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Abstract

Understanding how essential and toxic elements are distributed in cereal grains is a key to improving the nutritional quality of cereal-based products. The main objective of this work was to characterize the distribution of Cd and of nutrients (notably Cu, Fe, Mn, P, S and Zn) in the durum wheat grain. Laser ablation inductively coupled mass spectrometry and synchrotron micro X-ray fluorescence were used for micro-scale mapping of Cd and nutrients. A dissection approach was used to quantitatively assess the distribution of Cd and nutrients among grain tissues. Micro X-ray absorption near-edge spectroscopy was used to identify the Cd chemical environment in the crease. Cadmium distribution was characterized by strong accumulation in the crease and by non-negligible dissemination in the endosperm. Inside the crease, Cd accumulated most in the pigment strand where it was mainly associated with sulfur ligands. High-resolution maps highlighted very specific accumulation areas of some nutrients in the germ, for instance Mo in the root cortex primordia and Cu in the scutellum. Cadmium loading into the grain appears to be highly restricted. In the grain, Cd co-localized with several nutrients, notably Mn and Zn, which challenges the idea of selectively removing Cd-enriched fractions by dedicated milling process.

Main finding of the work

This work provides the first high-resolution maps of Cd distribution in durum wheat grain and, thereby, adds to our understanding of the processes involved in Cd loading and storage in cereal seeds.

Keywords

Cadmium; durum wheat grain; LA-ICP-MS; µXRF; µXANES

1. Introduction

Cadmium (Cd) is a heavy metal contaminant whose toxicity to humans has long been recognized. The European Food Safety Authority (EFSA) established a provisional tolerable weekly intake for Cd of 2.5 μg kg^{-1} body weight based on the long-term harmful effects of low doses of Cd on human health (EFSA 2011). Though the average dietary exposure is below this level in the European population, some subgroups including children, young women and vegetarians are more likely to frequently exceed it (Clemens *et al.* 2013). In Europe, almost one third of dietary exposure to Cd is via consumption of cereals (EFSA 2012). Durum wheat has a greater tendency to accumulate Cd in grains than common wheat and other cereals (McLaughlin and Singh 1999; Grant *et al.* 2008). The cadmium concentration is often above 0.1 mg kg^{-1} in durum wheat grains and can sometimes exceed the regulation limit of 0.2 mg kg^{-1} set by the European Union (EC 2008). Therefore, particular attention should be paid to reducing the level of Cd in durum wheat-based products (e.g. semolina, pasta).

Substantial efforts have already been made to understand how Cd is taken up, transported, and allocated to the grains in cereal crops (Clemens and Ma 2016; Maccaferri *et al.* 2019). However, little information is available on how Cd is distributed within the grain, even though the differential accumulation of an element among grain tissues may mirror its internal loading, storage and functions within the grain (Lombi *et al.* 2009). For instance, the transfer of zinc (Zn) from maternal to filial tissues was proposed as the main barrier to Zn loading into wheat grains, based on the much lower concentration of Zn measured in the endosperm than in the vascular bundles in the crease (Wang *et al.* 2011). In the same way, the involvement of sulfur (S) complexes in the loading of iron (Fe) into the grain was proposed based on the co-localization of these two elements in the nucellar projection (Singh *et al.* 2013). A clear picture of how Cd is distributed among tissues and how it is co-localized with nutrients in the grain is thus a key to developing agronomic strategies to improve the food safety of durum-wheat based products. Among nutrients, those to primarily focus on are Fe, Mn and Zn as they share transporters and loading pathways with Cd, and S since Cd has a high affinity for thiol groups and is often bound to S-containing ligands in plant tissues (Clemens *et al.* 2013).

Significant advances in imaging techniques make it possible to obtain high definition

elemental maps of wheat grains (Lombi *et al.* 2011). For instance, synchrotron micro X-ray fluorescence (µXRF) has been used to investigate the distribution of P, S, Mn, Fe, Cu and Zn in bread wheat (Singh *et al.* 2013; Ajiboye *et al.* 2015; De Brier *et al.* 2016). Laser ablation inductively coupled mass spectrometry (LA-ICP-MS) can also be used to identify the distribution of metals. This technique has been widely used in recent years to map trace elements such as As, Cu, Fe, Mn, Ni, Pb and Zn in grains of bread wheat (Lombi *et al.* 2009; Wu *et al.* 2013; Van Malderen *et al.* 2017), sunflower (Pessôa *et al.* 2017), maize (Imran *et al.* 2017) as well as in cocoa beans (Thyssen *et al.* 2018). The chemical analysis of dissected tissues is another way to investigate the distribution of elements in plant grains (Pieczonka and Rosopulo 1985; Kutman *et al.* 2011; Detterbeck *et al.* 2016). Chemical analysis provides quantitative information at the scale of the whole grain whereas imaging can only show the distribution of elements in a section of the grain.

Only a few studies have attempted to map the distribution of Cd in grains. This is probably because the level of Cd accumulation in plant grains is usually too low to be accurately quantified by imaging (Clemens and Ma 2016). Van Malderen *et al.* (2017) tried to map Cd in bread wheat grain with a Cd concentration of 0.05 µg g^{-1} but the signal intensity of Cd in some parts fell to the background level, preventing the precise delimitation of areas in which Cd accumulated. The only informative maps of Cd distribution were imaged on Cd-contaminated rice grains (Basnet *et al.* 2014; Yan *et al.* 2019) and in sunflower (Pessôa *et al.* 2017). In rice, Basnet *et al*. (2014) observed that Cd was distributed throughout the grain whereas, in sunflower, Pessôa *et al.* (2017) observed that Cd preferentially accumulated in the cotyledons. Today, a high-resolution map of Cd distribution in durum wheat grain is still lacking.

In the present study, LA-ICP-MS was set up to produce images of the localization of Cd and of six mineral nutrients (Cu, K, Mn, Mo, P and Zn) in transversal and longitudinal sections of mature durum wheat grains (including the embryo) at the micrometer scale spatial resolution. Synchrotron µXRF and micro X-ray absorption near-edge spectroscopy (µXANES) were also used to provide distribution maps of Cd and of nutrients (including Fe and S) at a sub-micrometer lateral resolution, and Cd speciation, respectively, particularly in the crease area. In addition to high-resolution maps, a grain dissection experiment was performed to provide quantitative information on Cd and nutrient concentrations in dissected grain tissues. The

objectives of the study were to (i) precisely characterize the spatial distribution of Cd in durum wheat grain, (ii) investigate the differences between the distribution of mineral nutrients based on current knowledge of their loading, storage and functions in the grain, and (iii) examine if there are Cd-enriched tissues that could be removed by adequate milling processes without having too much impact on the nutritional value of the durum wheat flour.

2. Materials and methods

2.1. Grain production

Mature grains of durum wheat (*Triticum durum* cv. *Sculptur*) were harvested from hydroponically grown plants exposed to 100 nM $Cd(NO_3)_2$. The composition of the nutrient solution and the hydroponic culture procedure are the same as those described in Yan *et al.* (2018) (for details see supplementary comment S1). The average concentration of Cd in the grains was 2.5 ± 0.1 µg g^{-1} dry weight (DW). As a control for mapping, grains with a background level of Cd (0.0024 ± 0.0019 µg g^{-1} DW) were collected from plants grown under the same conditions but with no Cd added to the nutrient solution. Neither Cd toxicity nor mineral deficiency symptoms was observed in plants over the whole culture period. The spikes were freeze-dried at -60 ˚C for 72 hours before the grains were harvested. All the grains used in this experiment were taken from this pool, whose characteristics are summarized in supplementary Table S1.

2.2. Preparation of thin sections by cryomicrotomy

Grains were soaked for 5 to 6 hours at 4 °C. For LA-ICP-MS, grains were then embedded in O.C.T compound (tissue-teck) at low temperature (~ -30 °C) and 40 µm-thick transversal (across root or leaf primordia of the embryo) as well as longitudinal (alongside the crease) sections were prepared by cryomicrotomy (Leica CM 1950). The thin sections were directly deposited on glass slides and air dried. Before LA-ICP-MS analysis, the structural integrity of the grain section was checked under the microscope (Fig. 1-2, supplementary Fig. S1). For µXRF and µXANES, 16 µm-thick sections were cut at ~ -50 °C with a cryomicrotome (Leica LN22) cooled with liquid nitrogen. The frozen sections were sandwiched between two Ultralene® films, and mounted directly on the sample holder immediately before measurement. They were

kept frozen (-170 °C) during transfer to the analysis chamber and throughout the synchrotron measurements using a passively cooled liquid nitrogen cryostat.

2.3. LA-ICP-MS imaging

Elemental analysis of the grain section was achieved by coupling an NWR 213 laser ablation system equipped with a TV2 ablation cell (ESI, Freemont, CA) with a 7700 cs ICP-MS (Agilent, Tokyo, Japan) with Pt cones. A helium flow of 800 mL min^{-1} was used to transport the ablated material directly into the dry plasma of the ICP-MS. ICP-MS parameters (torch position, carrier gas flow rate and ion lense voltage) were tuned for each set of experiments by ablating the 612 NIST glass reference material while monitoring the ^{238}U and ^{232}Th signals. Detection limits of monitored isotopes are commonly in the range of 0.0001-0.1 µg g^{-1} (Becker 2002). The grain sections were ablated using a Nd:YAG laser source at 213 nm with a 20 Hz repetition frequency, a 10 µm x 10 µm square laser beam, a fluency of 12 J cm^{-2} (20% of delivered energy), and a scan speed of 10 µm s^{-1}. Samples were entirely ablated in a line-by-line scan mode with a distance between lines of 20 µm (non-ablated lines). ^{114}Cd and other isotopes (^{31}P, ^{39}K, ^{55}Mn, ^{63}Cu, ^{64}Zn, ^{98}Mo) were measured with a sampling time of 1 s while maximizing the integration time for ^{114}Cd (0.59 s). A csv file reporting isotope intensities versus time (then converted to the position at the surface of the sample) was recorded for each ablated line. A specific program was designed and coded to merge the files and build the maps of the different isotopes. For each map, the relative intensity was calculated by dividing the raw signal by the maximum value.

2.4. Synchrotron µXRF imaging and µXANES analyses

Micro-XRF and Cd L_{III}-edge µXANES were performed on the ID21 scanning X-ray microscope at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The X-ray beam was monochromatized with a Si(111) two-crystal monochromator and focused using Kirkpatrick-Baez mirrors on the sample with a lateral resolution of 0.4 µm (V) x 1.4 µm (H). Measurements were made in a vacuum and in cryogenic conditions using a cryostat passively cooled with liquid nitrogen (Cotte *et al.* 2017). To avoid potassium interference (K-edge at 3608 eV) with Cd L lines, chemical mapping was performed in two stages: Cd-S-P-Mg at 3570 eV and Fe-Mn-Ca-K at 7200 eV. In both cases, maps were obtained by scanning the samples with a 2 µm step

(1 µm for the crease) and a counting time of 100 ms per point. The fluorescence signal was collected with a Silicon Drift Diode XRF detector (SGX Sensortech with an active area of 80 mm^2) and normalized by the incident photon intensity. Elemental mass fractions were calculated from fundamental parameters with the PyMca software package, applying pixel-by-pixel spectral deconvolution to hyperspectral maps normalized by the incoming flux (Sole *et al.* 2007). The incoming flux was monitored using a drilled photodiode previously calibrated to 3570 and 7200 eV. In PyMCA, the reference incoming flux was set at $5x10^{10}$ photons s^{-1}, and XRF detector parameters were set to an active area of 0.7746 cm^2 and 4.65 cm sample to XRF detector distance. Active area and sample to detector distance were calibrated using an AXO standard (http://www.axo-dresden.de/products/highprecision/reference.htm) that contains Fe and Pd as reference to the elements analysed in the wheat grains. The sample matrix was assumed to be cellulose/ice at a density of 1.5 g cm^{-3}. The sample thickness was set at 16 µm using a cryomicrotome. In order to filter out the pixels with poor signal to noise, each pixel was tested to have a signal in the detector element's region of interest $\geqslant 3.29 \times \sqrt{signal + background}$. After filtering the poor signal quality pixels, the calculation of limit of detection (LOD) was performed for each pixel and the highest LOD calculated was used to set the color scale bars in the elemental maps. The LOD maps and the elemental maps are shown in supplementary Fig. S4 and S5. The LOD values obtained were (in mg g^{-1}) 0.16 for Cd, 0.31 for S, 1.95 for P, 32 for Mg, 0.41 for Fe, 0.47 for Mn, 2.35 for Ca and 7.02 for K.

Cadmium L_{III}-edge µXANES spectra of the areas of interest of the grain section were recorded in fluorescence mode using a SDD XRF detector (SGX Sensortech with an active area of 80 mm^2) in the range 3520 and 3590 eV with an energy step of 0.5 eV and a counting time of 100 ms. The normalized spectra were compared to Cd-reference compounds we collected previously on the same beamline (Isaure *et al.* 2006; Isaure *et al.* 2015; Penen *et al.* 2017) and simulated by linear combination fits (LCFs) of the Cd references (fingerprint approach). The quality of the fit was estimated by the normalized residual sum of squares (*Nss*) and the precision on the proportion of Cd species was estimated to be 10% as described in Isaure *et al.* (2017).

2.5. Grain dissection and analysis of dissected grain fractions

Five fractions of wheat grain were considered: (i) the outer periphery (*OP*), which is

loosely attached to the grain and mainly corresponds to the pericarp epidermis, hypodermis, and degenerated parenchyma (Bechtel *et al*. 2009), (ii) the inner periphery *(IP*) comprising the remaining layers that strongly adhere to the endosperm (cross and tube cells, seed coat, nucellar remnants and the aleurone layer), (iii) the germ (*GM*) including the scutellum and the embryo axis, (iv) the starchy endosperm (*SE*), and (v) the crease (*CR*) tissues comprising the periphery layers embedded in the crease of the grain. Because the *IP* parts stick tightly to the starchy endosperm, it was impossible to experimentally separate the whole *IP* from the whole *SE* while being sure that their respective mineral contents were not affected by cross-contamination. Consequently, the concentrations of the element were determined on a subsample of the core of the starchy endosperm free from *IP* contamination. The quantity of a given element in the *IP* was estimated by calculating the difference between the quantity in the whole grain and the sum of the quantities in *OP*, *GM*, *CR* and *SE*. The concentration was then derived by dividing by the biomass of the *IP* (for details see supplementary comment S2).

The elemental composition of the fractions was determined as follows. A mixture of 0.8 mL of 30% H_2O_2 and 0.2 mL of 67.5% HNO_3 was added to each sample fraction. The mixture was left at room temperature overnight for preliminary digestion. Digestion was accomplished by heating the mixture in an oven using a temperature gradient of 60 ˚C for 30 min, 80 ˚C for 45 min, and 100 ˚C for 90 min. After cooling and filtering through 0.2-µm polycarbonate membrane filters, the solutions were diluted to 10 mL with ultrapure water. The concentrations of Cd, Zn, Cu, Mn, Mo, and Fe in the digests were determined by ICP-MS (7700x, Agilent Technologies), and those of Ca, K, Mg, and P were determined by ICP-OES (ACTIVA, Horiba Jobin Yvon) by the central analytical service of the University of the Basque Country. The results were validated using procedural blanks and the NIST Standard Reference Material 1573a. The recovery rates of reference values were higher than 90% for all elements.

3. Results

3.1. LA-ICP-MS elemental imaging in durum wheat grains

The maps of cadmium (^{114}Cd), zinc (^{64}Zn), copper (^{63}Cu), manganese (^{55}Mn), molybdenum (^{98}Mo), potassium (^{39}K), and phosphorus (^{31}P) distributions are shown

for the cross sections at the level of the leaf (Fig. 1) or root (Fig. 2) primordia and for the longitudinal section alongside the crease (supplementary Fig. S1). The germ, crease, grain periphery, and starchy endosperm (*SE*) were identified on the different maps. Two layers were visible at the grain periphery: the outer (*OP*) and the inner (*IP*) periphery. In the germ, it was possible to distinguish the scutellum facing the endosperm and the embryo axis, including the coleoptile and coleorhiza surrounding the leaf and root primordia.

The starchy endosperm generally had the lowest intensities and the *IP*, the germ and the crease had the highest, regardless of the element. In detail, the *OP* had a detectable signal mainly for Cd, Mn and Zn. Except at the bottom of the grain close to the root primordia (Fig. 2), high abundances of all the elements, particularly K, P, Cd, Mn and Zn, were found in the *IP* layers. In the crease, which is visible only on the radial cross-sections (Fig. 1, 2), the signal intensities of Cd, Zn and Mn was very high but the exact location varied with the element and with their position along the embryo axis. On the cross section made at the level of the shoot primordia (Fig. 1), Cd was abundant in all the crease layers, Zn was more abundant in the innermost layers, presumably the modified aleurone layer, whereas Mn was more abundant in the outermost layers, presumably the nucellar projection, the pigment strand and the vascular bundle area. Hotspots of Cd, Mn and Cu were identified on the cross section at the level of the root primordia (Fig. 2) and attributed to the nucellar projection and/or the pigment strand. Zinc was highly abundant in the crease tissues on each side of the endosperm cavity, but the signal intensity of Zn was lower in the location assumed to be the pigment strand and/or in the vascular bundle area.

In the germ, the scutellum was particularly homogeneously enriched in P, Cu, K and to a lesser extent in Cd (Fig. 1-2, supplementary Fig. S1). In the scutellum, Zn was preferentially localized in the part facing the endosperm whereas Mn tended to preferentially accumulate on the opposite side, facing the shoot or root primordia, although to a lesser extent. In the embryo axis, the root cortex (and possibly the epidermis) showed stronger signals of Cd<Zn<Mn<Mo. Markedly high intensities of molybdenum were observed in the periphery of the root primordia (Fig. 2, supplementary Fig. S1). Finally, the starchy endosperm had always the lowest signals. The lower abundances of elements in the *SE* than in the peripheral layers of the grain were clearly visible on the maps and in the signal intensity profile from the inside to

298 the outside of the grain (supplementary Fig. S2). The difference between these and the
299 other parts was (Zn, Mn, P, K) > (Cu, Mo) > Cd.
300 The accumulation of Cd in the *SE* was also visible when we compared the map of Cd
301 in a contaminated grain with that in the uncontaminated control sample
302 (supplementary Fig. S3). Figure 1 and supplementary Fig. S2 suggest a gradient in Cd
303 intensity *IP* > outer endosperm neighboring *IP* > core of the starchy endosperm. It can
304 be seen that the Cu signal in the endosperm was slightly higher around the crease (Fig.
305 1). Tentative grouping of the elements according to the most discriminating hotspots
306 could be as follows: Cd, Mn and Zn accumulated particularly in the crease; P, Cu and
307 K showed a stronger and more homogeneous accumulation in the scutellum; Mo was
308 preferentially located in the periphery of the root primordia.
309
310 *3.2. Synchrotron µXRF elemental imaging*
311 Elemental maps of a transversal section with a zoom on the crease are shown in
312 supplementary Fig. S4 and S5 and tricolor maps in Fig. 3. The highest concentration
313 of Cd was clearly located in the crease, where its concentration locally reached 600
314 µg g^{-1}. The high resolution lateral map of the crease pinpointed a “curved zone”
315 assumed to be the pigment strand (Drea *et al.* 2005) where Cd was particularly
316 concentrated (red arrows in Fig. 3). Several hotspots where Cd and P co-localized
317 were observed in the vascular bundle area (white arrows in Fig. 3). In contrast, no Cd
318 was detected elsewhere in the grain, probably because the concentration of Cd was far
319 lower in the other grain parts (endosperm, germ and peripheral tissues), as suggested
320 by LA-ICP-MS maps. Sulfur was mainly located in the crease - in the vascular bundle
321 area but also in the pigment strand and in the adaxial nucellar projection - as well as
322 in the root primordia and in the endosperm, particularly in the outward endosperm, in
323 line with results obtained by De Bier *et al.* (2016). High concentrations of P were
324 found in the aleurone layer, where P-enriched vacuoles were visible, and in the
325 scutellum. Iron was concentrated in the vacuoles of the aleurone cells (like P) and in
326 the abaxial nucellar projection. The outward scutellum was also enriched in iron.
327 Manganese showed a different pattern with preferential localization in the vascular
328 bundle area, in the outer periphery and, to a lesser extent, in the root primordia.
329 Calcium was distributed mainly as spots in the endosperm and in the outer periphery,
330 while K was mainly located in the aleurone layer and in the germ (notably in the

scutellum).

3.3. Cadmium speciation by Cd L_{III}-edge µXANES

Cadmium µXANES was performed on the distinct areas of the crease and compared to Cd-references to probe the Cd ligands. Spectra collected on the various Cd+P hotspots were averaged and showed an edge with a peak characteristic of O ligands (supplementary Fig. S6). The global spectrum was satisfactorily reproduced by 62% Cd-O and 27% Cd-cystein with O ligands provided by carboxyl ligands from organic acids. Although phosphate ligands produced poorer spectral agreement, it was difficult to unambiguously exclude these ligands. The averaged spectrum collected in Cd hotspots in the pigment strand was fitted with 43% Cd-O and 42% Cd-cystein while the averaged spectrum collected in the (Cd, S)-diluted areas of the vascular parenchyma was fitted with 27% Cd-O and 63% Cd-cystein. In contrast, no Cd absorption edge was detected in the nucellar projection, in the aleurone layer or in the endosperm because of their much lower concentration of Cd.

3.4. Concentrations and amounts of elements in the dissected grain parts

Figure 4 shows how the concentration of each element differed among the different grain parts. None of the elements studied were homogeneously distributed in the wheat grain. Three groups were distinguished among grain parts. The concentrations of *SE* were the lowest of all elements, clearly lower than the corresponding concentration of the whole grain (normalized concentration <1). The concentrations of the second group of grain tissues including the *GM* and *IP* were the highest of all the elements, 2.2 to 8.5 times higher than the concentration of the whole grain. Concentrations in the third group, *OP* and *CR*, were between the two, either above or below the concentration of the whole grain, depending on the element. It is worth noting that the crease had by far the highest concentrations of Cd, Mn and Zn, with concentrations respectively 16.6-, 19.2- and 17.7-fold higher than the corresponding concentration of the whole grain. Concentrations obtained by µXRF and chemical analyses of dissected tissues differed but were not inconsistent since µXRF gives a local concentration in an area of a few μm^2 while chemical analyses provides information on a bulk tissue that can be highly heterogeneous. Generally, the ranking of grain tissues according to the concentrations of a given element was consistent with

LA-ICP-MS and µXRF maps. In addition, analysis of dissected tissues revealed a tendency for concentrations of Cd, Cu and Mo to be relatively the highest in the *SE*. This characteristic may also be true for S, as suggested by µXRF maps (Fig. 3), but its concentration was not determined in dissected grain tissues in the present study.

Supplementary Fig. S7 shows how the amount of each element was partitioned among the different parts of the grain. *SE+IP* were the two main fractions that together contained between 52% (Mn) and 89% (Mg) of the total quantity of the grain. Hence, 69% of Cd in the grain was in *SE+IP*. Although *IP* represents only 10% of the grain dry weight, it accounted for between 22% (P) and 78% (Mg) of the total quantity of the element (30% of Cd). Conversely, *SE*, which is a main grain fraction based on biomass (83% of total, on average) accounted for only 25% (Ca) to 54% (Mo) of the total (39% of Cd). This reflects the high concentration of elements in the *IP* versus their very low concentration in the *SE*. The crease, which only accounted for 0.9% of grain DW, contained more than 15% of the Cd, Mn and Zn in the grain. The germ accounted for 3% of the grain biomass but contained more than a quarter of total P and 15-18% of total Cu, Mn, and Fe. The germ accumulated only 7% of total Cd. This is both the lowest contribution of the germ to the total quantity of an element and the lowest pool of Cd in the grain.

4. Discussion

4.1. Is nutrient accumulation in the crease evidence for their highly regulated loading into the seed?

In cereals, inorganic elements are mainly transported to the grain via the phloem (Frick and Pizzolato 1987). They are unloaded from the phloem all along the crease, transported through the apoplast and symplast of the chalaza and nucellar projection cells towards the endosperm cavity, a space filled with water and nutrients that are absorbed by the modified aleurone cells of the endosperm (Wang *et al.* 1995; Patrick and Offler 2001; Borg *et al.* 2009; Yu *et al*. 2015). In the early growth stage of the grain, it is possible that nutrients are also delivered to the endosperm from the whole pericarp through the seed coat by the dorsal and lateral bundles (Fisher 1990). However, this process is limited in time because the dorsal and lateral bundles degenerate and because the seed coat cuticulizes. The crease consequently becomes the main entry route for nutrients to the seed. In the crease region, part of the chalaza,

namely the pigment strand, differentiates into an apoplastic barrier through the accumulation of hydrophobic substances and cell wall lignification. One function of the pigment strand may be to limit the backflow of nutrients (but not of water) from the seed to the maternal tissue. The seed is then isolated from the pericarp, seed coat and nucellar remnants (Zee and O'Brien 1970). Nutrients can still be actively delivered to the seed via the symplastic route thanks to the transfer cells (Wang *et al.* 1995) that enable control of the delivery.

Metal transporters coded by the genes of metal homeostasis are not completely specific and can transport Zn, Fe, Mn, Cd, Pb and Cu with variable affinity, explaining why the distribution of some metals in grains is similar, as is the case of Mn and Zn here. For example, HMA(1,2,3,10) pump Cd/Zn/Co/Pb across the membrane against their electrochemical gradient whereas HMA(4 to 9) operate on Cu/Ag (Tauris *et al.* 2009; Bashir *et al.* 2016). From the transversal sections of LA-ICP-MS maps obtained in our work, and more specifically at the shoot primordia level, it is possible to infer that Zn was more efficiently transferred from the phloem to the aleurone layer than Mn whose signal was higher in maternal tissues than in the aleurone layer. Based on current knowledge of putative metal-chelates in phloem (Harris *et al.* 2012; Khan *et al.* 2014; Flis *et al.* 2016), one possible explanation is that yellow strip-like transporters can transport the Zn-nicotianamine (Zn-NA) chelates from the phloem to the endosperm cavity and/or from the endosperm cavity to aleurone cells (Palmgren *et al.* 2008) but no - or very little - Mn, which has a much lower affinity for NA than Zn (pK= 8.8 for Mn, pK= 17.4 for Zn; Benes *et al.*, 1983).

4.2. Relationship between the distribution and the function of inorganic nutrients in durum wheat grain

The endosperm is the reserve tissue of the seed but was seen to have the lowest concentrations of elements. This apparent paradox is however sound from a functional point of view. Indeed, at maturity, and therefore during germination, *SE* is a dead tissue and if inorganic nutrients were stored in *SE*, their concentrations would be very high so that transport by diffusion could cover the requirements resulting from the growth of the embryo. Given the size of the endosperm (80% of the seed DW), high concentrations also mean huge amounts of inorganic nutrients, which may not be available. High concentrations of inorganic elements in the endosperm may also affect

starch synthesis and proper protein folding in which starch granules are embedded (Mills *et al.*, 2005). Therefore, it is functionally more efficient to store inorganic nutrients in the aleurone layer (Regvar *et al.* 2011) because at germination, the aleurone cells are alive and can actively transfer nutrients to the germ (Bechtel *et al.* 2009). Aleurone cells (which are part of the *IP* fraction) have countless globoids of phytic acid combined with metal elements such as Zn, Mn, Cu, Fe, and Mg (O'Dell *et al.* 1972; Cakmak 2007; Ficco *et al.* 2009; De Brier *et al.* 2016) allowing the storage of both P and metals. The germ also contained high concentrations of elements, which can be interpreted as a starter pool for germination and for the initial growth of the seedling tissues. Although *SE* had the lowest concentrations of elements, likely reflecting the concentrations required for its metabolism, *SE* was on average the second pool of elements based on quantities, due to the important contribution of this tissue to the grain DW. Compared to the *IP*, *SE* is a significant pool of elements that can be mobilized more slowly, along with the mobilization of C and N reserves of the starch/protein matrix.

It is striking that the great majority of Cu accumulation took place in the scutellum and secondarily in the *IP*. This matches the distribution of P and is consistent with the high affinity of Cu for phytates, higher than that of Cd and Mn but equivalent to that of Zn (Crea *et al.* 2008). The ability of copper to change its redox state in biological conditions makes the control of its reactivity particularly important. Furthermore, in plants, excluding Cu sorbed onto the root apoplast, most Cu is contained in the plastocyanin of chloroplasts for photosynthesis, which is not among the plant's top priorities during early germination. From a functional point of view, it makes sense that Cu is stored in the scutellum and not in the embryo axis contrary to Zn and Mn, which are involved in many more enzymes during early germination, in particular those required for growth, protein synthesis, cell elongation, ion absorption and resistance to stress (Broadley *et al.* 2007; Millaleo *et al.* 2010). For example, Zn is required as a cofactor in over 300 enzymes (Palmgren *et al.* 2008). Interestingly, Zn and Mn were highly concentrated in root primordia, which are the first organs to grow. Molybdenum, which is required for both nitrogen assimilation and phytohormone synthesis (Bittner 2014), was also intensely located in the root cortex primordia. Hence, the dissection data and the maps suggest particularly well integrated control of distribution among the grain tissues consistent with the fact that metals are essential

463 for plant metabolism but also highly reactive and possibly toxic.
464
465 *4.3. Cadmium distribution, loading and storage in durum wheat grain*
466 To our knowledge, this is the first time that Cd has been mapped in wheat grain. In
467 general, the distribution of Cd in the grain closely mirrored that of Zn and was
468 characterized, notably, by very strong accumulation in the crease. The results of
469 dissection showed that the concentration of Cd was on average 16-fold higher in the
470 crease than in the whole grain, reaching 40 µg g^{-1}. The LA-ICP-MS and µXRF maps
471 of the cross sections showed that Cd was not evenly distributed inside the crease and
472 that distribution varied with the cutting axis. The µXRF distribution map obtained
473 from a cut made at the level of root primordia showed that, on this axis, the vast
474 majority of Cd accumulated in maternal tissues, notably in the pigment strand where it
475 co-localized with S, and where its concentration locally reached 600 µg g^{-1}. This
476 suggests that Cd loading in nucellar projection cells strongly limits its allocation to
477 the seed, perhaps even more so than that of Mn.
478 Speciation of Cd by µXANES suggested various possible Cd ligands in the crease,
479 probably corresponding to different forms of Cd transport or storage. It seems that Cd
480 is nearly half bound by thiols in the Cd-enriched pigment strand while in (Cd, S)-
481 diluted areas of the vascular parenchyma this proportion increases to 63%. Assuming
482 that the speciation of Cd in the vascular parenchyma mirrors that in the phloem
483 vessels, this would mean that Cd is significantly associated with S ligands in the
484 phloem of durum wheat, in agreement with the literature. Indeed, thiols were
485 identified as Cd ligands in the phloem of the Cd hyperaccumulator *Arabidopsis*
486 *halleri* and of the Cd non-accumulator *Arabidopsis lyrata* (Isaure *et al.* 2015). Thiol
487 ligands were also found in the phloem of *Brassicacea napus* where Cd was assumed
488 to be transported mainly as phytochelatin-Cd and glutathione-Cd complexes
489 (Mendoza-Cozatl *et al.* 2008). Based on this result, one possible explanation for the
490 strong accumulation of Cd in the maternal tissues of the crease is that transporters
491 involved in metal loading in the nucellar projection and/or metal unloading in the
492 endosperm cavity (such YSL and ZIP proteins) do not carry any (or only very few)
493 metals bound to thiol-containing peptides such as Cd.
494 LA-ICP-MS maps showed that Cd was relatively more abundant in the endosperm
495 than most other elements. The dissection results confirmed that the normalized

concentration of Cd in the endosperm was higher than that of most nutrients, notably Fe and Zn, and showed that 40% of the total Cd in the grain was stored in the *SE*. This pattern may be related to the protein network of the *SE* that embeds starch granules including glutenins and gliadins (Mills *et al.* 2005). Indeed, Cd has a high affinity for S-containing compounds and, accordingly, the distribution pattern of Cd shown in Fig. 1 mirrors that of S in Fig. 3. In rice grains, Cd is associated with S-containing amino acids including cysteine and methionine (Wei *et al.* 2017). The thiol groups of these amino acids are involved in the disulfide bonds that control protein folding and may be preferential sites of Cd binding and retention thereby explaining the relatively high concentration of Cd in the endosperm. Consistent with this hypothesis, the inward decreasing gradient of Cd in the endosperm may reflect the protein gradients in the *SE*, which are more concentrated in the subaleurone layer than in the inner parts (Tosi *et al.* 2011).

4.4. Development of new milling technologies to reduce the level of Cd in durum wheat products

New milling technologies for grain could improve the sanitary quality of durum products by removing the grain tissues that are rich in Cd. However, since there is no tissue in the grain of durum wheat that selectively accumulates Cd, a choice will need to be made between reducing the concentration of Cd and maintaining the nutritional quality of the grain. If the priority is to reduce the grain Cd concentration, for instance because the grain Cd concentration is high or because the grains are intended to be consumed by a population particularly exposed to Cd, the best option would be to remove all fractions except the starchy endosperm, which means producing “white semolina”. According to the calculations made based on our dissection experiment (supplementary Table S2), this would reduce the concentration of Cd by more than 50% but would simultaneously cause huge nutrient losses, particularly of Mg, Fe, Mn and Zn. If the priority is to reduce the grain Cd concentration while minimizing the concomitant loss of inorganic nutrients, the best option would be to selectively remove the outer periphery and the crease, which means saving the *SE+IP* (with or without the germ) for the downstream transformation. This would reduce the Cd concentration by 24% while limiting the loss of P, K, Mg, Cu, Fe and Mo to less than 10%. Only the concentrations of Mn (-29%) and Zn (-21%) would be significantly

reduced.

5. Conclusions

In this study, we combined two high-resolution imaging approaches (LA-ICP-MS and µXRF) with a dissection approach to describe the distribution of Cd and seven inorganic nutrients in durum wheat grain. The three approaches made it possible to identify high accumulation of Cd in the crease, and inside the crease, mainly in maternal tissues. Loading of Cd into the grain is therefore largely constrained, as is that of Fe and Zn. To control the accumulation of Cd in the grain without affecting that of Zn and Fe (or vice versa), the limiting stages of their loading into the grain and the associated physiological processes will have to be identified. The similarities observed between the µXRF maps of Cd and S distribution, particularly in the endosperm, as well as the results of µXANES showing that Cd is mainly linked to sulfur ligands in the vascular bundle area, suggest that sulfur fertilization could be a way to reduce the allocation of Cd to the grain as well as its accumulation in the endosperm in durum wheat. Molecular speciation measurements of Cd such as those conducted by Persson et al. (2016) will deepen the understanding of processes behind endosperm Cd accumulation. The preferential accumulation of Cd in certain grain tissues also opens the way for reducing the Cd content of durum wheat-derived products by adapting milling processes. However, because Cd accumulates in the same parts of the grain as several nutrients (including Zn) these adaptations will also alter the nutritional quality of the resulting flour.

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Figure captions

Figure 1. LA-ICP-MS distribution maps of ^{114}Cd, ^{64}Zn , ^{63}Cu, ^{55}Mn, ^{98}Mo, ^{39}K and ^{31}P in mature durum wheat grains sectioned transversally at the position of the leaf primordia. The color scale represents different signal intensities, blue corresponds to the lowest relative intensity and yellow to the highest. The optical image (upper left) shows the different grain tissues visible in this transversal section: *OP*, outer periphery (likely the outer pericarp); *IP*, inner periphery (likely the aleurone layer, the seed coat, the inner pericarp and other maternal peripheral layers); *SE*, starchy endosperm; *col*, coleoptile; *lp*, leaf primordia; *sl*, scutellum; and within the crease (*cr*) region: *mal*, modified aleurone layer; *np*, nucellar projection; *vb*, vascular bundle area.

Figure 2. LA-ICP-MS distribution maps of ^{114}Cd, ^{64}Zn , ^{63}Cu, ^{55}Mn, ^{98}Mo, ^{39}K and ^{31}P in mature durum wheat grains sectioned transversally at the position of the root primordia. The color scale represents different signal intensities, blue corresponds to the lowest relative intensity and yellow to the highest. The optical image (upper left) shows the different grain tissues visible in this transversal section: *OP*, outer periphery (likely the outer pericarp); *SE*, starchy endosperm; *coz*, coleorhiza; *rp*, root primordia; *sl*, scutellum; *cr*, crease.

Figure 3. Synchrotron µXRF tricolor distribution maps of Cd-S-P, Cd-Mg-P, Fe-Mn-Ca and Fe-Mn-K in mature durum wheat grains sectioned transversally at the position of the root primordia. A more detailed map shows the distribution of Cd and P within the crease and the areas where µXANES spectra were collected (white arrows indicate typical positions of P and Cd hotspots, red arrows indicate typical positions of Cd hotspots, black arrows indicate typical Cd diluted areas). The optical image (upper left) shows the different grain tissues visible in this transversal section: *OP*, outer periphery (likely the outer pericarp); *SE*, starchy endosperm; *al*, aleurone layer; *rp*, root primordia; *sl*, scutellum. Within the crease (upper right), *vb* stands for vascular bundle area, *ps* for pigment strand and *np* for nucellar projection (either abaxial or adaxial). The maps of Cd, S, P and Mg were collected at an incident energy of 3570 eV, a step size of 2 µm (1 µm for the crease area) and a dwell time of 100 ms per point. The maps of Fe, Mn, Ca and K maps were collected at an incident energy of

7200 eV, a step size of 2 µm and a dwell time of 100 ms per point. Tricolor maps are displayed as quantitative maps in mg g^{-1}.

Figure 4. Concentrations of P, Ca, K, Mg, Cd, Cu, Fe, Mn, Mo, and Zn measured in the following dissected grain tissues: germ (*GM*), outer periphery (*OP*), inner periphery (*IP*), starchy endosperm (*SE*) and crease (*CR*). The concentration of a given element in a tissue was normalized by dividing the concentration of the tissue by that of the whole grain.