RESEARCH PAPER

Megapixel imaging of (micro)nutrients in mature barley grains

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Abstract

Understanding the accumulation and distribution of essential nutrients in cereals is of primary importance for improving the nutritional quality of this staple food. While recent studies have improved the understanding of micronutrient loading into the barley grain, a detailed characterization of the distribution of micronutrients within the grain is still lacking. High-definition synchrotron X-ray fluorescence was used to investigate the distribution and association of essential elements in barley grain at the micro scale. Micronutrient distribution within the scutellum and the embryo was shown to be highly variable between elements in relation to various morphological features. In the rest of the grain, the distribution of some elements such as Cu and Zn was not limited to the aleurone layer but extended into the endosperm. This pattern of distribution was less marked in the case of Fe and, in particular, Mn. A significant difference in element distribution was also found between the ventral and dorsal part of the grains. The correlation between the elements was not consistent between and within tissues, indicating that the transport and storage of elements is highly regulated. The complexity of the spatial distribution and associations has important implications for improving the nutritional content of cereal crops such as barley.

Key words: Barley, iron, micronutrients, zinc.

Introduction

Cereal grains are a key source of metabolizable energy in almost every diet. As such they also represent one of the major pathways through which humans acquire mineral nutrients. However, the content of micronutrients, such as Fe and Zn, in the grains of cereal crops is often low. This results in micronutrient malnutrition affecting more than half of the world’s population, with staggering economic and social costs (FAO, 1997). For this reason the Copenhagen Consensus Conference (www.copenhagenconsensus.com) lists micronutrient deficiencies, especially for Fe and Zn, as a top priority issue. The importance of barley usage for human consumption worldwide is significantly smaller than that of wheat and rice. However, barley represents a traditional food source in some areas of the world and its use has been advocated in regional diets (e.g. Bere and Brug, 2009).

In the last decade an increased research effort has tried to address the issue of low micronutrient content of cereal grains by various means, ranging from fertilization to conventional breeding and biotechnologies (e.g. Cakmak, 2008). The ability of cereals to supply micronutrients depends on the concentrations of these elements in the grain, their bioavailability (which is related to their speciation), and their distribution within the grains (e.g. White and Broadley, 2009). This latter aspect is important as a considerable portion of cereal grains are processed, for instance milled, before consumption.
The distribution of micronutrients in cereal grains has been investigated by several authors. The simplest approach is based on the analysis of grain fractions obtained by successive cycles of abrasive milling. For instance, Liu et al. (1974, 2007) investigated the distribution of P and micronutrients in four low phytic acid barley isolines. The grain fractions were obtained by dehulling followed by three successive milling cycles. The information obtained is of substantial value in terms of human nutrition, but provides limited information regarding micronutrient distribution at the tissue level.

Information about micronutrient distribution can be achieved by in situ staining or spectroscopic techniques. Ozturk et al. (2006) visualized the distribution of Zn in wheat by staining, using diphenyl thiocarbazone (DTZ). The results showed that the red Zn–DTZ complex was mainly present in the aleurone and embryo regions. When providing useful information, it should be noted that staining methods only target specific metals and are based on chemical reactions between the histological dye and the metal of interest. These reactions are subject to competitive exchange equilibrium with endogenous ligands and are usually considered able to visualize only the labile metal ions (McRae et al., 2009).

Among the spectroscopic investigations, Mazzolini et al. (1985) used proton-induced X-ray emission (PIXE) to assess quantitatively the distribution of macro and micronutrients in wheat seeds. However, this study focused on the embryo region and utilized a rather large beam size for their analyses (10–12 μm). Ockenden et al. (2004) used scanning and transmission electron microscopy (SEM/TEM), in combination with energy dispersive X-ray microanalysis (EDX), to investigate the distribution of P and micronutrients in barley low phytic acid genotypes. This study focused on aleurone and scutellum cells, and provided subcellular information. However, SEM/TEM-EDX techniques are characterized by relatively high detection limits (e.g. Lombi et al., 2010) and did not provide detailed micronutrient distribution maps. Another detailed investigation of Se distribution in wheat and of As in rice was recently reported by Moore et al. (2010) who utilized nanosecondary ion mass spectrometry (nano-SIMS). In this case the lateral resolution (<100 nm) allowed the subcellular distribution to be visualized, but the analyses were limited to regions of only a few μm². Recently, laterally resolved synchrotron X-ray fluorescence spectroscopy (μ-XRF) has been used to assess the distribution of As and micronutrients in rice grains (Meharg et al., 2008; Lombi et al., 2009; Takahashi et al., 2009; Williams et al., 2009; Wirth et al., 2009). In contrast to the studies reported above, these μ-XRF investigations provided elemental maps for various elements in whole grain sections. However, even in these cases, the lateral resolution, dictated by the step size used, was 10–15 μm due to the acquisition time required (typically 0.5–1 s pixel⁻¹; Lombi and Susini, 2009).

In the present study, advantage was taken of a recently developed XRF detection system (Ryan et al., 2009) to obtain megapixel high-definition trace element images of cross- and longitudinal sections of barley grains. Elemental maps are presented with lateral definition improved ~100 times, compared with previous synchrotron studies. This information is required in order to better understand the processes of micronutrient storage in the grains. Also, this information is needed to identify areas of the grains that should be targeted for detailed molecular analyses such as those based on laser capture microdissection and microarray analysis recently reported for barley by Tauris et al. (2009) and Borg et al. (2009). Finally, detailed spatial distribution information could be used to optimize grain processing methodologies.

Materials and methods

Plant material and sample preparation

Grains of barley (Hordeum vulgare L. cv. Golden Promise) were harvested from greenhouse-grown plants cultivated in a mixture of soil and sand supplemented with 0.15 kg m⁻³ Osmocote plus (Scotts Company, UK). The grains were rapidly rinsed three times with milli-Q water to remove surface contaminants and thereafter freeze dried. A subsample of 50 g, i.e. >1000 grains, was pulverized in a titanium mill and used as whole grain control material. For determination of total elemental concentrations in different grain tissues, 35 randomly selected grains were divided into five batches and their hull and embryo removed manually by use of a teflon-coated scalpel. Thereafter, each batch of seven grains was polished by high-speed shaking at 30 Hz in a ball mill (MM301, Retsch, Germany) mounted with a rack containing microcentrifuge tubes loaded with 200 mg of acid-washed quartz sand (Fluka 84878, 40–150 mesh SiO₂). Before use, the sand had been additionally purified with 5% HNO₃ and dried. Polishing was performed in six repeated cycles each of 150 s duration. Between each cycle, the remaining weight of the seven grains was recorded and they were moved to new tubes. In parallel, the mixture of abraded material and sand was weighed. This procedure allowed collection of six fractions of abraded material consisting of different proportions of pericarp, testa, aleurone, and endosperm in mixture with sand. After the final cycle of polishing, the rest of the grain, representing the core endosperm, was rapidly washed three times with milli-Q water in order to remove surface dust. Following drying, all samples, together with control samples of pure sand and pulverized whole grain, were digested for 80 min at 140 °C in a closed microscaled microwave digestion system (Multiwave 3000, Anton Paar GmbH, Graz, Austria) equipped with a 64 position rotor (64MG5, Anton Paar GmbH, Graz, Austria). The digestion medium consisted of a mixture of 125 μl of 30% H₂O₂ and 250 μl of 65% HNO₃. All digests were diluted to 3.5% HNO₃ and analysed by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500ce, Agilent Technologies, UK). The results were validated using certified reference material NIST 8436 (durum wheat) and true blanks. Results for pure sand were subtracted from those for the abraded samples. Five independent replicates were analysed for each of the nine grain fractions (hull, embryo, pure endosperm, and six samples representing a gradient across the testa–aleurone-endosperm interface). The satisfactory micronutrient recoveries shown in Table 1 indicate that neither significant micronutrient contamination nor losses occurred during the process, in agreement with previous validations (Hansen et al., 2009).

Laterally resolved μ-XRF analysis (μ-XRF)

For μ-XRF, thin sections of the grains were prepared in order to overcome issues related to the penetration of the X-ray beam.
Table 1. Dry matter and elemental distribution (% of total content) in barley grains (mean ± SE, n=5)

| Element | Dry Matter | Mg  | K   | Mn  | Fe  | Cu  | Zn  |
|---------|------------|-----|-----|-----|-----|-----|-----|
| Hull    | 7.4±0.2    | 3.0±0.3 | 15±1.0 | 4.0±2.0 | 13±2.5 | 3±0.1 | 2±0.2 |
| Embryo  | 2.7±0.1    | 5.0±0.4 | 11±0.5 | 30±1.4 | 4.0±4.0 | 12±0.5 | 13±0.8 |
| TAE1    | 3.6±0.3    | 15±0.5 | 13±0.4 | 10±0.5 | 14±1.4 | 10±0.5 | 13±0.1 |
| TAE2    | 3.8±0.7    | 22±2.3 | 16±1.4 | 7±1.0  | 17±0.9 | 10±1.2 | 14±1.2 |
| TAE3    | 3.7±0.6    | 14±0.9 | 11±1.8 | 5±0.2  | 11±1.7 | 7±0.7  | 10±0.8 |
| TAE4    | 3.4±0.3    | 9±0.3  | 7±0.3  | 4±0.3  | 8±1.4  | 7±0.5  | 7±0.5  |
| TAE5    | 2.9±0.3    | 6±0.3  | 5±0.4  | 4±0.3  | 7±1.7  | 5±0.3  | 5±0.7  |
| TAE6    | 2.6±0.2    | 3±0.1  | 3±0.1  | 3±0.1  | 4±0.7  | 4±0.6  | 3±0.2  |
| Endosperm | 65.6±1.2  | 7±0.7  | 19±1.8 | 34±3.2 | 18±2.5 | 43±4.3 | 25±3.0 |
| Recovery| 96±0.4     | 84±6.1 | 101±3  | 102±10 | 97±10  | 102±9  | 90±4  |

* TAE1–TAE6, testa–aleurone-endosperm gradient.

Results

Total concentrations (ICP-MS)

About 96% of the initial mass was recovered after fractionation of barley grains into nine fractions: hull, embryo, core endosperm, and six samples across the testa–aleurone-endosperm interface (Table 1). The largest fraction was the core endosperm, accounting for 65.6% of the total grain weight. The six samples representing a gradient across the testa–aleurone-endosperm interface each accounted for 2.6–3.8% of the total weight (Table 1).

The recoveries of the six elements Mg, K, Mn, Fe, Cu, and Zn ranged from 84% to 102%, measured as the ratio between the accumulated content of the nine grain fractions and that of the whole grain (Table 1). Generally, the embryo and the external parts of the grain (TAE1–TAE3 in Table 1) were enriched in nutrients in comparison with the endosperm.

Endosperm and hull concentrations were quite similar for Mg, Mn, Cu, and Zn, while K and Fe had a much higher abundance in the hull relative to the endosperm (Fig. 1). The embryo had the highest concentration of Mn, Zn, and Cu of all tissues, while Fe and Mg concentrations were highest in the aleurone-containing subfractions. Compared with the whole grain, the embryo had a >8-fold higher Mn concentration and contained >30% of the total Mn. Embryo concentrations of Zn and Cu were 4- to 5-fold higher than in the whole grain.

The six subfractions of the testa–aleurone-endosperm interface generally showed a negative gradient in micro-nutrient concentrations directed towards the endosperm (Fig. 1). In contrast, the macronutrients Mg and K had the highest concentrations in the second subfraction (sample TAE2) containing the outer aleurone layer. In total, the six subfractions across the testa–aleurone–endosperm interface accounted for 69% of the Mg content, 62% of Fe, 58% of Zn, 55% of K, 44% of Cu, and 34% of the Mn content of the whole grain (Table 1). The remaining endosperm, constituting ~66% of the grain mass, only contained 7% of the total Mg content, while the corresponding values for Fe, Zn, and Cu were 18, 25, and 43%, respectively.

Elemental distribution (μ-XRF)

The elemental distribution maps reported in Figs 2, 4, and 5 clearly indicate the potential of the Maia detector to collect megapixel high-definition trace element imaging maps of relatively large objects. For instance, the longitudinal maps reported in Fig. 2 are 18.4 Mpixel images (8.3×3.3 mm collected at 1.25 μm lateral resolution) and were collected in ~3 h. In contrast, some of the most detailed elemental maps of cereal grains, such as those recently published by Lombi et al. (2009), are ~40 Kpixel in size (collected at 25 μm lateral resolution) and required ~7 h of collection time.

Another advantage provided by the Maia detector is that 100% of the grain section area was sampled in this experiment as the beam size and pixel or step size were the same. As a result of the complete sampling of the specimen...
in this study, elemental maps maintain a considerable definition even when enlarged. For instance, in Fig. 3, a large image of the embryo, an important part of the grain not previously investigated at high resolution in barley, is presented. In contrast, in previous studies, the discrepancy between beam size and step size often translated into an undersampling of the specimens. For instance, Takahashi et al. (2009) utilized a beam of $1.1 \mu m^2$ to map rice grains with a step size of 10–50 $\mu m$. This translates into an effective sampling of only 1–0.4% of the thin section of the rice grain.

The elemental distribution maps for a longitudinal section are reported in Fig. 2. The distributions of the elements detected by $\mu$-XRF (Cu, K, Fe, Mn, and Zn) in the two longitudinal and the two cross-sections were very similar. Therefore, only one set of images for each orientation is reported here.

The most striking feature is the lack of a signal for micronutrients in large parts of the endosperm. It is also evident that K was the only element analysed that was highly represented in the hull. The distribution of Cu and Zn appeared to be similar, with both elements present in the embryo, around the perimeter of the grain, and in the crease area in the ventral part of the grain. This similarity between the distribution of Cu and Zn was mirrored in the total concentration analyses reported in Fig. 1. Iron was found mainly in the external parts of the grain and in the scutellum. Mn was mainly distributed in the outer ventral part of the grain and in the embryo, in correspondence with the vascular bundle (the pigment strand which is part of the vascular bundle is clearly visible in the microphotograph of Fig. 2).

A line scan across the grain, away from the embryo region, is reported in Fig. 3. This line scan was obtained by laterally averaging a box (reported in the microphotography in Fig. 2) with a width of 105 $\mu m$ (84 pixels) and a length of 2200 $\mu m$ across the grain. The thickness of the aleurone

![Fig. 1. Concentrations of Mg, K, Mn, Fe, Cu, and Zn in grain tissue fractions of barley. TAE1–TAE6 represent an inwards-directed sample gradient across the testa–aleurone–endosperm interface. Values are means ±SE (n=5).](image-url)
layer (see insert in Fig. 3) varies between 100 µm and 200 µm in these barley seeds. In the ventral side, and faintly in the dorsal side of the grain, it can be seen that Mn was the element that extended most outwards and that this distribution coincided with the testa. Cu, Fe, and Zn in the dorsal side of the grain were localized within a narrow band of ~150 µm which most probably corresponds to the aleurone layer. In contrast, in the ventral side, the distribution of Cu, Zn, and, to a lesser extent, Fe was much wider over ~600 µm, suggesting that these elements may not be limited to the aleurone layer. However, it should be noted that this longitudinal section was cut very near the centre of the grain, and the folding of the aleurone layer along the crease could explain this result.

An enlargement of the elemental map for Fe, Zn, and Mn for the embryo and scutellum is reported as an RGB map (Zn in red, Fe in green, and Mn in blue) in Fig. 4. Cu distribution is not shown in this figure but it followed very closely the distribution of Zn (Fig. 2). Figure 4 demonstrates how the high definition of the analyses can be used to investigate details of the micronutrient distribution associated with small morphological features. Zn is distributed along the scutellum region, while Fe appears much more concentrated in the ventral part of the scutellum. Mn
was localized in a narrow band in the scutellum in proximity to the embryo. Fe was largely absent from the root and shoot primordia. Higher concentrations of Mn were observed in the primordial roots compared with the embryonic shoots. Mn in the root primordia was mainly localized in the external part of the radicula (embryonic root) and in the calyptra (root cap). Zn was present in the calyptra and in the coleorhiza (root sheath). Zn and Cu (Fig. 2) appeared to be the dominant elements in the shoot primordia, with some localized areas where Mn was also present. In comparison with the rest of the grain (Fig. 2), Mn was concentrated in the embryo and this result is in line with the ICP-MS analysis showing that 30% of the Mn was present in the embryo (which represented only 2.7% of the grain dry matter).

The elemental maps obtained from a cross-section of the grain are reported in Fig. 5. In all cases, with the exception of K, high concentrations were observed in the area corresponding to the vascular bundle. This is particularly evident for Mn and Fe. Fe, however, appeared also to be distributed along the whole perimeter of the grain. This cross-section is particularly useful to resolve differences in the distribution of Cu and Zn. In both cases these elements were not only restricted to the perimeter of the grain but a substantial presence into the endosperm adjacent to the vascular bundle could be observed. These results are in line with those obtained by physical separation and ICP-MS analyses (Table 1).

Discussion

Chemical analysis of physically separated grain fractions

Cu, K, Mn, and Zn concentrations in the hulled grains (Fig. 1) were generally in agreement with previous results,
while Fe concentrations were higher (Liu et al., 1974, 2007). The higher Fe concentration was not due to surface contamination, for example with soil, since the Fe content in the hull only accounted for 13% of the total grain content (Table 1) and hull Al concentrations were extremely low (data not shown). The differences must therefore be ascribed to contrasts in the cultivation conditions and genotype.

Liu et al. (1974, 2007) conducted a polishing fractionation procedure of barley grain similar to that performed in the present work. The abraded material amounted in both cases to ~35% of the grain weight even though the degree of polishing is a function of both the method used and the hardness of the grains. Both studies also showed that a large proportion of the Mn was found in outer grain parts that were removed in the first steps of polishing. The other micronutrients decreased from the outer to the inner parts of the grain, consistent with a significant proportion being confined to globoids in the aleurone layer, probably complexed by phytate. A detailed investigation conducted by Ockenden et al. (2004) using SEM/TEM-EDX revealed that Zn and/or Fe were present in globoids in the aleurone and scutellum tissues.

**Distribution of (micro) nutrients by µ-XRF**

Even though the distribution of elements obtained by physical separation is very similar to previous results obtained by Liu et al. (1974, 2007), the high-definition images collected by µ-XRF provide much greater details of the spatial distribution of micronutrients.

Only a few studies have addressed the distribution of (micro)nutrients in the embryo. This is possibly due to the spatial complexity of this part of the grain and the lack of sensitivity of analytical equipment. Mazzolini et al. (1985) investigated the distribution of (micro)nutrients in wheat grains by PIXE. However, the beam size utilized (10–12 µm in diameter) did not allow high-definition distribution maps. Mazzolini et al. (1985) also reported average elemental concentrations in the radicula, coleorhiza, scutellum, and leaf primordia based on an integration of the X-ray spectra in these areas. The largest Mn concentration was found in the radicula, and this agrees well with the data presented in Fig. 4 where, however, a distinct gradient between the external and the central parts of the radicula can be observed. The present results again agree with the study of Mazzolini et al. (1985) that identified the scutellum as the main organ for Fe accumulation. However, the high-definition image reported in Fig. 4 shows how Fe is preferentially concentrated in the ventral part of the scutellum. This result is very similar to the Fe distribution in rice grains recently reported by Takahashi et al. (2009). The concentration of Zn in the different parts of the embryo reported by Mazzolini et al. (1985) was relatively constant (410–600 mg kg⁻¹), and this again is in agreement with the results reported in Fig. 4. However, even in this case the enhanced definition obtained in the present study highlights marked differences in the pattern of distribution; notably, Zn is much more concentrated in the tip of the root primordia. Interestingly, Takahashi et al. (2009) observed elevated concentrations of Zn in the root tip of rice grains 36 h post-germination. It is possible that the presence of Zn in the root tip before germination and root elongation could not be detected by Takahashi et al. (2009) since the step size used was 10–50 µm. The presence of Zn in meristematic tissues can be explained by its role in protein synthesis, and membrane structure and function (Cakmak 2000). The physiological processes controlling the distribution of elements within the cereal grain are not well understood. Gradients may result from the distribution and expression pattern of ion transporters that remain poorly characterized (Karley and White. 2009; Tauris et al. 2009). Furthermore, properties related to complexation may contribute to gradients in elemental distribution within plant organs (Conn and Gillham, 2010).

In the rest of the grain, Cu and Zn appear to be the most mobile among the elements investigated. Ozturk et al. (2006), using a staining technique, suggested not only that Zn distribution is limited to the aleurone layer but that this element is also present in the outer parts of the endosperm which is known to be rich in proteins. The present study suggests that Zn and Cu are also present in the ventral part of the endosperm (Figs 2, 3, and 5). In contrast, both elements seem to be limited to the aleurone layer in the lateral and dorsal parts of the grain. This may be related to a difference in the transport from the aleurone to the subaleurone endosperm cells in various parts of the grain or to protein gradients in the grain. A similar distribution pattern for Zn and Cu was observed by Lombi et al. (2009) in rice grains. This pattern also mirrored the distribution of S in the rice grains, indicating a possible complexation of these elements by thiol groups of cysteine-containing proteins. These findings are also supported by SEC-ICP-MS analyses of barley by Persson et al. (2009).

It is generally reported that Fe mainly accumulates in the aleurone layer (Mazzolini et al., 1985; Ockenden et al., 2004) where Fe is thought to be complexed by phytate (Lott, 1984; Persson et al., 2009). Figure 5 similarly suggests a much more limited distribution of Fe in the endosperm compared with Zn and Cu. However, it should be noted that the spectroscopic techniques employed in previous studies, such as SEM/TEM-EDX, are significantly less sensitive than µ-XRF and therefore diffuse lower concentrations of micronutrients in other parts of the grain may not have been revealed in those studies. As argued by Borg et al. (2009), the evidence that ferritin is present in isolated amyloplasts (Balmer et al., 2006) may indicate that Fe localization may not be completely restricted to the aleurone layer. The present study shows that a part of the Fe is present in the ventral part of the endosperm. This finding is supported by the ICP-MS results (Table 1) showing that approximately one-fifth of the Fe was present in the core endosperm. A more detailed investigation at the cellular and subcellular level is required to confirm these results. This could probably be achieved by nano-SIMS, which was recently demonstrated for other elements by Moore et al. (2010).
The aim of the present study was to provide some information regarding the physiological processes involved in micronutrient transport by investigating the relationships between the element distributions reported in Fig. 2 for Cu, Fe, and Zn. Association plots were generated for these elements which clearly indicate how important various physiological processes are in controlling the relative distribution of these elements across different tissues.

The association plot in Fig. 6 shows the relationship between Zn and Fe. In this case it is evident that the Zn/Fe ratio in the endosperm (correlation ‘family’ b) and in the part of the embryo relative to the root and shoot primordia (correlation ‘family’ c) was larger than the ratios observed in the perimeter of the grain (aleurone) and in the scutellum (correlation ‘family’ a). This may indicate that the bottleneck for Fe transport to the endosperm may not be related to the unloading from the vascular bundle or uptake from the filial tissue, as Fe is present in similar concentrations to Zn in the aleurone and scutellum. Therefore, it can be speculated that storage of Fe in phytate granules or in protein storage vacuoles (Kim et al., 2006) in the aleurone may be a key process limiting its transfer to the endosperm. It is possible that the Fe transported to the endosperm from the aleurone layer is complexed by nicotianamine, as was shown in the rice endosperm by Lee et al. (2009). Tauris et al. (2009) found that YSL9 was expressed in both the aleurone and endosperm. These transporters belong to the Yellow Stripe Like transporter family whose members are known to translocate Fe–nicotianamine complexes.

The association plot in Supplementary Figs S1 and S2 available at JXB online show the relationship between Cu and Zn/Fe for all data points reported in Fig. 2. The relationship between Cu and Fe concentrations reported in Fig. S1 is similar to what was observed between Zn and Fe. The strong correlation between Cu and Zn concentrations (Fig. S2) in ‘family a’ refers to the areas relative to the vascular bundle, the perimeter of the grain (i.e. the aleurone layer), and the embryo. The pixels associated with the other correlation groups (b and c) are localized in the crease region in the ventral part of the grain. These areas are characterized by larger Cu/Zn ratios in comparison with the Cu/Zn ratio found in the remaining part of the grain (correlation ‘family a’). It is likely that different transporters are responsible for the relative differences in the distribution of Cu and Zn. For example, Tauris et al. (2009) reported that HMA8, a P1B-type heavy metal ATPase, which is known to transport Cu, is highly expressed in the transfer cells and aleurone. The same authors also showed that genes encoding Zn transporters belonging to the ZIP family were transcribed in various grain tissues.

**Conclusions**

Large gradients in the distribution of (micro)nutrients were present both within and between different tissues of the barley grain. The gradients were especially evident in the embryo and scutellum regions. Of particular importance in terms of nutrition is the finding that Zn, Cu, and, to some extent, Fe were also present in the endosperm but that this distribution was different between the ventral and dorsal part of the grain.

This study demonstrates the potential of high-definition μ-XRF for the investigation of the distribution of (micro)-nutrients in cereal grains. In fact, this technique combined a μm scale lateral resolution, workable collection times which allow whole grain mapping, and high sensitivity. The information that can be generated by this technique can be utilized to optimize grain processing. Furthermore, it has the potential to provide important information in terms of grain physiology, especially when combined with molecular biology techniques. For instance, detailed elemental maps such as those reported here could be utilized to target specific areas for detailed molecular
investigations. Finally, this spectroscopic methodology is ideally suited to provide a functional characterization of transgenic lines and mutants.

**Supplementary data**

Supplementary data are available at JXB online.

Figure S1. Association plot showing the relationship between Fe and Cu of semi-quantitative concentrations for all data points reported in Fig. 2. The pixel positions of the ‘families’ of correlations selected in the association plots are highlighted in green in the Zn map reported to the right.

Figure S2. Association plot showing the relationship between Zn and Cu of semi-quantitative concentrations for all data points reported in Fig. 2. The pixel positions of the ‘families’ of correlations selected in the association plots are highlighted in green in the Cu map reported to the right.

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