Methods

Subcellular dynamics studies of iron reveal how tissue-specific distribution patterns are established in developing wheat grains

Sadia Sheraz1, Yongfang Wan2, Eudri Venter3, Shailender K. Verma4, Qing Xiong4, Joshua Waites4,5, James M. Connorton4,5, Peter R. Shewry2, Katie L. Moore1 and Janneke Balk4,5

1School of Materials and Photon Science Institute, University of Manchester, Manchester, M13 9PL, UK; 2Department of Plant Sciences, Rothamsted Research, Harpenden, AL5 2JQ, UK; 3Bioimaging facility, Department of Computational and Analytical Sciences, Rothamsted Research, Harpenden, AL5 2JQ, UK; 4Department of Biological Chemistry, John Innes Centre, Norwich, NR4 7UH, UK; 5School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK

Summary

- Understanding the mechanisms of iron trafficking in plants is key to enhancing the nutritional quality of crops. Because it is difficult to image iron in transit, we currently have an incomplete picture of the route(s) of iron translocation in developing seeds and how the tissue-specific distribution is established.
- We have used a novel approach, combining iron-57 (57Fe) isotope labelling and nanoscale secondary ion mass spectrometry (NanoSIMS), to visualize iron translocation between tissues and within cells in immature wheat grain, Triticum aestivum.
- This enabled us to track the main route of iron transport from maternal tissues to the embryo through the different cell types. Further evidence for this route was provided by genetically diverting iron into storage vacuoles, with confirmation provided by histological staining and transmission electron microscopy energy dispersive X-ray spectroscopy (TEM-EDS). Almost all iron in both control and transgenic grains was found in intracellular bodies, indicating symplastic rather than apoplastic transport. Furthermore, a new type of iron body, highly enriched in 57Fe, was observed in aleurone cells and may represent iron being delivered to phytate globoids.
- Correlation of the 57Fe enrichment profiles obtained by NanoSIMS with tissue-specific gene expression provides an updated model of iron homeostasis in cereal grains with relevance for future biofortification strategies.

Introduction

As widely consumed staple crops, cereals are important sources of mineral micronutrients, including iron and zinc which are essential for human health. However, deficiencies in these minerals affect large parts of the global population (WHO, 2013, 2015). The daily requirements for bioavailable iron and zinc are often not met in cereal-based diets for two reasons. First, iron and zinc are unevenly distributed in the grain, accumulating at high concentrations in the embryo (germ) and outer layers (bran), which are removed during polishing or milling. By contrast, both minerals are low in the starchy endosperm which constitutes about 70% of the grain and is preferentially consumed in human diets (as white wheat flour and polished rice, for example). Second, most of the iron and zinc in the embryo and aleurone layer is bound to phytic acid, forming insoluble complexes that have poor bioavailability. Attempts to biofortify cereal grains are focussing on increasing the total amounts of iron and zinc, changing their distribution and reducing phytic acid levels (Vasconcelos et al., 2017; Cominelli et al., 2020).

The molecular pathways of iron and zinc uptake from the soil into plant roots are relatively well understood, but we know little about mineral loading into the seeds and subsequent distribution to different tissues (Mari et al., 2020). Recent isotope labelling studies in Arabidopsis suggested that virtually all the iron in seeds was remobilized from senescing leaves (and other organs) with only an indirect contribution from uptake by the roots (Pottier et al., 2019). Studies using isotope pulse labelling have not yet been conducted in wheat, but a time course of mineral partitioning indicated that 77% of the iron in mature grain is remobilized from the shoot (Garnett & Graham, 2005), and that this process is regulated by NAC transcription factors (Uauy et al., 2006; Waters et al., 2009; Borrill et al., 2019).

Physical separation between the tissues of the mother plant and the seed (the filial generation) means that iron is secreted and then taken up again by the developing zygote (Mari et al., 2020). Nutrients are delivered to the developing grain via the vascular bundle which runs through the ventral crease in wheat, and the nucellar projection. The latter is all that remains from the nucellus which
once surrounded the embryo sac, and comprises a dense group of transfer cells along the top of the vascular bundle (see Bechtel et al. (2009) for a description of wheat grain development). In the early stages of grain development, nutrients are secreted from the maternal transfer cells into a cavity that will fill up as the endosperm of the developing seed expands. The rapidly dividing endosperm cell mass differentiates into specialized cell types (Olsen, 2020), most notably a single outer layer of aleurone cells which differ from the other endosperm cells in lacking starch and accumulating protein, lipids, minerals and phytic acid. Periclinal divisions of the aleurone cells continue to form several layers of subaleurone cells which accumulate protein but not minerals. The aleurone cells that are in contact with the nucellar transfer cells in the tip of the crease differentiate to have a special function in nutrient transport. For this reason they are often called endosperm transfer cells, but to avoid confusion with the nucellar transfer cells in the tip of the crease we will use the term modified aleurone here, which is also commonly used in the literature (Evers, 1970; Borg et al., 2009).

The distribution of iron in biological materials has been visualized using histological staining, X-ray fluorescence (XRF), micro-proton induced X-ray emission (µ-Pixe) and laser-ablation indcursively coupled plasma mass spectrometry (LA-ICP-MS). When applied to cereal grains, these techniques showed accumulation of iron in the crease, the aleurone layer and the scutellum of the embryo in wheat (Neal et al., 2013; Singh et al., 2013; De Brier et al., 2016) and a similar pattern in other cereals (Takahashi et al., 2009; Iwai et al., 2012; Detterbeck et al., 2020). Nanoscale secondary ion mass spectrometry (NanoSIMS) has also become a significant tool to visualize minerals at the subcellular level due to its unique capabilities of high spatial resolution (50 nm), high sensitivity (ppm and ppb for some elements) and detection of trace elements and isotopes (for example see Malherbe et al., 2016; Kopittke et al., 2020). During NanoSIMS analysis, the sample surface is impacted with a high-energy primary ion beam which causes sputtering of the surface and ejection of atoms and small molecules. Some of this sputtered material becomes ionized, referred to as ‘secondary ions’, which are detected and analysed by mass in a double focussing mass spectrometer. The instrument has two primary ion sources to generate either a caesium ion (Cs+) beam, used for analysis of negative secondary ions, or an oxygen ion (O2−) beam to analyse positive secondary ions. Due to the design of the NanoSIMS, the secondary ions must have an opposite polarity to the primary ions. Up to seven secondary ions can be detected simultaneously but these must be selected before acquiring the data (Hoppe et al., 2013). As the NanoSIMS operates under ultra-high vacuum, careful sample preparation is required to avoid redistribution of elements from their in vivo location (Grovenor et al., 2006).

Very few genes have been characterized to date that influence the distribution pattern of iron in seeds. One of those is the vacuolar iron transporter (VIT). Distruption of this gene in Arabidopsis resulted in relocation of iron from provascular strands to the abaxial (lower) epidermis in the embryo which occupies most of the seed volume (Kim et al., 2006). In rice, mutation of either the VIT1 or VIT2 paralogue leads to iron accumulation in the embryo and depletion in the large endosperm of the grain (Zhang et al., 2012; Bashir et al., 2013; Che et al., 2021). Conversely, overexpression of TaVIT2 in the starchy endosperm of wheat grain leads to iron accumulation in this tissue and > two-fold more iron in white flour (Connorton et al., 2017).

Many questions remain regarding the mechanisms that determine how iron is translocated from the maternal vascular bundle into the developing seed and how this element is distributed within the seed as tissues differentiate and expand. To address these questions, we combined iron isotope labelling with NanoSIMS to compare the dynamics of iron distribution in developing wheat grains between a control and a TaVIT2 overexpressing line. Our results revealed that the major route of iron is from the nucellar transfer cells through a zone of endosperm cells between the crease and the embryo, but that this pathway is disrupted by overexpression of TaVIT2. Different cell types displayed specific patterns of isotope-enriched vesicles and globoids, highlighting the different roles of each cell type in iron translocation.

Materials and Methods

Plant material and growth

Transformation of wheat lines (Triticum aestivum var. Fielder) with pBract202-TaVIT2 and their initial characterization have been described in the literature (Connorton et al., 2017; Hayta et al., 2019). As a control, we used the offspring of a plant regenerated after transformation that tested negative for the transgene. This line, 22-15, did not accumulate iron in the endosperm and white flour fraction (Supporting Information Table S1; Connorton et al., 2017). All analyses were carried out using the T3 generation, except for the germination tests for which T4 grain was used. Plants were grown in a glasshouse kept at approximately 20°C with 16 h of light. Plants were watered as required.

T-DNA copy number and TAIL-PCR

To estimate the numbers of T-DNA copies in individual plants, quantitative real time polymerase chain reaction (PCR) analysis was carried out similar to the approach taken by (Bartlett et al., 2008) using DNA from seedlings of the T4 generation. Thermal asymmetric interlaced (TAIL) PCR was performed essentially as described in Wu et al. (2015), except that recombinant Taq polymerase purified from Escherichia coli was used (Engelke et al., 1990). All primers used are listed in Table S2.

Element analysis by ICP-OES and ICP-MS

Preparation of flour fractions and element analysis were essentially carried out as previously described (Tosi et al., 2011; Connorton et al., 2017). See Supporting Information Methods S1 for details.

Isotope labelling and sample preparation for microscopy

Iron-57 in the form of 57Fe2O3 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added to a small volume of
concentrated hydrochloric acid and incubated at 37°C overnight until it was dissolved. The solution was diluted with water (H2O) to obtain 20 mM $^{57}$Fe in 1 M hydrochloric acid (HCl) and stored at 4°C. The $^{57}$Fe feeding solution (50 µM $^{57}$Fe, 0.5 mM sodium citrate and 10 mM MES-KOH pH 6.0) was freshly prepared by mixing 25 µl $^{57}$Fe-HCl stock solution, 50 µl 100 mM sodium citrate, 10 µl 1 M sodium hydroxide and 1 ml 100 mM MES buffer, made up to a final volume of 10 ml with H2O. After adjusting the pH to 6.0, 1 ml was filtered (porosity size 0.22 µm) and pipetted into a 1.5-ml Eppendorf tube attached to the ear with tape. A 10 µl glass microcapillary tube (Drummond, Sigma Aldrich, St Louis, MO, USA) was placed in the tube and the other end was inserted into the rachis (see Fig. 3a and Supporting Information Methods S1). The solution was taken up naturally by the wheat ear over the course of 12 h. The grains (40–50) from one ear were harvested after 24, 72 or 240 h and dissected into four parts: embryo, and three equal remaining sections (S1, S2 and S3), and then were freeze-dried for ICP-MS. Four grains from the middle of the ear were harvested for high pressure freeze-etching, sectioning and microscopy.

Transverse slices of the grain (0.1 mm thick, see Fig. 3 for positions) were infiltrated with 0.5 M MES-KOH pH 5.4, and frozen using a high-pressure freezer (HPM 100; Leica Microsystems, Milton Keynes, UK). Freeze substitution, embedding in LR White resin (R1281; Agar Scientific, Stansted, UK) and sectioning was as described in Moore et al. (2012, 2016).

Iron staining using the Perls’ method
Iron staining of tissue was performed as described in the literature (Meguro et al., 2007; Roschzttardtz et al., 2009) using the Perls’ method, enhanced with 3,3'-diaminobenzidine where indicated.

Transmission electron microscopy energy dispersive X-ray spectroscopy (TEM-EDS) analysis
The embedded wheat grain samples used for NanoSIMS were sectioned with a Leica UC7 ultramicrotome (Leica Microsystems, Vienna, Austria), and 100-nm ultrathin sections were mounted on 200-mesh copper TEM grids (Agar Scientific). Freeze substitution, embedding in LR White resin (R1281; Agar Scientific, Stansted, UK) and sectioning was as described in Moore et al. (2012, 2016).

Iron homeostasis genes in wheat were taken from (Borrill et al., 2021). Gene expression was measured with bulk standards and particular care was taken to minimize the mass interference of $^{56}$Fe:$^{57}$H with $^{57}$Fe (Supporting Information Fig. S7). Before analysis of regions of interest, a depth profile was acquired from a bulk iron standard to check the measured $^{57}$Fe:$^{56}$Fe isotope ratio and compare it to the natural isotope ratio of 2.3%. The detector for $^{57}$Fe was set slightly to the left of the centre of the peak to avoid the mass interference of $^{56}$Fe:$^{57}$H. This resulted in a slightly lower measured isotope ratio than natural, with an average of 2.24% across all measurements (Fig. S7c). All regions of interest and subcellular features were however enriched by significantly more than the 0.06% difference in the ratio.

Regions of interest were selected using the charge-coupled device (CCD) camera on the NanoSIMS. The samples were coated with 40 nm of platinum (Pt) before loading into the instrument to minimize sample charging. The Pt was removed by repeatedly scanning a defocussed O^- beam (D1 = 0) over an area of 70 µm × 70 µm with a total dose of 2.55 to 3 × 10^{17} ions/cm^2. Following implantation, ion images were acquired using a focussed beam over an area of 50 µm × 50 µm with 256 × 256 pixels and dwell time of 2000 µs per pixel. Several hundred images of each region of interest were acquired and summed together to improve the statistics.

Data processing was conducted with FIJI software using the OpenMIMS plugin (Harvard, Cambridge, MA, USA). Image processing included drift correction, summing of images, selecting regions of interest (ROIs) and extracting counts from them and generating colour merge and hue saturation intensity (HSI) ratio images to show isotopic variation of $^{57}$Fe:$^{56}$Fe.

Bioinformatics
Iron homeostasis genes in wheat were taken from (Borrill et al., 2014) and supplemented with literature searches to a total of 232 genes including homologues. Publicly available RNA-sequencing (RNA-Seq) datasets of modified aleurone, starchy endosperm and aleurone layer enriched samples (Pfeifer et al., 2014) were mapped to the IWGSC ReFseq v1.1 gene models using KALLISTO v.0.43.1 (Bray et al., 2016). Gene expression was analysed using the R package Sleuth v.0.30.0 with default settings. This identified 213 transcripts from 67 wheat iron homeostasis genes that were expressed in at least one tissue. Transcript per million (TPM) means ± SD were calculated from four replicates in the RNA-Seq data set. To filter for real expression over noise, a cut-off of 1 TPM in at least one tissue was imposed, and a q-value of significance < 0.05 as calculated using the likelihood ratio test.
Statistical analysis

Statistical analysis was carried out using the software packages GraphPad or Excel.

Results

Genetic characterization of HMW-TaVIT2 line 22-19 for further study

Previously we generated 25 independent Agrobacterium-transformed wheat lines with one or more copies of the HMW-TaVIT2 transgene, placing TaVIT2 under the control of the starchy endosperm-specific promoter of the gene High Molecular Weight subunit of glutenin 1Dx5 (Connorton et al., 2017). Transcript levels of TaVIT2 in developing grain correlated well with the number of transgene copies (factor 0.2, \( R^2 = 0.597, P<0.01 \)). By contrast, the iron concentration in hand-milled flour was variable between lines with similar transgene copy numbers. To select one line for detailed study, we chose line 22-19 based on a representative pattern of iron staining in hand-dissected grain and a consistent two-fold increase in the iron concentration in the white flour fraction. T-DNA copy number analysis of the T1 generation indicated that line 22-19 had more than one insert of tandemly arranged T-DNAs. Further selection gave a stable pattern of 8 to 10 T-DNA copies in the T2, suggesting the line was homozygous for a tandem array of four copies and possibly heterozygous for another single copy insertion (Table S1).

To determine the position of the T-DNA insertion(s), TAIL-PCR was performed with nested primer sets for either the left border (LB) or right border (RB) T-DNA sequence, in combination with an arbitrary degenerate (AD) primer. A 1.1-kb PCR product generated with the LB primer set and the AD3 primer was found consistently in transgenic plants but not in the non-transgenic control. Part of the sequence of the PCR product matched the LB and the other part matched the 5’UTR of *TraesCS4D02G046700* (Fig. S1a,b). No specific PCR products were generated with the RB primer set with any of the four AD primers. The position of the T-DNA was verified with primers spanning the insertion site and the LB3 primer (Fig. S1c). Analysis of DNA from 12 T1 siblings showed that the selected line (22-19-4) was homozygous for this T-DNA insertion, in agreement with the segregation pattern of T-DNA copies in the T2 generation.

*TraesCS4D02G046700* encodes a Ubc enzyme variant (Uev), belonging to a small gene family conserved in all eukaryotes. The closest rice homologue is OsUEVIA (Os03g0712300) which has 85% amino acid identity (Wang et al., 2017). Transcript levels of the wheat UEV1A-4D gene are slightly increased in developing grain, but this was also the case for the 4A and 4B homeologues (Fig. S1d). Moreover, the wheat UEV1A-4A and -4D homeologues are identical in amino acid sequence and have highly similar expression patterns (expVIP database, wheat-expression.com). Thus, the T-DNA insertion in line 22-19 does not appear to disrupt the expression of an essential gene.

In TaVIT2 grain, the amount of iron is increased in a specific region of the endosperm and decreased in the embryo and aleurome cells

To further investigate differences in iron distribution as a consequence of overexpressing TaVIT2 in the endosperm, T3 grains from line 22-19-4-5 (henceforth called TaVIT2) and from control plants were cut longitudinally and at two transverse positions before staining with Perls’ reagent (Fig. 1). Similar to other TaVIT2 transformation events (Connorton et al., 2017), dense iron staining was found in the starchy endosperm surrounding the crease. Iron staining in this part of the endosperm was more intense in the proximal region close to the embryo than in the distal region (Fig. 1a, compare transverse section 1 and 2), in a zone of cells equivalent to the endosperm adjacent to scutellum (EAS) in maize kernels (Doll et al., 2020). By contrast, both the embryo and aleurome layer in TaVIT2 grain displayed a lower intensity of iron staining than in control grain. The difference in iron staining of the aleurome was also observed in higher magnification images using diamine benzidine (DAB)-enhanced Perls’ staining (Fig. 1b).

To quantify the decrease in iron content in the embryos, developing grains at 24 d post anthesis (dpa) were hand-dissected into two parts, embryo and the remaining tissues (rest), and the concentration of iron was measured by ICP-MS. Iron was significantly decreased, by 28.5%, in embryos of TaVIT2 grains compared to control embryos, with a concentration of 173 ± 20 mg kg\(^{-1}\) dry weight compared to 242 ± 9 mg kg\(^{-1}\) in control (\(P>0.03\), see Fig. 1c). However, germination tests of TaVIT2 grain showed no effect on germination or on early seedling growth in alkaline soil (Fig. S2).

The iron contents of the aleurome layer and endosperm of mature grains were estimated by analysis of pearling fractions. This technique uses abrasion to remove material from the outer layers to the inner core of the grain. Sequential cycles of pearling result in fractions enriched in embryo and pericarp (F1), aleurome (F2) and subaleurone (F3). The iron concentration in the F1 fraction of TaVIT2 grain was decreased by 26% compared to control grain, in agreement with the percentage decrease in dissected embryos. It should be noted that the absolute iron concentration in the F1 pearling fraction is much lower than in dissected embryos, because of dilution by dry matter from the bran and the embryo at maturity. The F2 fraction contained 36.6% less iron in TaVIT2 grain, 49.8 ± 3.2 mg kg\(^{-1}\) dry weight compared to 77.7 ± 0.4 mg kg\(^{-1}\) in control grain (Fig. 1d). The inner starchy endosperm (core) contained approximately two-fold more iron in TaVIT2 grain, whereas the iron concentration in the whole grain is similar in TaVIT2 and control grain, as noted previously (Connorton et al., 2017; Balk et al., 2019). The distribution of other metals such as manganese and zinc were little affected by TaVIT2 overexpression, except for small increases in the F1 fraction (Fig. S3). The distribution of phosphorus in TaVIT2 grain was not affected, resulting in a lower phosphorus : iron ratio in the central endosperm (Fig. S3). Thus, overexpression of TaVIT2 in the endosperm does not affect the amount of iron mobilized from the maternal plant into the grain,
but does affect the distribution of iron within the grain: iron accumulates in a specific region of the endosperm at the expense of iron translocation to the embryo and aleurone.

**TaVIT2 endosperm cells accumulate iron in clusters of vesicles and globoids**

Based on its function as a vacuolar iron transporter, overexpression of *TaVIT2* is expected to lead to iron accumulation in vacuoles. To obtain information on the subcellular location of iron in TaVIT2 grain, semi-thin transverse grain sections were stained with Perls’-DAB (Fig. 2). Dense granules of dark staining were observed in the endosperm of TaVIT2 grain, but not in control grain (Fig. 2a,b). The region where intense iron staining was found corresponded with the blue Perls’ staining in hand-cut sections of TaVIT2 grain (Fig. 1a). Higher magnification images showed that the iron staining was confined to clusters of small round bodies of approximately 0.5–2 μm in diameter in the cytoplasm of endosperm cells (Figs 2c, S4). There appeared to be no association with other cell organelles, such as starch grains or the nucleus.

TEM identified several distinct electron-dense morphological structures in the endosperm region of interest in TaVIT2 grain, which were absent from control grain. The most abundant morphologies were particles forming the outline of vesicles 0.2–0.8 μm in diameter but lacking any trace of a membrane (Fig. 2d,f); and clusters on the outside of membrane-bound vesicles 0.5–2.3 μm in diameter (Figs 2e, S5). Dispersed particles and aggregates of smaller particles were also observed but were less abundant (Fig. S5a). Elemental analysis by EDS indicated that all four electron-dense morphologies in TaVIT2 grain contained iron, whereas iron was not detectable outside these areas (Fig. S5). The two most abundant types of iron-rich morphologies seen in TEM were also distinguishable by NanoSIMS (Fig. 2e,f). The NanoSIMS images were aligned with Perls’-DAB staining applied to adjacent sections. While the two morphologies looked identical with Perls’-DAB staining, this technique revealed that the smaller type are surrounded by an intracellular membrane (Fig. 2f, white arrow), indicating they are iron globoids inside a larger vacuole.

Accumulation of iron in a specific region of the starchy endosperm but not throughout, raises the question whether this coincides with a local abundance of iron or is due to localized expression of the *TaVIT2* transgene. The *TaVIT2* transgene is expressed using the *HMW Glu-1Dx5* promoter, which is active in the entire starchy endosperm during grain filling as shown by promoter-GUS studies (Lamacchia et al., 2001). To verify that the expression pattern of *HMW-TaVIT2* is similar, we carried
The NanoSIMS and Perls’-DAB images are from adjacent sections. In (e), nanoSIMS images were acquired using the caesium ion (Cs⁺) beam to detect FeO⁻ (Moore et al., 2012), but the mass of ⁵⁷Fe⁺⁻O⁻ is nearly indistinguishable from the ⁵⁶Fe⁺⁻O⁻H⁻ ion. However, the positive ions ⁵⁷Fe⁺⁺ (m/z = 56.9354) and ⁵⁶Fe⁺⁺H⁺ (m/z = 56.9428), generated with the oxygen ion (O⁺⁻) beam, are sufficiently separated to allow reliable mapping of the ⁵⁷Fe⁺⁺ signal (Fig. 2).

To directly target the iron isotope to the developing grains, a 1 ml solution of 50 µM ⁵⁷Fe³⁺ and 0.5 mM citrate was fed into the base of the rachis (stem of the flowering spike) using a glass microcapillary tube (Fig. 3a). Feeding was performed on wheat ears around 18 dpa, to coincide with the mid-stage of grain filling and iron mobilization (Waters et al., 2009; Beasley et al., 2019), and high activity of the HMW promoter driving expression of TaVIT2 (Lamacchia et al., 2001). Following a 12 h isotope feeding pulse, the optimal chase time was determined experimentally, by ICP-MS measurement of ⁵⁷Fe in the embryo and rest of the grain at 24, 72 and 240 h (10 d), counting from the start of feeding. The relative abundance of ⁵⁷Fe increased from background levels (2.12%) to c. 5% after 24 h and to c. 8% after 72 h (Fig. 3b). Sampling the grain 10 d after isotope labelling showed no further increase in ⁵⁷Fe enrichment, either because the grain filling period had come to an end or because nonlabelled iron from the senescing leaves was mobilized at a sufficient rate to dilute the remaining isotope. Capillary insertion had no significant effect on grain development (Moore et al., 2016) and the total iron concentration in ‘fed’ and ‘nonfed’ samples was similar (data not shown).

The highest concentration of ⁵⁷Fe accumulating over 72 h was in the embryos (Fig. 3c), indicating that most of the iron taken up at this stage is partitioned there. Interestingly, the increase in ⁵⁷Fe enrichment was similar in proximal and distal parts of the grain, suggesting a constant rate of translocation to all grain tissues (Fig. 3c, inset). Overexpression of TaVIT2 resulted in a

out in situ hybridizations of 21 dpa grain. Hybridization with an antisense TaVIT2 probe showed intense positive staining in all parts of the endosperm of TaVIT2 grain, especially in the subaleurone cells, matching the expected pattern of HMW Glu-1Dx5 promoter activity. In control grain, positive staining but of weaker intensity was seen in the aleurone cell layer, where endogenous TaVIT2 is expressed. There was no signal in any tissue with the sense probe which served as negative control (Fig. 5a).

In summary, accumulation of iron-dense vesicles, representing iron trapped by overexpression of TaVIT2, indicates that the starchy endosperm region between the maternal transfer tissue and the embryo is a major transport route of iron in developing wheat grain.

The rate of iron transport to the embryo is decreased in TaVIT2 grain

To study how iron is translocated into the developing grain at (sub)cellular resolution, we designed an experimental protocol for iron isotope labelling and subsequent NanoSIMS analysis (Fig. 3a). Iron-57 (⁵⁷Fe) was chosen as a stable isotope over iron-54 and iron-58 because its mass differs sufficiently from other abundant elements in biological material and it has a relatively low natural abundance of 2.12%. By contrast, iron-54 has a natural abundance of 5.8% which would make it harder to detect enrichment after pulse-labelling. Although iron-58 has a very low natural abundance (0.28%) it is difficult to separate from nickel-58 (68.1% natural abundance) by mass. To detect iron by NanoSIMS, previously we used the caesium ion (Cs⁺) beam to detect FeO⁻ (Moore et al., 2012), but the mass of ⁵⁷Fe⁻⁻O⁻ is nearly indistinguishable from the ⁵⁶Fe⁻⁻O⁻H⁻ ion. However, the positive ions ⁵⁷Fe⁺⁺ (m/z = 56.9354) and ⁵⁶Fe⁺⁺H⁺ (m/z = 56.9428), generated with the oxygen ion (O⁺⁻) beam, are sufficiently separated to allow reliable mapping of the ⁵⁷Fe⁺⁺ signal (Fig. 2).
Iron is translocated in dynamic intracellular vesicles

Next, we exploited the combined methods of $^{57}$Fe pulse labelling and NanoSIMS to investigate iron dynamics within cells. Previous NanoSIMS analysis of $^{56}$Fe$^{16}$O$^-$ in durum wheat grains at 16 dpa showed that iron was concentrated in phosphate-containing globoids in the aleurone, whereas it was uniformly distributed in a starchy endosperm cell located 100 µm from the aleurone (Moore et al., 2012). Detection of $^{56}$Fe$^{16}$O$^-$ ions with the $^-$ beam showed a similar pattern of iron-containing globoids in the aleurone cells of bread wheat at 21 dpa (Figs 5a, S8). Interestingly, $^{57}$Fe labelling revealed two populations of iron-dense structures, a population with 20–30% $^{57}$Fe enrichment 0.1–1 µm in diameter and a population with c. 5% enrichment which were generally larger in diameter (0.5–2.5 µm) (Figs 5b, S8). The first population had virtually no associated PO signal, whereas the second population had a strong PO signal likely derived from phytic acid. In several instances, the $^{57}$Fe-rich structures are seen abutting larger globoids and were possibly merging (Fig. 5a, white arrow heads in aleurone image). Overexpression of TaVIT2 in the endosperm led to a dramatic decrease in $^{57}$Fe entering the aleurone between 20 and 22 dpa.

Transfer cells of the nucellar projection connect the vascular bundle from the mother plant to the grain, and thus play an important role in nutrient transport. The pattern of iron-rich bodies was comparable in transfer cells of control and TaVIT2 grain, showing a combination of disperse iron-rich vesicles and large clusters filling up most of the small cell volume (Figs 6, S9). The percentage $^{57}$Fe enrichment was slightly, but non-significantly, decreased in transfer cells of TaVIT2 grain compared to control (Figs 4, 6), as expected in this maternal tissue through which iron passes before it reaches the grain.
In the modified aleurone cells adjacent to the transfer cells, the pattern of iron-rich bodies was different from that in aleurone cells around the periphery of the grain (Figs 5a, 6). Modified aleurone cells in both control and TaVIT2 grain showed an abundance of $^{57}\text{Fe}$-enriched vesicles, ranging from 6 to 20% enrichment in the control and 6–15% in TaVIT2. These patterns correlate with the specific function of modified aleurone cells, namely nutrient transfer rather than nutrient storage. Endosperm-specific expression of TaVIT2 did have a negative influence on the iron dynamics in modified aleurone cells, intermediate to the suppression of iron translocation into the maternal transfer cells and EAS.

Starchy endosperm cells between the crease and embryo (EAS) contained only few iron-enriched vesicles 5–7 µm in diameter, whereas cells in the ‘cheeks’ of the developing grain showed a diffuse pattern of iron (Figs 5a, 6). In TaVIT2 grain, large clusters of $^{56}\text{Fe}$ vesicles and globoids were found but with little or no enrichment in $^{57}\text{Fe}$. It is likely that the cells are saturated with iron before our time point of investigation and that iron transport into the cell is decreased. However, at the periphery of the EAS we observed a cluster of vesicles with smaller, $^{57}\text{Fe}$-rich vesicles on the outside and larger, $^{56}\text{Fe}$ + PO containing vesicles towards the centre of the cluster (Fig. 7). Again, instances of abutting vesicles suggest that fusion is taking place, with ‘newer’ $^{57}\text{Fe}$ being delivered to ‘older’ $^{56}\text{Fe}$ stored with PO.

The scutellum contained a large number of iron-rich, membrane-bound vesicles (Fig. 6), in agreement with iron accumulation seen by XRF (Neal et al., 2013; Singh et al., 2013; De Brier et al., 2016). Cells in the outer cell layers of the scutellum, adjacent to endosperm cells that are undergoing programmed cell death, tended to have a higher enrichment with $^{57}\text{Fe}$. The plasmalemma of the embryo also showed a high density of iron-rich vesicles. In the TaVIT2 overexpressing grain, the density of the iron vesicles was similar to control grain (Fig. 6), but $^{57}\text{Fe}$ enrichment was dramatically decreased (Fig. 4), in agreement with the lower total iron content of TaVIT2 embryos (Fig. 3c).

In summary, the presence of iron-rich organelles in most cell types (except the starchy endosperm) and their heterogeneity in $^{57}\text{Fe}$ enrichment suggest that vesicle-mediated transport is a major route of cell-to-cell iron translocation.

**Discussion**

To gain insight into iron translocation and distribution into developing cereal grains, we developed a protocol for $^{57}\text{Fe}$ labelling combined with NanoSIMS. This revealed how different cell types contribute to iron translocation with remarkable differences in their cell biology. A summary of the findings is presented in Fig. 8. The main flux of iron is from the maternal transfer cells to the embryo through a specific zone of the starchy endosperm, as shown by $^{57}\text{Fe}$ enrichment in embryos of control grain (Fig. 3c) and entrapment of this iron flux when TaVIT2 is over-expressed under the control of an endosperm-specific promoter (Figs 1–4). A role in nutrient transport for this part of the endosperm, previously termed EAS, has been suggested based on transcriptomics analysis in maize, which showed a marked upregulation of transporters for sugars, amino acids and some metal transporters in this tissue (Doll et al., 2020). The amount of iron travelling to the aleurone layer at the periphery of the endosperm was also strongly affected by TaVIT2 overexpression (Figs 4, 5), although no iron accumulation was observed in tissues adjacent to the aleurone, and the route of this pool of iron therefore remains unclear.

NanoSIMS analysis of $^{57}\text{Fe}$ provided subcellular detail of how iron is trafficked through the cell by vesicles and vacuolar globoids. Subcellular imaging of $^{57}\text{Fe}$ was facilitated by the recent addition of the RF O-source to the NanoSIMS allowing measurement of positively charged iron ions with discrimination of
The observed cell-type specific patterns of iron uptake and storage are likely to be underpinned by differential gene regulation. Vesicle-mediated transport of iron has been reported in erythrocytes, where a large flux of iron is delivered to the mitochondria for haem biosynthesis (Hamdi et al., 2016). Our study suggests that vesicle transport could also be involved. Vescicle-mediated transport of iron may be involved in the iron uptake and storage in aleurone cells. The observed cell-type specific patterns of iron uptake and storage are likely to be underpinned by differential gene regulation.
Fig. 6 Nanoscale secondary ion mass spectrometry (NanoSIMS) analysis of iron-57 ($^{57}$Fe) enrichment along the vertical axis in wheat grain. Light microscopy (LM) images of a transect through a control and a TaVIT2 grain with 50 µm × 50 µm images acquired by NanoSIMS showing the $^{57}$Fe : $^{56}$Fe ratio. The position of the transect is indicated in the cartoon image, top left. The scan areas appear as dark patches in the LM image. The $^{57}$Fe enrichment is represented by a false colour scale from 2.3 to 15%.
expression. An RNA-Seq study by Pfeifer et al. (2014) investigated gene expression in tissue samples enriched in modified aleurone cells, starchy endosperm and aleurone cells of wheat grains at 20 dpa. We reanalysed this data set to extract the expression levels of wheat genes likely to be involved in iron homeostasis, based on sequence homology to genes in rice and Arabidopsis (Fig. S10). Ferritin and VIT genes were noticeably upregulated in the aleurone tissue, but not in the modified aleurone or starchy endosperm, in agreement with our NanoSIMS data (Fig. 5). Two different NAS genes were upregulated in aleurone and starchy endosperm, TaNAS9 and TaNAS6, respectively. TaNAS9 is orthologous to OsNAS3 (Bonneau et al., 2016), but there is no direct orthologue of TaNAS6 in rice. Enzymes encoded by NAAT and DMAS work sequentially to convert NA to a 3″-oxo intermediate and then to deoxymugineic acid (Beasley et al., 2017). Transcripts of the TaNAAT2 triad are enriched in the modified aleurone but not in the other two tissues. This suggests that the modified aleurone converts NA to the 3″-oxo intermediate, which could be further converted to deoxymugineic acid by TaDMAS1 expressed in all three tissues. Wheat homologues of the mugineic acid exporter OsTOM2 are also expressed in all three tissues, but the homologue of the NA exporter OsENA2 is primarily expressed in aleurone cells.

Elevated aleurone expression of a wheat gene triad related to OsPEZ1, encoding a phenolics exporter active in the xylem (Ishimaru et al., 2011), suggests that enhancing the solubility of iron in cell walls may facilitate iron uptake as well. Uptake of iron-NA complexes is mediated by yellow stripe-like (YSL) transporters, of which seven different paralogues are expressed in the investigated wheat grain tissues. The iron-NA transporter ZmYSL2 was recently shown to be important for iron import into the embryo and aleurone cells in maize kernels (Zang et al., 2020), and this gene may correspond to TraesCS2D02G387800 in wheat (Fig. S10c). Transcripts of the wheat homologues of OsYSL6 and OsYSL9 are also enriched in

Fig. 7 Heterogenous composition of iron-rich vesicles. (a) Nanoscale secondary ion mass spectrometry (NanoSIMS) images of endosperm cells at the periphery of the endosperm adjacent to scutellum (EAS) in TaVIT2 grain, showing the distribution of sodium-23 (23Na), 31P16O and 56Fe (left) and the 57Fe : 56Fe ratio (middle). The same area in an adjacent 1 µm section stained with Perls’-diamine benzidine (DAB) is shown on the right. A cell wall runs through the middle of the image. s, starch grain. (b) Relationship between the 57Fe enrichment and 31P16O signal in iron-rich vesicles. The size of each data point corresponds to the area of the vesicle. (c) Relationship between the 56Fe and 31P16O signal from the iron-rich vesicles.
aleurone cells. Interestingly, a rice mutant line of *OsYSL9* had decreased amounts of iron in the embryo, but increased contents in the polished grain (mostly endosperm), indicating that *OsYSL9* plays a role in iron translocation from the EAS to the embryo (Senoura et al., 2017).

Dynamic iron studies, gene expression data and functional genetics will be invaluable for developing new biofortification strategies. Our isotope labelling study indicated that endosperm-specific expression of *TaVIT2* was successful as a biofortification strategy because iron is captured from the large flux going through the EAS and retained there, starting before 20 dpa (Figs 3, 4). Interestingly, previous studies overexpressing ferritin under the same promoter did not lead to accumulation of significant amounts of iron in the endosperm (Neal et al., 2013). A possible reason for this difference is that ferritin is a facultative iron store, which can release iron as easily as taking it up. In *TaVIT2* grain, iron accumulated in a relatively small part of the endosperm, and the challenge will be to direct more iron into the ‘cheeks’ of the grain. Moreover, increasing total grain iron, rather than redistributing it, would be necessary. It was recently shown that the latter can be achieved by overexpressing the rice *NAS2* gene under the constitutive *UBIQUITIN* promoter (Beasley et al., 2019). Because of different modes of action of the *OsNAS2* and *TaVIT2*, combining these two transgenes is likely to have an additive effect, increasing iron in wholemeal and white flour fractions simultaneously. Moreover, higher NA levels should also raise the concentration of zinc and lead to higher bioavailability of both micronutrients (Beasley et al., 2019). Other genes, such as those encoding grain-specific YSL and PEZ transporters, may also be interesting candidates for biofortification. Isotope labelling and imaging can make an important contribution in understanding cell-specific processes of iron homeostasis, helping to inform such biofortification strategies.

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**Author contributions**

SS, YW, EV, SKV, QX, JW and JMC performed experiments and analysed data. JMC, PRS, KLM and JB planned the research. KLM and JB wrote the manuscript.

**ORCID**

Janneke Balk https://orcid.org/0000-0003-4738-1990
James M. Connorton https://orcid.org/0000-0002-9379-5599
Katie L. Moore https://orcid.org/0000-0003-1615-7232
Peter R. Shewry https://orcid.org/0000-0001-6205-2517
Eudri Venter https://orcid.org/0000-0002-8102-2403
Shailender K. Verma https://orcid.org/0000-0002-4192-933X
Joshua Waites https://orcid.org/0000-0001-9899-4557
Yongfang Wan https://orcid.org/0000-0002-9374-5014

**Data availability**

The data that support the findings of this study are available in the Supporting Information of this article and, if not there, available from the corresponding author upon reasonable request.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Insertion site of the T-DNA in line 22-19.

**Fig. S2** Germination of TaVIT2 grain is similar to control grain.

**Fig. S3** Manganese, zinc and phosphorus in pearling fractions of wheat grain.

**Fig. S4** Additional images of enhanced Perls’ staining of TaVIT2 grain.

**Fig. S5** TEM and EDS analysis of TaVIT2 grain.

**Fig. S6** Expression pattern of the *HMW-TaVIT2* transgene.

**Fig. S7** NanoSIMS data from the iron standard.

**Fig. S8** NanoSIMS images and chemical iron staining of aleurone cells.

**Fig. S9** NanoSIMS images 50 μm × 50 μm of transfer cells.

**Fig. S10** Transcript abundance of iron homeostasis genes in selected wheat grain tissues.

**Fig. S11** NanoSIMS images of iron in cell walls.

**Methods S1** Additional information on Materials and Methods.

**Table S1** Copy number analysis and iron concentration in white flour of *HMW-TaVIT2* wheat lines.

**Table S2** Oligonucleotides used in this study.

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