are both low in the endosperm. TaVIT2, but not TaVIT1, was able to rescue growth of a yeast mutant lacking the vacuolar iron transporter. TaVIT2 also complemented a manganese transporter mutant, but not a vacuolar zinc transporter mutant. By over-expressing *TaVIT2* under the control of an endosperm-specific promoter, we achieved a > 2-fold increase in iron in white flour fractions, exceeding minimum legal fortification levels in countries such as the UK. The anti-nutrient phytate was not increased and the iron in the white flour fraction was bioavailable in-vitro, suggesting that food products made from the biofortified flour could contribute to improved iron nutrition. The single-gene approach impacted minimally on plant growth and was also effective in barley. Our results show that by enhancing vacuolar iron transport in the endosperm, this essential micronutrient accumulated in this tissue bypassing existing homeostatic mechanisms.

Introduction

Iron is essential for plant growth and needed for a range of cellular processes involving electron transfer or redox-dependent catalysis (Kobayashi and Nishizawa, 2012). However, excess levels of iron are toxic to cells and therefore organisms have evolved tight regulation and storage mechanisms. Plants store iron in ferritin or sequestered in vacuoles, with different species and tissues favouring one storage mechanism over another (Briat et al., 2010). Iron stored in seeds provides for essential iron enzymes during germination before the seedling develops a root and is able to take up iron independently.

Iron is also an essential micronutrient for human nutrition, and over a billion people suffer from iron-deficiency anaemia (WHO, 2008). Seeds such as rice, wheat and pulses are a major source of iron, especially in diets that are low in meat. To combat iron deficiency, more than 84 countries have legislation for chemical fortification of flours milled from wheat, corn and rice with iron salts or iron powder (www.ffinetwork.org/global_progress/index.php). A more sustainable approach is biofortification, or increasing the intrinsic micronutrient content of crops through traditional breeding or transgenic technology (Vasconcelos et al., 2017).

A key gene involved in iron loading in seeds, *VACUOLAR IRON TRANSPORTER1 (VIT1)*, was first identified in Arabidopsis (Kim et al., 2006), as a homolog of yeast *Ca^{2+}-SENSITIVE CROSS-COMPLEMENTER (CCC1)*, which transports iron into yeast vacuoles (Li et al., 2001) and manganese into Golgi vesicles (Lapinskas et al., 1996). *VIT1* is highly expressed in ripening Arabidopsis seeds, and targets iron to the vacuoles of the endodermis and veins of the

embryo (Kim et al., 2006; Roschzttardtz et al., 2009). Expression of *VIT1* also increases the manganese content of yeast cells (Kim et al., 2006), and it has a supporting role in manganese transport in Arabidopsis embryos (Eroglu et al., 2017). The VITs form a unique transporter family, found in plants, fungi and protists such as the malarial parasite *Plasmodium falciparum*, but they are absent from metazoans (Slavic et al., 2016). VITs in plants share a high degree of sequence similarity and the capacity to transport iron, but their biological functions may differ. For example, *TgVIT1* in tulips is involved in petal colour determination (Momonoi et al., 2009). Due to their roles in iron storage, VITs are potentially good candidates for iron biofortification. Indeed, expression of *VIT1* from Arabidopsis controlled by a *PATATIN* promoter enhanced the iron content of cassava tubers 3 – 4-fold (Narayanan et al., 2015). Given the promise for biofortification it is surprising that very few VITs from crop species have been characterized, particularly in cereals. Two *VIT* genes have been identified in rice, *OsVIT1* and *OsVIT2*. The genes showed different expression patterns throughout the plant and in response to iron, but were similar with respect to yeast complementation results. Knockout mutants accumulated more iron in the embryo, but this part of the grain is lost during processing to obtain white rice. The effect of overexpressing the *OsVIT* genes was not tested, and in fact virtually nothing is known about the wider physiological effects of overexpressing *VIT* in plants (Ravet et al., 2009).

For biofortification of cereal crops, simply increasing the iron content in grains is unlikely to increase their nutritional quality. Micronutrients are concentrated in the aleurone and seed coat, which are commonly removed in the production of polished rice or white wheat flour. The aleurone is also rich in phytate (myo-inositol-1,2,3,4,5,6-hexakisphosphate), a phosphate storage molecule that is a major inhibitor of iron bioavailability in wholegrain products (Hurrell and Egli, 2010). On the other hand, phytate is low in the endosperm (O'Dell et al., 1972), therefore this tissue should be targeted to increase bioavailable dietary iron in cereal food products. Previous biofortification strategies in wheat include overexpression of ferritin, which increased iron levels 1.6 – 1.8-fold but with large variations per line (Singh et al., 2017). Because ferritin is localized in plastids, iron transport into plastids also needs to be upregulated, and this may be a limiting factor in cereal grains. Elegant NanoSIMS (Nanoscale Secondary Ion Mass Spectrometry) studies showed that iron was concentrated in small vacuoles in the wheat aleurone, colocalising with phosphorus - most likely in the form of phytate, but that some also localized in patches in the endosperm (Moore et al., 2012). Other biofortification strategies have focussed on increasing the mobility of iron through overexpression of nicotianamine synthase genes for the production of chelator molecules to translocate iron(II) and other divalent metals (Singh et al., 2017).

Here, we identified and functionally characterized *TaVIT1* and *TaVIT2*, the two *VIT* paralogs found in the genome of bread wheat (*Triticum aestivum)*. The *VIT* genes differ in expression patterns and their ability to complement yeast metal transporter mutants. Based on these findings we selected *TaVIT2* for overexpression in the endosperm of wheat and barley, resulting in more than twice as much iron in white flour fractions but little impact on plant growth and

grain number. Our results suggest that by drawing iron into vacuoles in the endosperm, existing homeostasis mechanisms can be bypassed for a successful biofortification strategy.

Results

Wheat has two functionally differentiated *VIT* paralogs

The newly sequenced and annotated wheat genome (Clavijo et al., 2017) offers the opportunity to make a complete inventory of putative metal transporters in wheat (Borrill et al., 2014). We found that wheat has two *Vacuolar Iron Transporter* genes (*TaVIT1* and *TaVIT2*) on chromosome groups 2 and 5, respectively. As expected in hexaploid wheat, each *TaVIT* gene is represented by 3 copies (homoeologs) from the A, B and D genomes which share 99% identity at the amino acid level (Table S1, Figure S1). TaVIT1 and TaVIT2 have ~87% amino acid identity with their closest rice homolog, OsVIT1 and OsVIT2, respectively. Phylogenetic analysis suggests an early evolutionary divergence of the two *VIT* genes, as there are two distinctly branching clades in the genomes of monocotyledonous species, in contrast to one clade in dicotyledons (Figure 1a). The gene expression profiles of *TaVIT1* and *TaVIT2* were queried across 418 RNA-seq samples (Table S2). All homoeologs of *TaVIT2* were in general more highly expressed than *TaVIT1* homoeologs (Figure 1b). In the grains, *TaVIT1* and *TaVIT2* are both expressed in the aleurone, correlating with high levels of iron in this tissue which is removed from white flours during the milling process. In contrast, expression of *TaVIT1* and *TaVIT2* is very low in the starchy endosperm, the tissue from which white flour is extracted. Taken together, differences in phylogeny and expression pattern suggest that TaVIT1 and TaVIT2 may have distinct functions.

TaVIT2 facilitates transport of iron and manganese

To test if the TaVIT proteins transport iron, the 2BL *TaVIT1* homoeolog and 5DL *TaVIT2* homoeolog, hereafter referred to as *TaVIT1* and *TaVIT2* respectively, were selected and expressed in yeast lacking the vacuolar iron transporter Ccc1. The Δ*ccc1* yeast strain is sensitive to high concentrations of iron in the medium because of its inability to store iron in the vacuole. *TaVIT2* fully rescued growth of Δ*ccc1* yeast exposed to a high concentration of $FeSO_4$, but *TaVIT1* was no different from the empty vector control (Figure 2a). Yeast Ccc1 can transport both iron and manganese (Lapinskas et al., 1996). Therefore, we carried out yeast complementation using the Δ*pmr1* mutant, which is unable to transport manganese into Golgi vesicles and cannot grow in the presence of toxic levels of this metal (Lapinskas et al., 1995). We found that expression of *TaVIT2* in Δ*pmr1* yeast partially rescued the growth impairment on high concentrations of $MnCl_2$, indicating that TaVIT2 can transport manganese (Figure 2b). We also tested if *TaVIT1* and *TaVIT2* are able to rescue growth of the yeast Δ*zrc1* strain, which is defective in vacuolar zinc transport, but neither *TaVIT* gene was able to rescue growth on high zinc concentrations (Figure 2c).

Western blot analysis showed that both proteins were produced in yeast, but that TaVIT1 and TaVIT2 might differ in their intracellular distribution (Figure 2d). TaVIT2 was abundant in vacuolar membranes, co-fractionating with the vacuolar marker protein Vph1. TaVIT1 was also

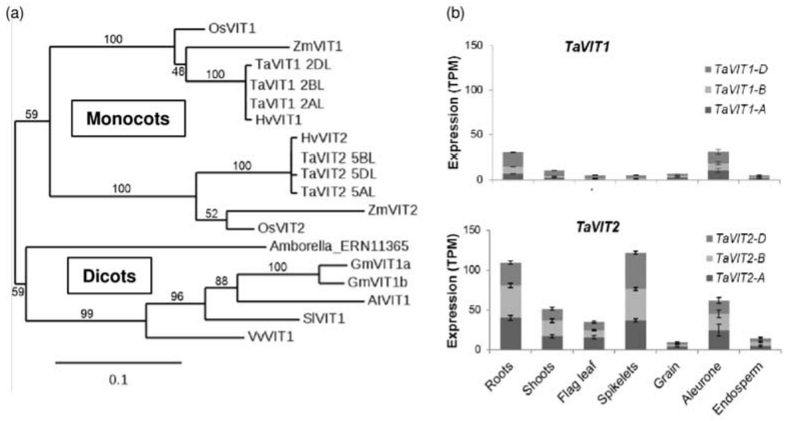

Figure 1 The wheat genome encodes two *VIT* paralogs with different expression patterns. (a) Phylogenetic tree of *VIT* genes from selected plant species: *At, Arabidopsis thaliana*; *Gm, Glycine max* (soybean); *Hv, Hordeum vulgare* (barley); *Os, Oryza sativa* (rice); *Sl, Solanum lycopersicum* (potato); *Ta, Triticum aestivum* (wheat); *Vv, Vitis vinifera* (grape); *Zm, Zea mays* (maize). Numbers above or below branches represent bootstrapping values for 100 replications. (b) Gene expression profiles of *TaVIT1* and *TaVIT2* homoeologs using RNA-seq data from expVIP. Bars indicate mean transcripts per million (TPM) ± SEM, full details and metadata in Table S2.

found in the vacuolar membrane fraction, but based on higher abundance in the total fraction, it appeared that most of the TaVIT1 protein was targeted to other membranes. Closer inspection of the amino acid sequences revealed that TaVIT2 contains a universally conserved dileucine motif for targeting to the vacuolar membrane (Bonifacino and Traub, 2003; Wang et al., 2014), which is absent from TaVIT1 (Figure S1b). Therefore, TaVIT1 may be able to transport iron, but will not complement Δ*ccc1* yeast. Instead, we tested if TaVIT1 was able to complement the

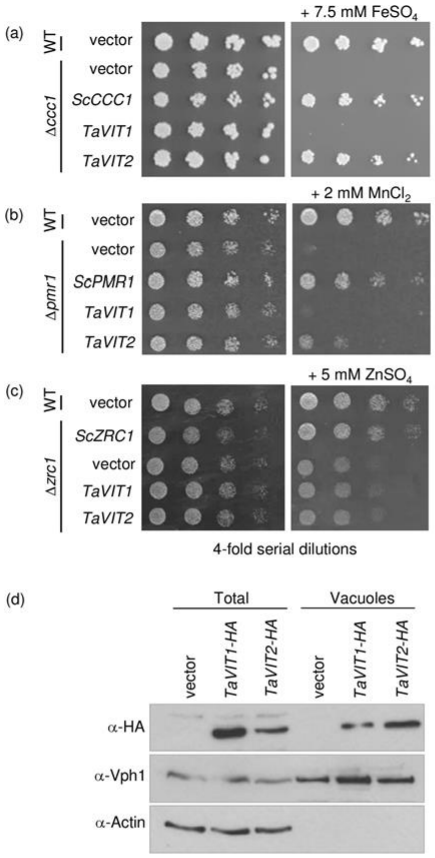

Figure 2 TaVIT2 facilitates iron and manganese transport. (a,b,c) Yeast complementation assays of *TaVIT1* and *TaVIT2* in Δ*ccc1* (a), Δ*pmr1* (b) and Δ*zrc1* (c) compared to yeast that is wild type (WT) for these three genes. The yeast (Sc) *CCC1*, *PMR1* and *ZRC1* genes were used as positive controls. Cells were spotted in a 4-fold dilution series and grown for 2-3 days on plates ± 7.5 mM $FeSO_4$ (Δ*ccc1*), 2 mM $MnCl_2$ (Δ*pmr1*) or 5 mM $ZnSO_4$ (Δ*zrc1*). (d) Immunoblots of total and vacuolar protein fractions from yeast cells expressing haemagglutinin (HA)-tagged *TaVIT1* or *TaVIT2*. The HA-tag did not inhibit the function of TaVIT2 as it was able to complement Δ*ccc1* yeast (data not shown). Vhp1 was used as a vacuolar marker and the absence of actin shows the purity of the vacuolar fraction.

Δ*fet3* yeast mutant, which is defective in high-affinity iron transport across the plasma membrane. Δ*fet3* mutants cannot grow on medium depleted of iron with the chelator BPS, but expression of *TaVIT1* rescued growth under these conditions (Figure S2). These data indicate that both TaVIT1 and TaVIT2 are able to transport iron, but that their localization in the cell may differ.

Over-expression of *TaVIT2* in the endosperm of wheat specifically increased the iron concentration in white flour

The functional characterization of TaVIT1 and TaVIT2 suggested that TaVIT2, as a *bona fide* iron transporter localized to vacuoles, is a good candidate for iron biofortification. We placed the *TaVIT2* gene under the control of the wheat endosperm-specific promoter of the *High Molecular Weight Glutenin-D1* (*HMW*) gene (Lamacchia et al., 2001) and transformed the construct together with a hygromycin resistance marker into the wheat cultivar 'Fielder' (Figure 3a). A total of 27 hygromycin-resistant plants were isolated and the copy number of the transgene was determined by qPCR. There were ten lines with a single copy insertion, and the highest number of insertions was 30. The transgene copy number correlated well with expression of *TaVIT2* in the developing grain (R^2 = 0.60, $p < 0.01$, Figure 3b; Figure S3). *TaVIT2* expression was increased 3.8 ± 0.2-fold in single copy lines and more than 20-fold in lines with multiple transgenes compared to non-transformed controls.

Mature wheat grains from transgenic lines and non-transformed controls were dissected with a platinum-coated blade and stained for iron using Perls' Prussian Blue. In non-transformed controls, positive blue staining was visible in the embryo, scutellum and aleurone layer, but the endosperm contained little iron (Figure 4). In lines over-expressing *TaVIT2* the Perls' Prussian Blue staining was visibly increased, in particular around the groove and in patches of the endosperm. To quantify the amount of iron, grains from individual lines were milled to produce wholemeal flour, which was sieved to obtain a white flour fraction, followed by element analysis using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Figure 5a, Table S3). Iron levels were consistently enhanced 2-fold in white flour, from 9.7 ± 0.3 µg/g in control lines to 21.7 ± 2.7 µg/g in lines with a single copy of *HMW-TaVIT2* ($p < 0.05$). Additional transgene copies resulted in a similar 2-fold increase in iron, whereas lines with ≥ 20 copies contained 4-fold more iron than controls, to 41.5 ± 8.2 µg/g in white flour ($p < 0.05$). The iron content of wholemeal flour of single insertion lines was similar to control lines, but increased up to 2-fold in high copy lines ($p < 0.01$). No statistically significant differences were found for other metals in single-copy *HMW-TaVIT2* wheat grains, such as zinc, manganese and magnesium (Table S3), nor for the heavy metal contaminants cadmium and lead (Table S4). In lines with ≥ 20 copies of *HMW-TaVIT2* significant increases in all elements except Mn and Pb were seen ($p < 0.05$), presumably as a secondary effect.

White flour has an improved iron:phytate ratio and the iron is bioavailable

Because the high phytate content of cereal grains inhibits bioavailability of minerals, we measured phytate levels in *TaVIT2* over-expressing lines, but found no significant increase in phytate in white flour (Figure 5b), although there was a slight increase in phosphorus (Table S3). There was also a small increase in phytate in wholemeal flour produced from those lines. Considering the 2-fold increase in iron, the iron:phytate molar ratio was improved 2-fold in white flour of *HMW-TaVIT2* lines, but unaffected in wholemeal flour (Figure 5c).

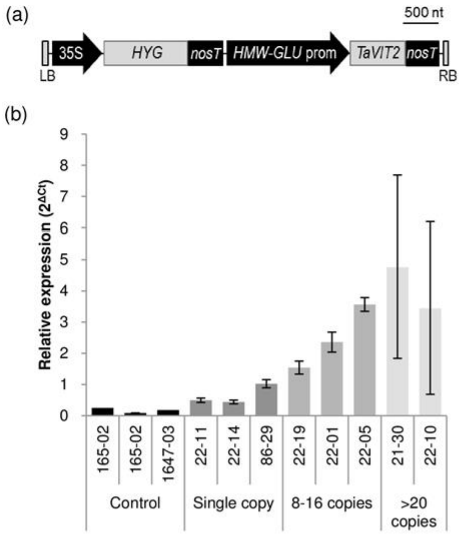

Figure 3 Expression of *TaVIT2* in cisgenic lines.
(a) Diagram of the transfer-DNA construct: LB, left border; 35S, CaMV 35S promoter; *HYG*, hygromycin resistance gene; *nosT*, *nos* terminator; *HMW-GLU* prom, high molecular weight glutenin-D1-1 promoter; *TaVIT2*, wheat *VIT2-D* gene; RB, right border. (b) Relative expression levels of *TaVIT2* in developing grains at 10 days post anthesis as determined by quantitative real-time PCR and normalized to housekeeping gene *Traes_4AL_8CEA69D2F*. Plant identification numbers and copy number of the *HMW-TaVIT2* gene are given below the bars. Bars indicate the mean ± SEM of 3 independent biological replicates.

To investigate the potential bioavailability of the iron, flour samples were subjected to simulated gastrointestinal digestion and the digests applied to Caco-2 cells, a widely used cellular model of the small intestine (Glahn et al., 1998). For the purpose of this experiment, the availability of iron was maximized by treating the samples with phytase and by exposing the cells directly to the digestate after heat-inactivation of the lytic enzymes. The increase in ferritin protein in Caco-2 cells after exposure to the digestate was used as a surrogate measure of iron availability. Iron

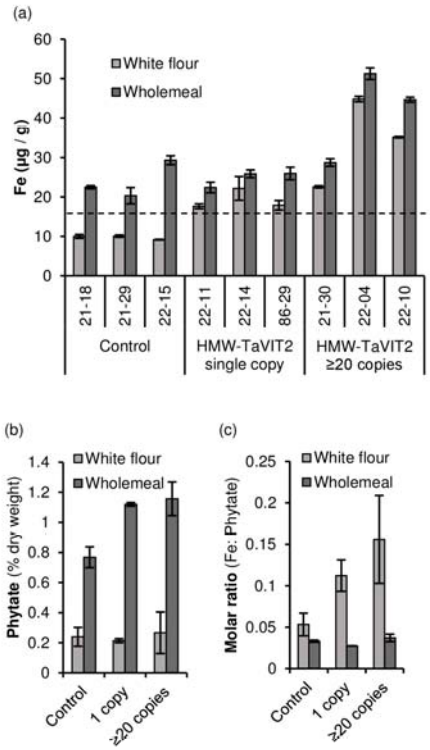

Figure 5 Iron and phytate content of flour milled from *HMW-TaVIT2* wheat lines. (a) Iron concentrations in white and wholemeal flour from 3 control and 6 *HMW-TaVIT2* lines. Bars represent the mean of 2 technical replicates and the deviation of the mean. White flour from *HMW-TaVIT2* lines has significantly more iron than control lines (n = 3-4, $p < 0.05$; see Table S3 for all data). The dotted line at 16.5 µg/g iron indicates the minimum requirement for wheat flour milled in the UK. (b) Phytate content of white and wholemeal flour of control and *HMW-TaVIT2* expressing wheat. Bars represent the mean of 2 biological replicates ± deviation of the mean. (c) Molar ratio of iron:phytate in control and *HMW-TaVIT2* expressing lines. Bars represent the mean of 2 biological replicates and the deviation of the mean.

from white flour was taken up by the Caco-2 cells, and more ferritin production was observed in cells exposed to samples from*TaVIT2*-overexpressing lines, however the values were variable between wheat lines (Figure S4). In contrast, the iron in wholemeal flour, although twice as high as in white flour, was not available for uptake, as previously noted (Eagling et al., 2014). Further analysis of breads baked from these flours is necessary to confirm overexpression improves

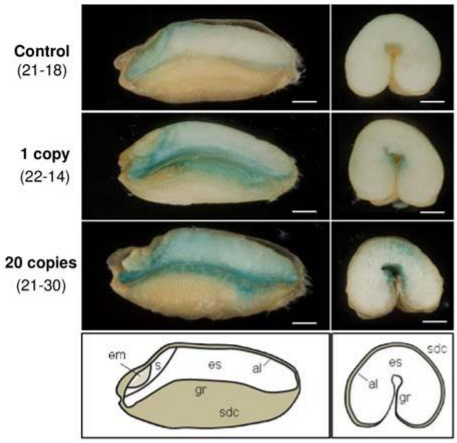

Figure 4 Perls' Prussian Blue staining for iron in grains transformed with *HMW-TaVIT2*. Grains from T_0 wheat plants were dissected longitudinally (left) or transversely (right). em, embryo; s, scutellum; sdc, seed coat; es, endosperm; al, aleurone, gr, groove. The transgene copy number and line number are indicated on the far left. Scale bars = 1 mm.

iron bioavailability. These data suggest that relocating iron into the endosperm may be more effective than increasing total iron in the grain as a biofortification strategy.

The high-iron phenotype has little impact on plant growth and is maintained in T_2 grains

To investigate if *TaVIT2* over-expression affected plant growth, we measured plant height, tiller number, grain size, number of grains per plant and thousand-grain weight in *TaVIT2* over-

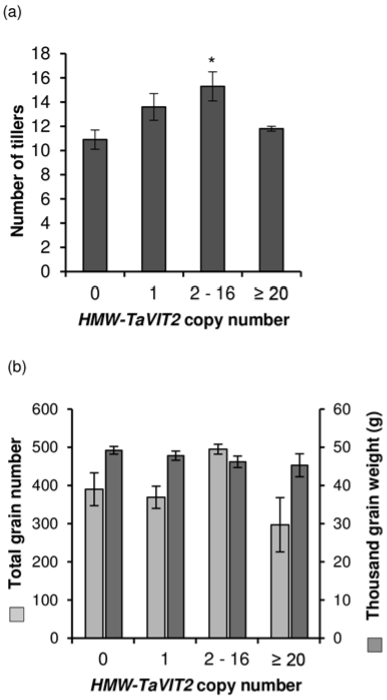

Figure 6 Growth parameters of *HMW-TaVIT2* wheat.
(a) Number of tillers and (b) seed output of T_0 wheat plants with indicated *HMW-TaVIT2* copy numbers. Bars indicate mean ± SEM of the following numbers of biological replicates: zero gene copies, n = 9; 1 gene copy, n = 10; 2 -16 gene copies, n = 9; ≥ 20 gene copies, n = 6. Further details given in Table S5. Asterisk indicates significant difference from negative control (One-way ANOVA with Tukey post-hoc test, * $p < 0.05$).

expressing lines and controls. None of these growth parameters were negatively affected by the *HMW-TaVIT2* transgene in the T_0 generation grown in controlled environment rooms (Figure 6 and Table S5). Conversely, a statistically significant increase in tiller number was seen in plants containing 2 – 16 copies of the *HMW-TaVIT2* transgene, to 15.3 ± 1.2 compared to the control of 10.9 ± 0.8 ($p < 0.05$, ANOVA, Table S5). Analysis of further generations and field trials are required to confirm this effect and its potential impact on yield.

Seed from the first T_0 transformant obtained (line 27-02, containing 2 copies *of HMW-TaVIT2*) was planted in a greenhouse to investigate the high-iron trait in the next generation (T_1). The *HMW-TaVIT2* transgene segregated in a 3:1 ratio (χ^2=0.29). Growth of plants in the greenhouse was very different from controlled environmental chambers, but there were no significant differences in growth and yield component traits for *HMW-TaVIT2* plants compared to wild-type segregants or non-transformed controls (Table S6). The iron levels were overall higher in grain from greenhouse-grown plants, even so T_2 grain contained a 2-fold increase in iron in the white flour fraction ($p < 0.05$, Table S6). Taken together, endosperm-specific over-expression of *TaVIT2* has no major growth defects and the iron increase showed a similar trend in the next generation despite different growth conditions.

Expression of *HMW-TaVIT2* in barley increases grain iron and manganese content

We also transformed barley (*Hordeum vulgare* cv. Golden Promise) with the *HMW-TaVIT2* construct. The 12 transgenic plants had either 1 or 2 copies of the transgene and were indistinguishable from non-transformed controls with regards to vegetative growth and grain development. Staining with Perls' Prussian Blue showed that, similarly to wheat, there was more iron in transformed grains than controls, and this tended to accumulate in the sub-aleurone region of the endosperm. To quantify the iron and other metals, lines B2 (1 copy) and B3 (2 copies) were selected for ICP-OES analysis and found to contain 2-fold more iron than the control in both white and wholemeal flour (Figure 7). The white flour produced from barley contained relatively high levels of phosphorus, suggesting that there was some aleurone present, so the differences in minerals between white and wholemeal flours are not as pronounced as in wheat. Interestingly, in barley there was also a 2-fold increase in manganese levels (Figure 7). These results indicate that the ability of TaVIT to transport manganese, as observed in yeast (Figure 2b), can be operational in plant tissue. Overall, our results indicate that endosperm-specific over-expression of *TaVIT2* is a successful strategy for increasing the iron content in different cereal crop species.

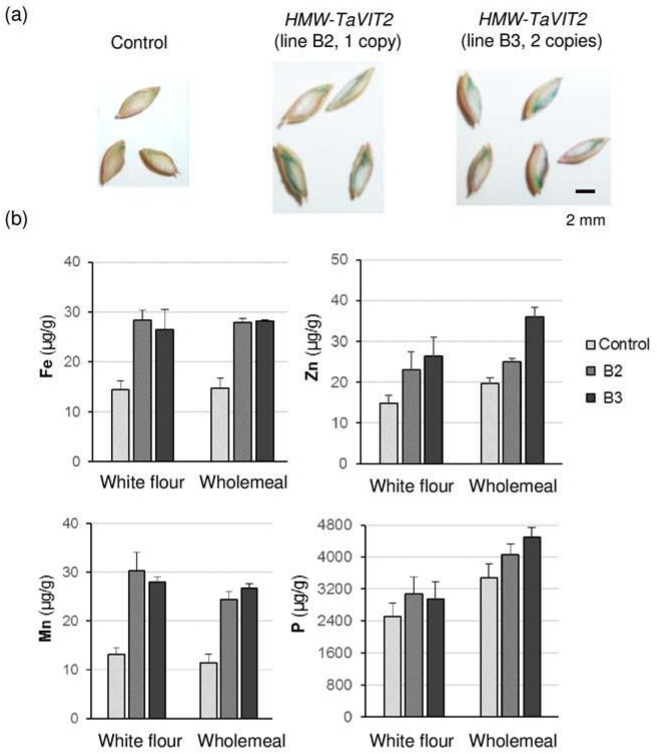

Figure 7 Endosperm-specific over-expression of *TaVIT2* in barley. The *TaVIT2-5DL* gene from wheat under the control of the wheat *HMW-GLU-1D-1* promoter (see Figure 3a for full details) was transformed into barley (*Hordeum vulgare* var. Golden Promise). Positive transformants were selected by hygromycin. (a) Mature barley T_1 grains of a control plant and two transgenic lines stained with Perls' Prussian blue staining for iron. (b) Element analysis in white and wholemeal flours from a control and two *HMW-TaVIT2* over-expressing barley plants. The values are the mean of 2 technical replicates. Error bars represent the deviation of the mean.

Discussion

The recently sequenced wheat genome greatly facilitates gene discovery in this economically important but genetically complex crop species. In a previous analysis (Borrill et al., 2014) we identified over 60 putative metal transporters, and started with the functional characterization of VITs. We selected *TaVIT1-B* and *TaVIT2-D* for our studies. Each TaVIT gene has three

homoeologs but these share 99% amino acid identity, and those amino acids that differ are not conserved, therefore we believe that our results are representative for all three homoeologs.

While TaVIT1 and TaVIT2 are ~87% identical with OsVIT1 and OsVIT2, we found remarkable differences. Each rice VIT has the dileucine motif involved in vacuolar targeting and GFP fusion proteins showed vacuolar localisation when transiently expressed in Arabidopsis protoplasts (Zhang et al., 2012). In wheat, only TaVIT2 has the dileucine motif and this correlated with vacuolar localization of TaVIT2 in yeast. Another striking difference between rice and wheat VITs is the yeast complementation results. *OsVIT1* and *OsVIT2* partially complemented mutants in iron transport (Δ*ccc1*) and zinc transport (Δ*zrc1*). In wheat, only *TaVIT2* showed complementation of Δ*ccc1* and we saw no evidence of Zn transport, similar to the metal specificity of the yeast homolog. The growth defect of Δ*ccc1* was completely rescued by *TaVIT2*, indicating efficient iron transport in contrast to only weak complementation by the rice *VIT* genes. The production of the rice VIT proteins in yeast was unfortunately not verified by Western blot analysis (Zhang et al., 2012). Our initial experiments showed that wheat *TaVIT1* was poorly expressed in yeast, so the sequence was codon-optimized to remove codons that are rare in *Saccharomyces cerevisiae* (Figure S5). This greatly improved expression of *TaVIT1* to even higher levels than *TaVIT2*, but *TaVIT1* still did not complement the yeast mutants in Fe, Zn or Mn transport. *TaVIT1*, however, did complement the Δ*fet3* yeast mutant (Figure S2). Yeast *FET3* is part of a complex directing high-affinity Fe transport across the plasma membrane (Askwith et al., 1994). This suggests that TaVIT1 is indeed a functioning iron transporter but that it mainly localizes to a membrane other than the tonoplast..It will be interesting to identify the amino acid residues that determine metal specificity and/or localization in the VIT family. However, currently there is no crystal structure of any of the VIT family members and no other good structural homology models. Recently, a first glimpse into the transport mechanism was provided, showing that Plasmodium VIT1 is a H^+ antiporter with strong selectivity for Fe^{2+} (Slavic et al., 2016; Labarbuta et al., 2017).

Over-expression of the vacuolar iron transporter *TaVIT2* in wheat endosperm was very effective in raising the iron concentration in this tissue. We hypothesize that increased sequestration of iron in the vacuoles creates a sink which then upregulates the relocation of iron to that tissue. If the tissue normally stores iron in vacuoles rather than in ferritin, proteins and chelating molecules for iron mobilisation into the vacuole will already be present. For a sink-driven strategy, timely expression of the gene in a specific tissue is essential: if the protein is produced constitutively, for example using the CaMV 35S promoter, then it will draw iron into all tissues, not in one particular tissue. Interestingly, knock-out mutants of *VIT1* and *VIT2* in rice accumulated more iron in the embryo (Zhang et al., 2012). A likely scenario is that iron distributed to the developing rice grain cannot enter the vacuoles in the aleurone (Kyriacou et al., 2014), and is thus diverted to the embryo. The finding further supports the idea that VITs play a key role in iron distribution in cereal grains. An additional advantage of endosperm-

specific expression is that possible growth defects in vegetative tissues are likely to be avoided, as found in our studies.

Wheat and barley transformed with the same *HMW-TaVIT2* construct showed surprising differences in the accumulation of iron and manganese. Wheat had a 2-fold increase in iron in the endosperm only, whereas barley contained 2-fold more iron in whole grains. Barley grains also contained 2-fold more manganese, but this element was not increased in wheat, even though TaVIT2 was found to transport both iron and manganese in yeast complementation assays. It is possible that the wheat *HMW* promoter has a different expression pattern in barley. If the promoter is activated in the aleurone cells in addition to the endosperm, this may lead to the observed higher iron concentrations in whole barley grains. The pattern of promoter activity can be further investigated with reporter constructs or by in-situ hybridization specific for the transgene. It is also possible that wheat and barley differ in iron and manganese transport efficiency from roots to shoots, thus affecting the total amount of iron and manganese that is (re)mobilized to the grain.

In the Americas, Africa and Asia, iron fortification of flours ranges from 30 to 44 µg/g. In Europe, only the UK has a legal requirement for fortification: white and brown flours must contain at least 16.5 µg/g iron. We have now achieved this iron concentration in white flour produced from the single-copy *HMW-TaVIT2* lines described here. More copies of *TaVIT2* increased iron levels further, but resulted in accumulation of other metals. Moreover, with ≥ 20 transgene copies there were fewer grains per plant. Combining endosperm-specific *TaVIT2* overexpression with constitutive *NAS* over-expression may be one suitable approach to increase grain iron levels further. A combination strategy using over-expression of *NAS2* and soybean ferritin increased iron levels in polished rice more than 6-fold, from 2 µg/g to 15 µg/g in the field (Trijatmiko et al., 2016). However, combining *NAS* and *FER* over-expression in wheat did not show a synergistic effect: constitutive expression of the rice *NAS2* gene resulted in 2.1-fold more iron in grains and 2.5-fold more iron in white flour, but coupled with endosperm-specific expression of *FER*, grain iron content was only 1.6 - 1.8-fold increased, similar to *FER* alone (Singh et al., 2017). As noted before, iron in wheat is mostly stored in vacuoles rather than ferritin, so increasing iron transport into vacuoles combined with increasing iron mobility is likely to be more effective. Nicotianamine is also reported to improve the bioavailability of iron (Zheng et al., 2010), which is a major determinant for the success of any biofortification strategy.

On a societal level, a major question is whether wheat biofortified using modern genetic techniques will be accepted by consumers. Our strategy used wheat genetic material (promoter and coding sequence), and could therefore be considered cisgenic. The *HMW-TaVIT2* lines also contain DNA from species other than wheat, such as a hygromycin resistance gene of bacterial origin, but these regions can be removed using CRISPR technology, leaving only wheat DNA. In addition, the wheat lines described here are valuable tools to identify processes

regulating iron content of the grain. Identification of the transcription factors that control *VIT* expression would be helpful, but none have been identified so far in any plant species. Once more genetic components of the iron loading mechanism into cereals have been identified, these can be targets of non-transgenic approaches such as TILLING (Krasileva et al., 2017).

Experimental procedures

Identification of wheat *VIT* genes, phylogenetic analysis and analysis of RNA-seq data

The coding sequences of the wheat *VIT* genes were found by a BLAST search of the rice *OsVIT1 (LOC_Os09g23300)* and *OsVIT2 (LOC_Os04g38940)* sequences in Ensembl Plants (http://plants.ensembl.org). Full details of the wheat genes are given in Table S1. Sequences of *VIT* genes from other species were found by a BLAST search of the Arabidopsis *AtVIT1 (AT2G01770)* and rice *VIT* sequences against the Ensembl Plants database. Amino acid alignments were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The tree was plotted with BioNJ with the Jones-Taylor-Thornton matrix and rendered using TreeDyn 198.3. RNA-seq data was obtained from the expVIP database (Borrill et al., 2016; http://www.wheat-expression.com). Full details of the data-sets used are given in Table S2.

Yeast complementation

Coding DNA sequences for the wheat 2BL *VIT1* homoeolog (TRIAE_CS42_2BL_TGACv1_129586_AA0389520) and the 5DL *VIT2* homoeolog (TRIAE_CS42_5DL_TGACv1_433496_AA1414720) were synthesized and inserted into pUC57 vectors by Genscript (Piscataway, NJ, USA). The wheat *VIT* genes were first synthesized with wheat codon usage, but *TaVIT1* was poorly translated in yeast so was re-synthesized with yeast codon usage including a 3x haemagglutinin (HA) tag at the C-terminal end. Untagged codon-optimized *TaVIT1* was amplified from this construct using primers *TaVIT1co-Xba*I-F and *TaVIT1co-EcoR*I-R (see Table S7 for primer sequences). *TaVIT2-HA* was cloned by amplifying the codon sequence without stop codon using primers *TaVIT2-BamH*I-F and *TaVIT2*(ns)-*EcoR*I-R, and by amplifying the HA tag using primers HAT-*EcoR*I-F and HAT(Stop)-*Cla*I-R. The two DNA fragments were inserted into plasmid p416 behind the yeast *MET25* promoter (Mumberg et al., 1995).

Genes *ScCCC1*, *ScFET3*, *ScPMR1* and *ScZRC1* were cloned from yeast genomic DNA, using the primer pairs *ScCCC1-BamH*I-F and *ScCCC1-EcoR*I-R, *ScFET3-Xba*I-F and *ScFET3-Xho*I-R, *ScPMR1-Spe*I-F and *ScPMR1-Xho*I-R, and *ScZRC1-Xba*I-F and*ScZRC1-EcoR*I-R, respectively. Following restriction digests the DNA fragments were ligated into vector p416-*MET25* and confirmed by sequencing. All constructs were checked by DNA sequencing.

The *Saccharomyces cerevisiae* strain BY4741 (MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) was used in all yeast experiments. Either wild-type (WT), Δ*ccc1* (Li et al., 2001), Δ*zrc1* (MacDiarmid

et al., 2003), Δ*pmr1* (Lapinskas et al., 1995) or Δ*fet3* (Askwith et al., 1994) was transformed with approximately 100 ng DNA using the PEG/lithium acetate method (Ito et al., 1983). Complementation analysis was performed via drop assays using overnight cultures of yeast grown in selective synthetic dextrose (SD) media, diluted to approximately 1×10^6 cells/ml, spotted in successive 4 × dilutions onto SD plates containing appropriate supplements. Plates were incubated for 3 days at 30°C. Total yeast protein extraction was performed by alkaline lysis of overnight cultures (Kushnirov, 2000).

Preparation of vacuoles from yeast

Preparation of yeast vacuoles was performed using cell fractionation over a sucrose gradient (Hwang et al., 2000; Nakanishi et al., 2001). Briefly, 1 L yeast was grown in selective SD media to an OD_{600} of 1.5-2.0 then centrifigued at 4000 *g* for 10 min, washed in buffer 1 (0.1 M Tris-HCl pH 9.4, 50 mM β-mercaptoethanol, 0.1 M glucose) and resuspended in buffer 2 (0.9 M sorbitol, 0.1 M glucose, 50 mM Tris- 2-(N-morpholino)ethanesulfonic acid (MES) pH 7.6, 5 mM dithiothreitol (DTT), 0.5 × SD media). Zymolyase 20T (Seikagaku, Tokyo, Japan) was added at a concentration of 0.05% (w/v) and cells were incubated for 2 h at 30°C with gentle shaking. After cell wall digestion, spheroplasts were centrifuged at 3000 *g* for 10 min and then washed in 1 M sorbitol before being resuspended in buffer 3 (40 mM Tris-MES, pH 7.6, 1.1 M glycerol, 1.5% (w/v) polyvinylpyrrolidone 40,000; 5 mM EGTA, 1 mM DTT, 0.2% (w/v) bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 × protease inhibitor cocktail (Promega)) and homogenized on ice using a glass homogenizer. The homogenate was centrifuged at 2000 *g* for 10 min at 4°C and the supernatant was transferred to fresh tubes, while the pellet was resuspended in fresh buffer 3 and centrifuged again. The supernatants were pooled and centrifuged at 150,000 *g* for 45 min at 4°C to pellet microsomal membranes. For preparation of vacuole-enriched vesicles the pellet was resuspended in 15% (w/w) sucrose in buffer 4 (10 mM Tris-MES pH7.6, 1 mM EGTA, 2 mM DTT, 25 mM KCl, 1.1 M glycerol, 0.2% (w/v) BSA, 1 mM PMSF, 1 × protease inhibitor cocktail) and this was layered onto an equal volume of 35% (w/w) sucrose solution in buffer 4 before centrifugation at 150,000 *g* for 2 h at 4°C. Vesicles were collected from the interface and diluted in buffer 5 (5 mM Tris-MES pH 7.6, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 × protease inhibitor cocktail). The membranes were centrifuged at 150,000 *g* for 45 min at 4°C and resuspended in a minimal volume of buffer 6 (5 mM Tris-MES pH 7.6, 0.3 M sorbitol, 1 mM DTT, 1 mM PMSF, 1 × protease inhibitor cocktail). Vesicles were snap-frozen in liquid nitrogen and stored at -80°C.

Generation of transgenic plant lines

The *TaVIT2* gene was amplified using primers TaVIT2-*Nco*IF and TaVIT2-*Spe*IR and cloned into vector pRRes14_RR.301 containing the promoter sequence comprising nucleotides -1187 to -3 with respect to the ATG start codon of the *GLU-1D-1* gene, which encodes the high-

molecular-weight glutenin subunit 1Dx5 (Lamacchia et al., 2001). The promoter-gene fragment was then cloned into vector pBract202 containing a hygromycin resistance gene and LB and RB elements for insertion into the plant genome (Smedley and Harwood, 2015). The construct was checked by DNA sequencing. Transformation into wheat (cultivar Fielder) and barley (cultivar Golden Promise) were performed by the BRACT platform at the John Innes Centre using *Agrobacterium*-mediated techniques as described previously (Wu et al., 2003; Harwood et al., 2009). Transgene insertion and copy number in T_0 plants were assessed by iDNA Genetics (Norwich, UK) using qPCR with a Taqman probe. For the T_1 generation, the presence of the hygromycin resistance gene was analysed by PCR with primers Hyg-F and Hyg-R.

Plant growth and quantitative analysis

The first generation of transgenic plants (T_0) were grown in a controlled environment room under 16 h light (300 µmol m^{-2} s^{-1}) at 18°C / 8 h dark at 15°C with 65% relative humidity. The next generation (T_1) were grown in a glasshouse kept at approximately 20°C with 16 h light. Wheat and barley plants were grown on a mix of 40% medium grade peat, 40% sterilized soil and 20% horticultural grit, and fertilized with 1.3 kg/m^3 PG Mix 14+16+18 (Yara UK Ltd, Grimsby, UK) containing 0.09% Fe, 0.16% Mn and 0.04% Zn. Ears from wheat and barley plants were threshed by hand and grain morphometric characteristics, mass and number were determined using a MARVIN universal grain analyser (GTA Sensorik, GmbH, Neubrandenburg, Germany).

RNA extraction and qRT-PCR

Samples of developing grain were taken at 10 days post anthesis and frozen in liquid nitrogen. RNA extraction was performed using phenol/chloroform extraction (Box et al., 2011). Developing grains were ground with a pestle and mortar under liquid nitrogen and mixed with RNA extraction buffer (0.1 M Tris-HCl, pH 8; 5 mM EDTA; 0.1 M NaCl, 0.5% (w/v) SDS, 1% (v/v) 2-mercaptoethanol) and Ambion Plant RNA Isolation Aid (ThermoFisher). Samples were centrifuged for 10 min at 15,000 *g* and the supernatant was added to 1:1 acidic phenol (pH 4.3):chloroform. After mixing and incubation at room temperature for 10 min, the upper phase was added to isopropanol containing 0.3 M sodium acetate. Samples were incubated at -80°C for 15 min and centrifuged for 30 min at 15,000 *g* at 4°C. The supernatant was discarded and the pellet was washed twice in 70% (v/v) ethanol and dried, before being resuspended in RNAse-free water. RNA was DNase treated using TURBO DNase-free kit (ThermoFisher) as per manufacturer's instructions, DNase inactivation reagent was added and the samples were centrifuged at 10,000 *g* for 90 s. Supernatant containing RNA was retained. RNA was reverse transcribed using oligo dT primer and Superscript II reverse transcriptase (ThermoFisher) according to manufacturer's instructions. Quantitative real time PCR was used to analyse expression of *TaVIT2* and the housekeeping gene (*HKG*) *Traes_4AL_8CEA69D2F*, chosen because it was shown to be the most stable gene expression across grain development in over

400 RNAseq samples (Borrill et al., 2016), using primer pairs qRT-*TaVIT2*-F, qRT-*TaVIT2*-R and qRT-*HKG*-F, qRT-*HKG*-R, respectively. Samples were run in a CFX96 Real-Time System (Bio-Rad) with the following conditions: 3 min at 95°C, 35 cycles of (5 s at 95°C, 10 s at 62°C, 7 s at 72°C), melt curve of 5 s at 65°C and 5 s at 95°C. *TaVIT2* expression levels were normalized to expression levels of the housekeeping gene and expressed as $2^{\Delta Ct}$.

Perls' Prussian Blue staining

Mature grains were dissected using a platinum-coated scalpel and stained for 45 mins in Perls' Prussian blue staining solution (2% (w/v) potassium hexacyanoferate (II); 2% (v/v) hydrochloric acid), then washed twice in deionized water.

Flour preparation, element analysis and phytate determination

Barley grains were de-hulled by hand and all grains were coarsely milled using a coffee grinder then ground into flour using a pestle and mortar. White flour fractions were obtained by passing the material through a 150 µm nylon mesh. Flour samples were dried overnight at 55°C and then digested for 1 h at 95°C in ultrapure nitric acid (55% v/v) and hydrogen peroxide (6% v/v). Samples were diluted 1:11 in ultrapure water and analysed by Inductively Coupled Plasma-Optical Emission Spectroscopy (Vista-pro CCD Simultaneous ICP-OES, Agilent, Santa Clara, CA, USA) calibrated with standards; Zn, Fe and Mg at 0.2, 0.4, 0.6, 0.8 and 1mg/l, Mn and P at 1, 2, 3, 4 and 5 mg/l. Soft winter wheat flour was used as reference material (RM 8438, National Institute of Standards and Technology, USA) and analysed in parallel with all experimental samples. Phytate levels were determined using a phytic acid (total phosphorus) assay kit (Megazyme, Bray, Ireland).

Bioavailability assays in Caco-2 cells

Caco-2 cells (HTB-37) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured as previously described (Rodriguez-Ramiro et al., 2017). Wheat flour samples were subjected to simulated gastrointestinal digestion as described (Glahn et al., 1998) with minor modifications. One gram of flour was added to 5 mL of pH 2 buffer saline-solution (140 mmol/L NaCl, 5 mmol/L KCl) followed by the addition of pepsin (0.04 g/mL). Ascorbic acid was added at a molar ratio of 1:20 to ensure complete solubilisation of released iron. Additionally, phytase (Megazymes, Bray, Ireland) was added to fully degrade phytate (myo-inositol hexakisphosphate). Samples were incubated at 37 °C on a rolling table for 90 min. Next, the pH of the samples was gradually adjusted to pH 5.5, bile (0.007 g/mL) and pancreatin (0.001 g/mL) digestive enzymes were added, the pH adjusted to 7, and samples were incubated for an additional hour. At the end of the simulated digestion, samples were centrifuged at 3000 g for 10 min, the gastrointestinal enzymes heated-inactivated at 80 °C for 10 min, centrifuged as before, and the resultant supernatant was subsequently used for iron uptake experiments similar to Bodnar et al. (2013) with little modifications. A volume of 0.5 mL of wheat digestate

was diluted in 0.5 mL of Eagle's minimum essential medium (MEM), and applied over Caco-2 cell monolayers grown in collagen-coated 12-well plates. Samples were incubated for 2 hour at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. After incubation, an additional 0.5 mL MEM was added and cells were incubated for a further 22 hours prior to harvesting for ferritin analysis. To harvest the cells, the medium was removed by aspiration, cells rinsed with 18 Ω MilliQ H2O and subsequently lysed by scraping in 100 µl of Cellytic M (Sigma-Aldrich, UK). Cell pellets were kept on ice for 15 min and stored at -80 °C. For analysis, samples were thawed and centrifuged at 14,000 x g for 15 min. The supernatant containing the proteins was used for ferritin determination using the Spectro Ferritin ELISA assay (RAMCO, USA) according to the manufacturer's protocol. Ferritin concentrations were normalized to total cell protein using the Pierce Protein BCA protein assay (ThermoFisher Scientific, UK).

All experiments were performed using the following controls: a) a blank digestion without any wheat sample or added iron and b) a reference digestion of 50 µM of ferrous sulphate heptahydrate ($FeSO4{\bullet}7H_2O$) solubilized in 0.1 M HCl with 1000 µM of ascorbic acid.

Statistical analysis

Statistical analyses (F-test, ANOVA, Student's *t*-test, Kruskal-Wallis test, regression analysis, χ^2) were performed using Microsoft Excel 2010 and Genstat 18th Edition. Unless otherwise stated in the text *p*-values were obtained from Kruskal-Wallis tests with Dunnett post-hoc tests. When representative images are shown, the experiment was repeated at least 3 times with similar results.

Acknowledgements

We would like to thank James Simmonds for technical assistance; Wendy Harwood for wheat transformation; Alison Huttly at Rothamsted Research for the *HMW GLU-D1-1* promoter; Sylvaine Bruggraber at MRC-HNR and Graham Chilvers at UEA for element analysis; Tony Miller and Dale Sanders for helpful discussions. This work was funded by HarvestPlus (J.M.C., J.B. and C.U.) and by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant BB/J004561/1 (C.U. and J.B.) and DRINC2 grant BB/L025515/1 (I.R.R., S.F.T. and J.B.).

Conflict of interest

Authors declare no conflict of interest.

Supporting Information

Figure S1 Gene models and protein sequence of wheat vacuolar iron transporters.

Figure S2 *TaVIT1* complements a plasma membrane iron transport-deficient yeast mutant.

Figure S3 Correlation between *HMW-TaVIT2* transgene copy number and expression of *TaVIT2.*

Figure S4 Ferritin formation in Caco-2 cells incubated with phytase-treated flour digestates.

Figure S5 Alignment of yeast codon-optimized *TaVIT1* DNA sequence with original sequence.

Table S1 Wheat *VIT* genes identified in this study.

Table S2 Expression analysis of *TaVIT* genes.

Table S3 Element analysis of control and *HMW-TaVIT2* wheat lines.

Table S4 Heavy metals in control and *HMW-TaVIT2* wheat lines.

Table S5 Architectural and yield components of control and *HMW-TaVIT2* T_0 transformants.

Table S6 Architectural and yield components of T_1 plants segregating from a *TaVIT2* over-expressor T_0 plant.

Table S7 List of primers.

Figure legends

Figure 1 The wheat genome encodes two *VIT* paralogs with different expression patterns. (a) Phylogenetic tree of *VIT* genes from selected plant species: *At, Arabidopsis thaliana*; *Gm, Glycine max* (soybean); *Hv, Hordeum vulgare* (barley); *Os, Oryza sativa* (rice); *Sl, Solanum lycopersicum* (potato); *Ta, Triticum aestivum* (wheat); *Vv, Vitis vinifera* (grape); *Zm, Zea mays* (maize). Numbers above or below branches represent bootstrapping values for 100 replications. (b) Gene expression profiles of *TaVIT1* and *TaVIT2* homoeologs using RNA-seq data from expVIP. Bars indicate mean transcripts per million (TPM) ± SEM, full details and metadata in Table S2.

Figure 2 TaVIT2 facilitates iron and manganese transport. (a,b,c) Yeast complementation assays of *TaVIT1* and *TaVIT2* in ⊗*ccc1* (a), ⊗*pmr1* (b) and ⊗*zrc1* (c) compared to yeast that is wild type (WT) for these three genes. The yeast (Sc) *CCC1*, *PMR1* and *ZRC1* genes were used as positive controls. Cells were spotted in a 4-fold dilution series and grown for 2-3 days on plates ± 7.5 mM $FeSO_4$ (⊗*ccc1*), 2 mM $MnCl_2$ (⊗*pmr1*) or 5 mM $ZnSO_4$ (⊗*zrc1*). (d) Immunoblots of total and vacuolar protein fractions from yeast cells expressing haemagglutinin (HA)-tagged *TaVIT1* or *TaVIT2*. The HA-tag did not inhibit the function of TaVIT2 as it was able to complement ⊗*ccc1* yeast (data not shown). Vhp1 was used as a vacuolar marker and the absence of actin shows the purity of the vacuolar fraction.

Figure 3 Expression of *TaVIT2* in cisgenic lines. (a) Diagram of the transfer-DNA construct: LB, left border; 35S, CaMV 35S promoter; *HYG,* hygromycin resistance gene; *nosT*, *nos* terminator; *HMW-GLU* prom, high molecular weight glutenin-D1-1 promoter; *TaVIT2*, wheat *VIT2-D* gene; RB, right border. (b) Relative expression levels of *TaVIT2* in developing grains at 10 days post anthesis as determined by quantitative real-time PCR and normalized to housekeeping gene *Traes_4AL_8CEA69D2F.* Plant identification numbers and copy number of the *HMW-TaVIT2* gene are given below the bars. Bars indicate the mean ± SEM of 3 independent biological replicates.

Figure 4 Perls' Prussian Blue staining for iron in grains transformed with *HMW-TaVIT2*. Grains from T_0 wheat plants were dissected longitudinally (left) or transversely (right). em, embryo; s, scutellum; sdc, seed coat; es, endosperm; al, aleurone, gr, groove. The transgene copy number and line number are indicated on the far left. Scale bars = 1 mm.

Figure 5 Iron and phytate content of flour milled from *HMW-TaVIT2* wheat lines. (a) Iron concentrations in white and wholemeal flour from 3 control and 6 *HMW-TaVIT2* lines. Bars represent the mean of 2 technical replicates and the deviation of the mean. White flour from

HMW-TaVIT2 lines has significantly more iron than control lines (n = 3-4, p<0.001; see Table S3 for all data). The dotted line at 16.5 µg/g iron indicates the minimum requirement for wheat flour sold in the UK. (b) Phytate content of white and wholemeal flour of control and *HMW-TaVIT2* expressing wheat. Bars represent the mean of 2 biological replicates ± deviation of the mean. (c) Molar ratio of iron:phytate in control and *HMW-TaVIT2* expressing lines. Bars represent the mean of 2 biological replicates and the deviation of the mean.

Figure 6 Growth parameters of *HMW-TaVIT2* wheat.

(a) Number of tillers and (b) seed output of T_0 wheat plants with indicated *HMW-TaVIT2* copy numbers. Bars indicate mean ± SEM of the following numbers of biological replicates: zero gene copies, n = 9; 1 gene copy, n = 10; 2 -16 gene copies, n = 9; ≥ 20 gene copies, n = 6. Further details given in Table S5. Asterisk indicates significant difference from negative control (One-way ANOVA with Tukey post-hoc test, * $p<0.05$).

Figure 7 Endosperm-specific over-expression of *TaVIT2* in barley. The *TaVIT2-5DL* gene from wheat under the control of the wheat *HMW-GLU-1D-1* promoter (see Figure 3a for full details) was transformed into barley (*Hordeum vulgare* var. Golden Promise). Positive transformants were selected by hygromycin. (a) Mature barley T_1 grains of a control plant and two transgenic lines stained with Perls' Prussian blue staining for iron. (b) Element analysis in white and wholemeal flours from a control and two *HMW-TaVIT2* over-expressing barley plants. The values are the mean of 2 technical replicates.