Ecophysiologie de l'allocation du cadmium au grain chez le blé dur

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Ecophysiologie de l'allocation du cadmium au grain chez le blé dur

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Titre : Ecophysiologie de l'allocation du cadmium au grain chez le blé dur

Résumé

Le cadmium (Cd) est un élément toxique. Les activités humaines ont contaminé un large éventail de sols agricoles. L'exposition de l'homme au Cd se fait majoritairement par voie alimentaire, notamment à travers les aliments de base tels que les céréales. Le blé dur accumule naturellement plus de Cd dans ses grains que les autres céréales. Une fraction significative de la production française de blé dur dépasse la limite réglementaire européenne fixée pour le Cd. Il est donc nécessaire de réduire l'accumulation de Cd dans les grains de blé dur. Cette thèse portant sur l'écophysiologie de l'allocation du Cd aux grains chez le blé dur a pour ambition d'aider au développement de stratégies agronomiques visant à réduire le niveau de contamination en Cd du blé dur et de ses dérivés.

Dans un premier temps, nous avons étudié la relation entre la structure de la biomasse aérienne et l'allocation de Cd aux grains. Nous avons fait l'hypothèse que la répartition de la biomasse aérienne entre pailles et grains était un facteur déterminant de l'allocation du Cd aux grains. Huit cultivars Français de blé dur - de hauteur de paille contrastée - ont été cultivés en présence de Cd. Comme prévu, le principal facteur expliquant la différence d'accumulation de Cd dans le grain était la structure de la biomasse aérienne. Les cultivars allouant une plus grande proportion de leur biomasse aérienne aux pailles - autrement dit les cultivars à longue tige - avaient tendance à accumuler moins de Cd dans leurs grains, car les tiges et les feuilles sont des puits de Cd en concurrence avec les grains lors de leur remplissage.

Les minéraux importés dans les grains proviennent soit de leur absorption directe par la racine après l'anthèse, soit de leur remobilisation depuis des réserves constituées avant l'anthèse. La deuxième partie de ce travail a été consacrée à déterminer l'importance quantitative de ces deux « sources » pour le Cd chez le blé dur, et de préciser comment leur contribution relative varie entre cultivars et avec le niveau d'azote (N). Le traçage isotopique a été utilisé pour suivre le flux de Cd absorbé après l'anthèse. L'impact du niveau d'azote a été testé en privant la moitié des plantes de N après l'anthèse, sur deux cultivars montrant une capacité contrastée à accumuler le Cd dans leurs grains. La contribution de la remobilisation a été estimé à 50%, ce qui signifie que la moitié du Cd accumulé dans les grains provenait du Cd prélevé après l'anthèse. Le Cd a été remobilisé à partir des tiges, peut-être des racines, mais pas à partir des feuilles. La contribution de la remobilisation n'a pas varié entre les deux cultivars, de sorte qu'aucune relation entre la « source » de Cd et son niveau d'accumulation dans le grain n'a été mise en évidence. La privation d'azote en phase de

remplissage a stimulé la remobilisation de N sans affecter celle de Cd, ce qui suggère que la remobilisation de Cd est un processus indépendant de la sénescence.

En troisième lieu, nous avons examiné comment les caractéristiques d'allocation de Cd aux grains étaient modulées par le niveau d'exposition au Cd. Cette question a été traitée en comparant les flux de Cd entre organes en phase de remplissage mesurés sur des plants de blé dur exposés à 5 ou 100 nM de Cd. L'augmentation du niveau d'exposition au Cd a pénalisé la croissance des plantes après l'anthèse et semblait accélérer la maturation des grains. Étonnamment, la concentration de Cd dans les parties aériennes a augmenté en proportion directe avec la concentration de Cd en solution nutritive, ce qui suggère une absence de régulation de l'absorption et de la translocation de Cd sur la fenêtre d'exposition testée. Au sein des organes aériens, l'allocation de Cd aux grains semble même favorisée à 100 nM, en raison peut-être de la saturation - à ce niveau d'exposition -des tissus séquestreurs de Cd situés dans les nœuds. En moyenne, la contribution relative des deux « sources » de Cd n'a pas été affectée par le niveau d'exposition au Cd.

Enfin, nous nous sommes intéressés à la localisation de Cd dans le grain. La localisation de Cd ainsi que celles des nutriments minéraux a été cartographiée par LA-ICP-MS sur des coupes transversales et longitudinales de grains contaminés en Cd. Ce travail a fourni la première carte de localisation de Cd dans un grain de blé dur. La distribution de Cd s'est caractérisée par une forte accumulation de Cd dans le sillon et par une dissémination dans l'endosperme amylacé plus prononcée que celle de Fe et Zn.

Mots clés : Faible-dose cadmium, sécurité sanitaire, variabilité intraspécifique, remobilisation, marquage isotopique, ablation laser.

Unité de recherche

Interactions Sol Plante Atmosphère – UMR 1391

INRA de Bordeaux, 71 avenue Edouard Bourlaux, CS 20032 - 33882 Villenave d'Ornon, France

Title: Ecophysiology of cadmium allocation to grains in durum wheat

Abstract

Cadmium (Cd) is a toxic element. Human activities have contaminated a wide range of agricultural soils. Most of Cd entering human bodies is through the dietary intake, and especially through staple food like cereals. Durum wheat naturally accumulates more Cd in its grains than other cereals. A significant fraction of the French durum wheat production has been found to exceed the European regulatory limit set for Cd. There is thus a need to reduce the accumulation of Cd in durum wheat grains. This thesis is dedicated to a better understanding of the ecophysiology of Cd allocation to the grains in durum wheat, with the ambition of helping to find agronomic strategies to reduce the Cd contamination level of durum wheat products.

In first, we investigated the relationship between the aboveground partitioning of Cd and the shoot allometry. We hypothesized that the partitioning of shoot biomass between grains and straws is a driver of the allocation of Cd to the grains. Eight French durum wheat cultivars differing in their stem height were grown in presence of Cd. As expected, the main factor explaining the difference in their grain Cd was the shoot biomass partitioning. Cultivars allocating a higher proportion of their aerial biomass to the straws, i.e. long-stem cultivars, tended to accumulate less Cd in their grains because stems and leaves are sinks for Cd in competition with developing grains.

Minerals imported into cereal grains originate from either direct post-anthesis root uptake or from the remobilization of pre-anthesis stores. The second part of this work was dedicated to determining the quantitative importance of these two pathways for Cd in durum wheat, and how their relative contribution vary between cultivars and with the level of nitrogen (N) supply. Stable isotopic labelling was used to trace the flux of Cd taken up post-anthesis. The impact of N supply was tested by depriving half of the plants of N after anthesis, in two cultivars showing a contrasted ability to accumulate Cd in their grains. The contribution of Cd remobilization was around 50%, which means that half of Cd in grains originated from Cd taken up pre-anthesis. Cd was remobilized from stems, possibly from roots, but not from leaves. The contribution of remobilization did not vary between the two cultivars so that no relationship between the pathway and the level of accumulation of Cd in grain was evidenced. Post-anthesis N deprivation triggered the remobilization of N without affecting that of Cd, which suggests that Cd remobilization is a senescent-independent process.

In third, we investigated how the characteristics of Cd allocation to the grains was affected by the level of Cd exposure. This question was addressed by comparing the fluxes of Cd during grain

filling in durum wheat plants exposed to 5 or 100 nM Cd. Increasing the level of Cd exposure penalized the plant growth after anthesis and seemed to accelerate the grain maturation. Surprisingly, the concentration of Cd in shoots increased in direct proportion with the concentration of Cd in nutrient solution, which suggests an unrestricted uptake and root-to-shoot translocation of Cd at 100 nM. Aboveground, the allocation of Cd to the grains was even promoted at higher Cd exposure, maybe due to the saturation of Cd sequestrating tissues in stem nodes. On average, the relative contribution of the two pathways for grain Cd loading was not affected by the level of Cd exposure.

In last, we focused on how Cd was distributed within durum wheat grains. The localization of Cd and mineral nutrients was mapped by LA-ICP-MS on transversal and longitudinal sections of Cd-contaminated grains. This work provided the first map of Cd localization in durum wheat grains. Cd distribution was characterized by a strong accumulation of Cd in the crease and by a non-negligible dissemination in the starchy endosperm, as compared to Fe and Zn.

Key words: Low-dose cadmium, food safety, intraspecific variability, remobilization, stable isotopic labeling, laser ablation.

Research unit

Interactions Sol Plante Atmosphère – UMR 1391

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题目：硬粒小麦分配镉至籽粒的生理生态学研究

摘要

镉是一种有毒的重金属元素。全球有大面积的农田受到镉的污染。膳食，尤其谷物，是镉进入人体的主要媒介。相比其他谷物，硬粒小麦常常能在籽粒中积累更多的镉。在法国的一项调查发现，有相当一部分硬粒小麦的镉含量超过了欧洲标准。因此，我们需要想办法来降低镉在硬粒小麦籽粒中的积累。本论文就是要深入研究硬粒小麦分配镉至籽粒的生理生态学，希望能够为制定相应农艺策略提供理论依据，以降低硬粒小麦食品的镉污染。

在第一部分，我们调查了镉与生物质在茎秆各部位间分配的关系。我们假设秸秆与籽粒间相对的生物量会影响镉向籽粒的分配。试验选用了 8 个茎秆高度各异的法国硬粒小麦品种。与预期的一致，茎秆生物质的分配是影响籽粒镉含量的主要因素。秸秆生物量较高的品种，也就是茎秆高度较高的品种，其籽粒中镉的含量往往较低。这是因为茎、叶同发育中的籽粒一样，都是镉积累的库，相互竞争。

矿质元素能通过 2 条途径运送到籽粒中去：一是花后即籽粒发育过程中根系的吸收，二是动用营养器官在花前的积累（再分配）。第二部分工作就是要对这两条途径对硬粒小麦籽粒镉积累的重要性进行定量分析，并且探究他们的相对重要性是否会随不同品种、不同的氮素营养水平而变化。我们使用了稳定同位素标记的手段来区别这两条途径。试验选用籽粒镉积累能力高和低 2 镉硬粒小麦品种。在花后，对一半的试材，我们切断了氮供给。研究发现，再分配对籽粒镉的贡献约为 50%。也就是说，籽粒中约一半的镉是在种子开始发育前吸收的。镉的再分配发生在茎，也有可能在根，但不在叶。再分配途径对 2 个品种籽粒镉的积累有着相似的贡献，说明了这 2 条途径与种间籽粒镉积累水平的差异之间没有关联。切断花后氮的供给促进了氮的再分配而未影响镉的再分配，说明后者是一个独立于衰老的过程。

在第三部分中，我们研究了镉的暴露水平是如何影响镉向籽粒分配的。在 5 或 100 nM 的镉暴露水平下，我们比较了镉在硬粒小麦籽粒发育期间的流动。研究发现，镉暴露水平的提高抑制了植物在花后生长，同时加速了籽粒的成熟。出乎意料的是，茎秆镉浓度的增加和营养液中镉浓度的增加比例一致。这说明在 100 nM 镉的水平下，根系

的镉吸收及镉从根到茎的转运都没有受到限制。在茎秆中，镉暴露水平的提高甚至促进了镉向籽粒的分配——这可能是由于镉在茎节的贮存达到了饱和。平均来看，花后吸收和再分配两条途径对籽粒镉积累的相对贡献没有受到镉暴露水平的影响。

在最后一部分，我们关注镉在硬粒小麦籽粒中的分布情况。我们使用 LA-ICP-MS 对受镉污染的籽粒的纵向和横向切片中的镉及矿质营养元素进行了定位。通过这部分工作，我们提出了首张镉在硬粒小麦籽粒中的定位图。镉分布的特征包括在籽粒腹沟处有较强的积累，以及，相比于铁和锌，显著地向胚乳中扩散。

关键词：低剂量镉；食品安全；种内变异；再分配；稳定同位素标记；激光烧蚀

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Table of Content

Chapter 1
General introduction

1.1 CADMIUM AND HUMAN

1.1.1 Cadmium poisoning

Cadmium (Cd) is a heavy metal contaminant. Its toxic effects to human have long been recognized and have widely concerned governments, researchers, and the public (Holdaway and Wang, 2018; Nordberg, 2009). The European Union included Cd in the Directive on the restriction of the use of certain hazardous substances in electrical and electronic equipment (Directive 2002/95/EC). The US Agency for Toxic Substances and Disease Registry (ATSDR) sets Cd as the 7th among 275 toxicants in its Priority List of Hazardous Substances considering the public impacts of Cd poisoning (ATSDR, 2017).

While occupational exposure to high-dose of Cd causes acute toxic effects, for the general population, the threat of Cd to heath is mainly attributed to the long-term low-dose exposure (Clemens *et al.*, 2013). Cd can remain in human bodies for decades (10-30 years of biological half-life), hence a long-term exposure can cumulatively increase the body burden of Cd over time and may cause adverse effects. The kidney is the main organ accumulating Cd and is the main target of chronic Cd poisoning (Xu *et al.*, 2018). The Cd-caused health effects are renal dysfunction, osteoporosis, lung cancer, diabetic nephropathy, hypertension, periodontal disease, age-related macular degeneration, and cardiovascular diseases (Faroon *et al.*, 2012). Itai-itai disease is the most infamous case of mass Cd poisoning, which happened to people living in the Jinzū River basin of Japan. The people suffered from severe spinal and leg pains. These manifestations have been attributed to multiple fractures and distortion of the long bones, which were caused by Cd-induced calcium (Ca) metabolism disorder in combination with disorders in tubular reabsorption of Ca by kidney (Järup and Åkesson, 2009; Nordberg, 2009). Cd is also a cancer-causing agent. The International Agency for Research on Cancer (IARC) classified Cd in the Group 1 carcinogens with sufficient evidence supporting its carcinogenicity (IARC, 2018).

1.1.2 Anthropogenic cadmium contamination

Cadmium is a natural trace element. Its average content in earth's crust is 0.1 μg kg^{-1}. Human activities have widely increased the background level of Cd in the environment (Figure 1-1).

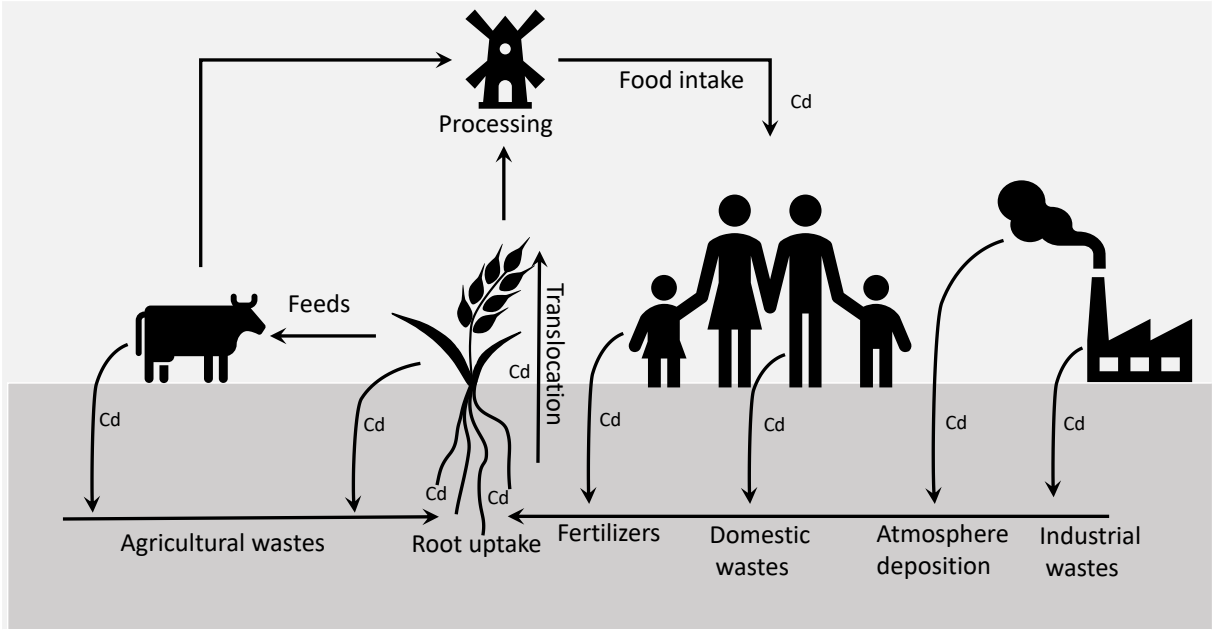

Figure 1-1 Cd flows in soils, crops and the food chain.

Cadmium is an important element used in industrial activities. Its main application is the manufacture of nickel-cadmium batteries, which accounts for 79% of the total consumption (source: International Cadmium Association (ICdA), data 2003). It is also intentionally added to pigments, plastic stabilizers, plating coats, and alloys. The high demand considerably boosted the production and consumption of Cd in the past century. The global refinery production of Cd has increased from about 6 000 tons $year^{-1}$ in 1950 to the 23 000 tons $year^{-1}$ in 2017 (source: the US Geological Survey, data 2018). The manufactures, uses, and wastes of Cd-containing products have significant impacts on Cd fluxes in the environment (Afolayan, 2018).

Mining and smelting Cd-containing ores, such as zinc (Zn) and phosphate (P) rocks, are sources of environmental contamination/pollution by Cd (McLaughlin and Singh, 1999). Cd in the wastewater and ore dusts do not only spread within the mining/smelting area, but could also discharge into the atmosphere, flow into rivers and percolate into soils resulting in a widespread contamination. The case of Itai-itai disease mentioned above is an example where Cd was discharged from the mine into the river water, which was then used to irrigate the paddy field. In the northeast of China, the use for irrigation of Cd-containing wastewaters from smelting caused

a serious and long-lasting contamination in some local agriculture productions (Liu and Zhen, 2008).

The repeated applications of agrochemicals, especially P fertilizers, causes significant anthropogenic inputs of Cd into agricultural soils. Cd naturally occurs in P rocks and contaminates the fertilizers through manufacturing unless a possible but high cost Cd removing process is carried out. In Europe, the applications of fertilizers is the second dominant source of Cd emissions to the environment (Pinot *et al.*, 2000). In France, about 54 tons Cd year^{-1} enter agricultural soils, predominantly (54%) from mineral fertilizers (ADEME/SOGREAH, 2007).

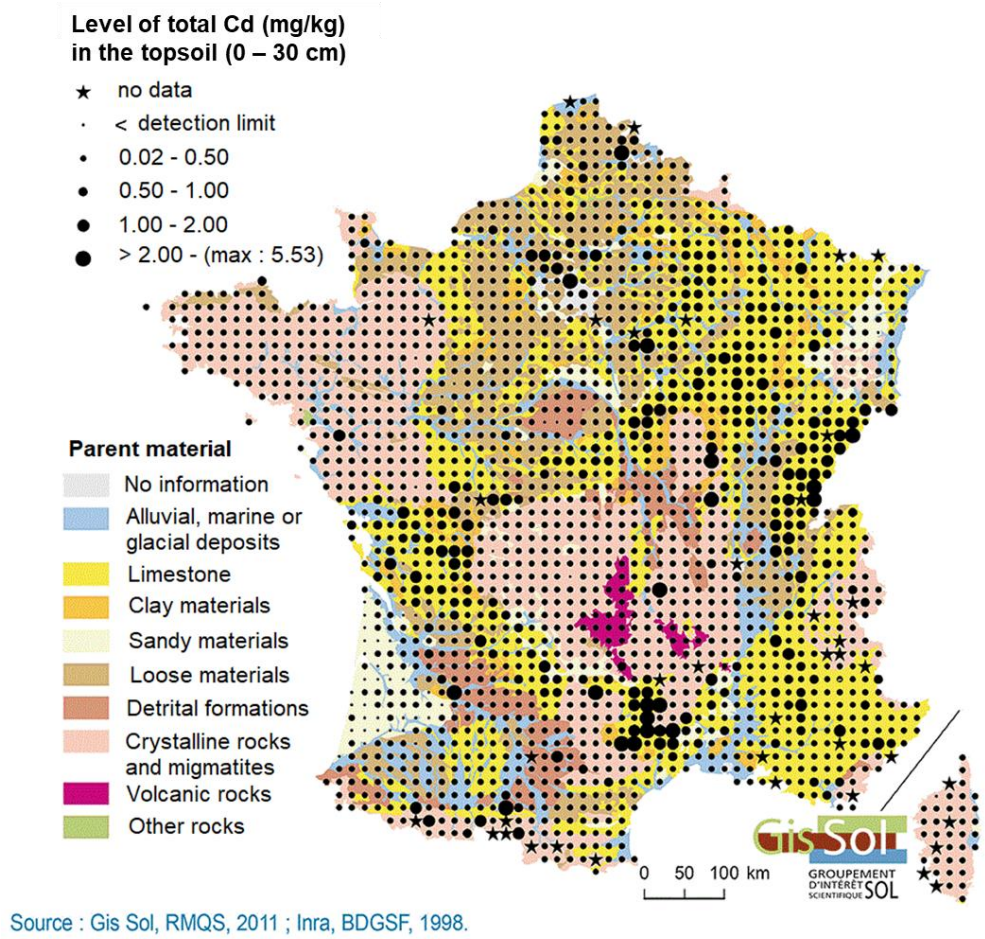

Figure 1-2 Concentrations of total Cd in the topsoil of France (translated from *https://www.gissol.fr*).

1.1.3 Cadmium in soils

Cadmium in soils is generally the main cause and the major source of Cd contamination for crops. The median level of total Cd in the topsoil is 0.15 mg kg^{-1} in Europe (van der Voet *et al.*, 2013) and 0.2 mg kg^{-1} in France (Figure 1-2; source Gis Sol-RMQS, data 2011). In France, in agricultural soils, the level of total Cd is a bit greater than the average for all top soils, namely 0.3

mg kg^{-1} for the median, but could be above 2 mg kg^{-1} (0.7% of frequency) in some hotspots (Mench and Baize, 2004). The content of soil in Cd increases in the following order: sandy soil < loam soil < clay soil, due to their different physico-chemical characteristics (McLaughlin and Singh, 1999). For instance, the median concentration of total Cd is 0.16 mg kg^{-1} in sandy soils while it is 0.44 mg kg^{-1} in clay soils and 0.56 mg kg^{-1} in heavy clay soils of France (Baize *et al.*, 2007). Moreover, the soil contents in Cd strongly depends on pedogenesis and tends to be higher in sedimentary rocks than in igneous rocks, but the latter one can importantly release Cd (Nagajyoti *et al.*, 2010).

Plant roots absorb Cd from the soluble fraction in the soil pore water. The soluble concentration of Cd was found to be a better indicator of Cd in mature durum wheat grains than the total soil Cd content (Viala *et al.*, 2017). In agricultural soils, the concentration of Cd in the pore water is generally in the nanomolar range (< 20 nM). The Cd in the soil pore water depends on the content of Cd that is exchangeable from the soil surfaces, which is depending on the soil physico-chemical properties, including soil pH, clay and organic matter contents, content of manganese oxides, redox potential, and type and content of organic and inorganic complexing ligands (McLaughlin and Singh, 1999; Nolan *et al.*, 2003).

Soil pollution is much more serious in newly industrialized countries, due to a rapid economic growth in combination with a long-period lack of awareness and of technics regarding environmental protection resulting in an inappropriate and poorly implemented environmental policy. For example, in China, the Ministry of Environmental Protection (MEP) reported that 19.4% of the farmlands have been polluted (according to the national standard in soil quality, GB 15618-1995), and 82.8% of them are polluted by heavy metals, Cd being the dominant metal (source MEP, data 2014).

1.1.4 Cadmium in foodstuffs

Food is the principal source (approx. 90%) of Cd exposure for non-smoking population (UNEP, 2010). In 1988, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) established a provisional maximum tolerable weekly intake for Cd of 7 µg kg^{-1} body weight (BW) (FAO/WHO, 1988). This tolerable limit was revised downwards to 5.8 µg kg^{-1} BW in 2010 (FAO/WHO, 2010). In Europe, in 2011, the European Food Safety Authority (EFSA) even recommended a lower weekly intake of 2.5 µg kg^{-1} BW in view of the up-to-date knowledge about chronic Cd toxicity (EFSA, 2011).

In average, the Cd exposure of the European population is close to the tolerable level, especially in some subgroups such as children and vegetarians, for which the levels of exposure are more frequently exceeded (Clemens *et al.*, 2013). In the French population, the tolerable weekly intake value set by the EFSA is exceeded by 0.6% of the adults and by 14.9% of the children (Arnich *et al.*, 2012). Meanwhile, the risk of Cd exposure increases with the deficiency of micronutrients, which is prevalent in developing regions, especially regarding iron (Fe) for young women. Fe deficiency in human, for example, may cause the upregulation of the divalent metal transporter 1 (DMT1) which also non-specifically mediates Cd^{2+} absorption and thereby can increase the accumulation of Cd (Vahter *et al.*, 2007). It is clearly a necessity of reducing dietary Cd exposure to improve the health of general population.

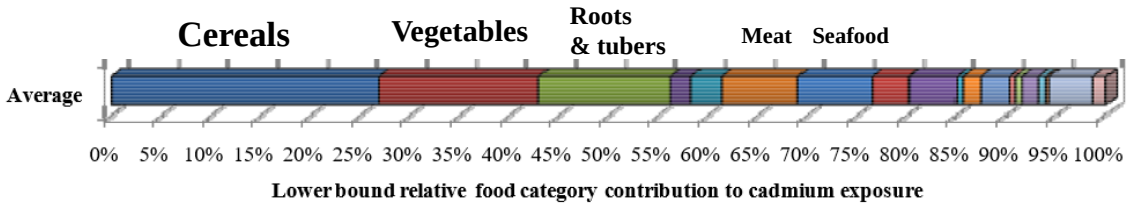

Figure 1-3 Contributions of different food categories (indicated by colors) to overall lower bound mean Cd exposure in the average population of Europe. The top 5 contributors are marked (extracted from EFSA 2012).

Cereals are staple foodstuffs. In Europe, about a quarter of the dietary exposure to Cd is due to cereals (Figure 1-3) (EFSA, 2012). In some Asian regions, such as the southern China and Japan, cereals could even account for more than 40% of Cd dietary intake (Gao *et al.*, 2006; UNEP, 2010). The European Union has set the threshold for the maximum levels of Cd in cereals: 0.2 mg kg^{-1} fresh weight (FW, i.e. the weight at harvest or to be consumed) for bran, germ, wheat and rice, and 0.1 mg kg^{-1} FW for other cereals (EC, 2008). However, following the EFSA recommendation of lowering the weekly intake of Cd from food, strong debates hold as to lower these threshold levels in foodstuffs, especially in cereals. A project was established by the Directorate-General for Health and Food Safety (DG SANCO) of the European Commission to lower the regulatory limit for Cd in durum wheat from 0.2 to 0.15 mg kg^{-1} (DGSanco, 2011). Reducing the accumulation of Cd in the edible part of cereal crops (i.e. grains) is a major issue and perhaps the most efficient way to protect the general population from Cd poisoning (Clemens *et al.*, 2013).

1.2 DURUM WHEAT

Cereals crops vary strongly regarding their abilities to absorb Cd and to accumulate it in the grain (Grant *et al.*, 2008). For instance, the content of Cd in aboveground parts generally follows the rank: oats < barley < common wheat < maize < durum wheat (Broadley *et al.*, 2001). It can be seen that durum wheat has a greater tendency to accumulate Cd in grains than other cereals (Figure 1-4) (McLaughlin and Singh, 1999). Consequently, the level of Cd in durum wheat is of special concern.

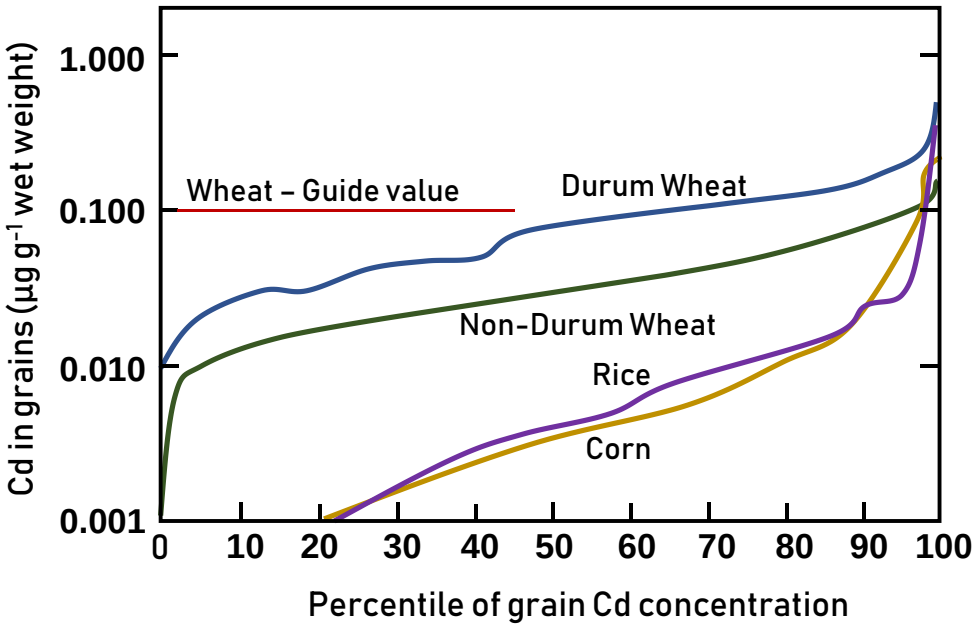

Figure 1-4 The statistical distribution of grain Cd concentration in cereal crops (extracted and redrawn from McLaughlin and Singh 1999).

1.2.1 The plant of durum wheat

Durum wheat (*Triticum turgidum* subsp. *durum*) is an annual *Poaceae* crop grown for its edible caryopses, i.e. grains. The grain is the hardest among all species of wheat, as indicated by the term 'durum', which means 'hard' in Latin. As an annual plant, the development of durum wheat plant can be divided into two stages: pre-anthesis or vegetative growth and the reproductive stage including flowering and grain filling (

Figure 1-5). Before anthesis, the plant accumulates dry matter in the vegetative organs, namely in roots, stems, and leaves. The inflorescence, also called the head of wheat, starts to emerge from the flag leaf sheath after tillering and during stem elongation. After anthesis and fertilization by pollen, the grain starts to fill, i.e., to accumulate dry matter. The endosperm cells divide and elongate rapidly in the early stage of the grain development in preparation for the substantial dry matter storage (Yu *et al.*, 2015a). The whole plant senescence is initiated during grain filling, the

leaves the closest to the developing grain senescing in last (Distelfeld *et al.*, 2014). The development of grain includes different steps of dry matter accumulation: milky stage and pasty stage. Afterwards, the grain filling/biomass accumulation stops at physiological maturity stage at which the grain reaches the maximum dry weight. Thereafter, only water is lost till harvest. Schnyder and Baum (1992) suggest that the physiological maturity of wheat is reached at 46% of kernel residual water (KRW). In the field, durum wheat grains are often harvested at a much lower water content for a safe storage. In France, the KRW at harvest is on average 12.1% (source: FranceAgriMer/Inland silos quality survey, data 2017).

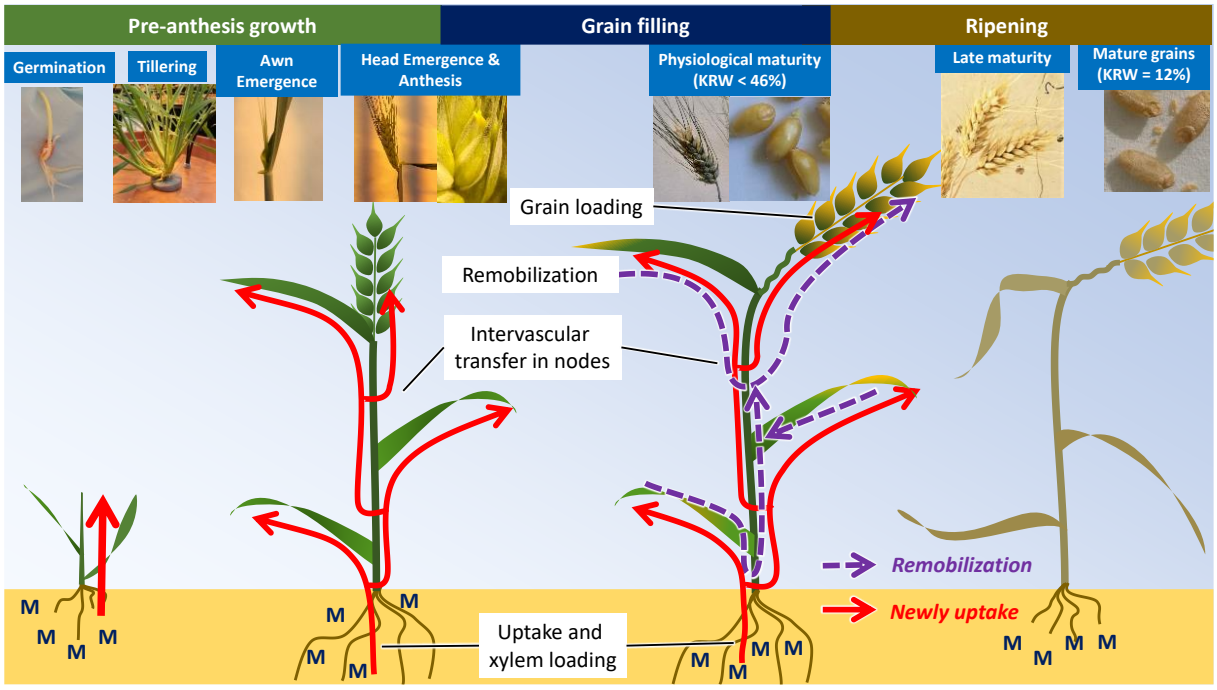

Figure 1-5 Schematics of key stages of durum wheat development and minerals transport from soils to grains during these stages.

Durum wheat is commonly cultivated in semiarid environments. The Mediterranean basin produces about 60% of the world durum wheat. In Europe, the great majority (> 80%) of the durum wheat production comes from the Mediterranean regions of Italy, Greece, Spain, and France (source: FranceAgriMer, data 2013). During the growing season, the climate of these areas is characterized by dryness and high temperature. More frequent drought and heat stresses are often associated with negative effects on grain filling, making the water supply often as a major limiting factor of the yield potential (Sissons *et al.*, 2012). For these reasons, breeding efforts have been widely directed towards improving the water-use efficiency and the yield potential of durum

wheat (Clarke *et al.*, 2010; Wang *et al.*, 2007). Due to its use in pastry industry, the protein content of grains is one of the major quality considerations for durum wheat. The low soil moisture conditions can restrict the availability of nitrogen (N) for the plant. Late N fertilization at the early stage of grain filling is a good strategy to improve the grain protein level.

1.2.2 Durum wheat production and consumption

France is the first producer and exporter of cereals in Europe and the second ranking exporter of wheat in the world (source: FranceAgriMer, data 2015). Durum wheat is one of the four main cereals in France following common wheat, maize and barley. The planted area of durum wheat in metropolitan France increased from 222 000 to 504 000 hectares between 1993 and 2011. In 2016, the area cultivated with durum wheat was 360 228 hectares (Figure 1-6), and the production was 1527 kilotons (source: Arvalis, data 2017). The main producing areas are the South-West (32% of the national production), the Center (26%), the Ocean-West area (24%), and the South-East (16%) of France. Numerous varieties of durum wheat are grown. In 2017, *Anvergur*, *Miradoux*, *Relief*, *Karur*, and *Casteldoux* were the five most widely planted cultivars in France, other important cultivars include *Sculptur* and *Pescadou* (source: FranceAgriMer/Inland silos quality survey, data 2017).

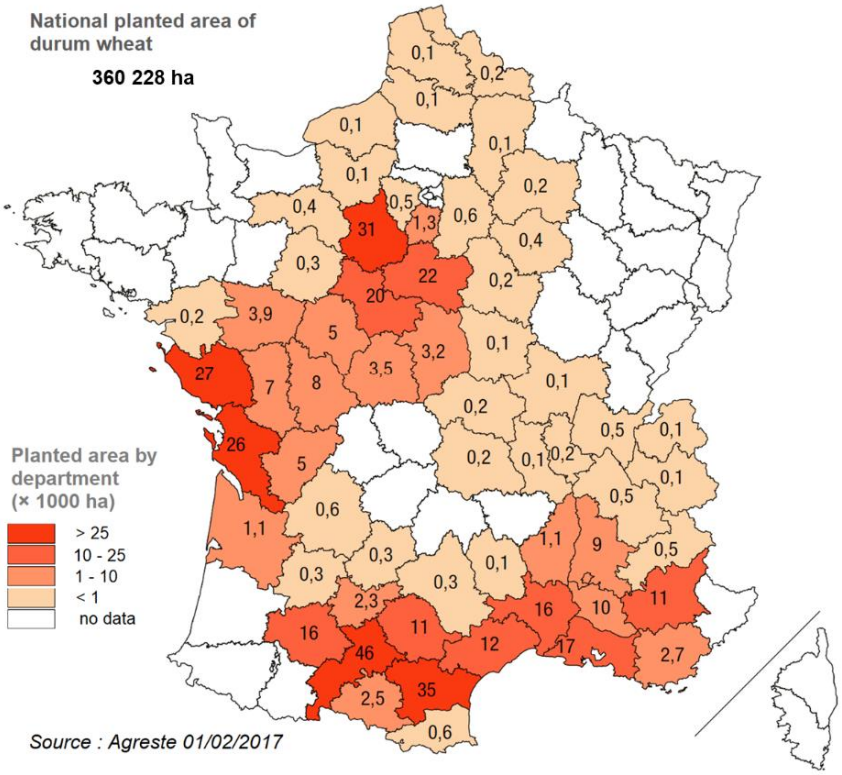

Figure 1-6 Planted area of durum wheat in France in 2016 (translated from *http://www.semencesdefrance.com*)

As a staple food, durum wheat-based foods supply people with carbohydrates, and to a lower extend they are a valuable source of protein, mineral nutrients, and dietary fibers. The general high contents of protein (14.8%, source: FranceAgriMer/Inland silos quality survey, data 2017) and gluten make durum wheat the best material to produce pasta. The grains are milled to produce durum semolina which is the raw material for pasta, macaroni, and couscous production (Sissons *et al.*, 2012). Durum wheat flour is produced from re-milled semolina, which can be used for baking applications. The milling by-products, namely the bran and the germ, are often added to foodstuffs in order to increase the dietary fiber contents and the nutrient value (Emanuelli *et al.*, 2014). Besides, durum wheat can be also consumed as whole grains after lightly dehulling and cracking.

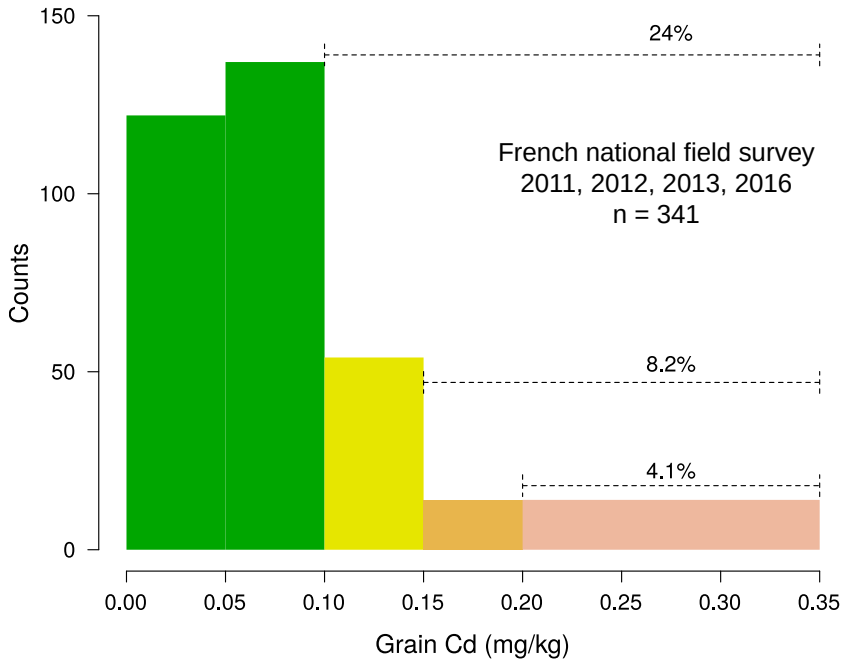

Figure 1-7 Distribution of Cd concentrations in durum wheat grains based on field investigations carried out in France (Nguyen *et al.*, 2017).

1.2.3 Cadmium in durum wheat

Concentrations of Cd in the grains of durum wheat can frequently be above 0.1 mg kg^{-1}, and sometimes exceed the limit of European and international trade regulations of 0.2 mg kg^{-1} (Grant *et al.*, 2008). The INRA-QUASAR (1998-2002) program investigated the level of Cd in 57 durum wheat grain samples over France. It ranged from 0.09 to 0.32 mg kg^{-1} dry weight (DW), and 15% of the samples were above the European maximum level for Cd in wheat grain (0.2 mg kg^{-1} wet weight ≈ 0.23 mg kg^{-1} DW considering 12% kernel residual water) (Mench and Baize, 2004). Two recent research projects (Arvalis-INRA project Cadur and ANR project CaDON) on a larger size of samples (n = 341) collected across 6 years showed that 4.1% of the durum wheat grains contain

Cd exceeding the European threshold of 0.2 mg kg^{-1} (Figure 1-7), while 24% were above 0.1 mg kg^{-1} which was the initial maximum level of the first draft for the revision of Cd limits in wheat (Nguyen *et al.*, 2017; DGSanco, 2011).

1.3 PHYSIOLOGY OF CADMIUM ALLOCATION TO GRAINS

The movement of Cd from soils to grains includes distinct mechanisms (

Figure 1-5). Briefly, Cd in soil is absorbed by roots as Cd^{2+} from the soil solution. Cd is then transported radially towards the stele and loaded into the xylem of the vascular bundles. After that, Cd is translocated upwards in the xylem sap by the transpiration stream and to a lower extend by the root pressure. Then, Cd is distributed among shoot organs. During grain filling, the Cd in vegetative tissues can be remobilized and allocated to developing grains (Uraguchi and Fujiwara, 2013). Cd has no biological functions for higher plants and moves in plants through pathways for mineral nutrients (Clemens, 2006). This is partly due to the lack of selectivity of the transporters that mediate nutrient transport. For example, Heavy Metal ATPase transporters (HMAs) are responsible for the root sequestration (e.g. HMA3) and for the xylem-to-phloem transfer (e.g. HMA2) of numerous heavy metals such as Zn and Cd (Sasaki *et al.*, 2014; Takahashi *et al.*, 2012; Yamaji *et al.*, 2013). The physiology of Cd allocation in plants is complex. Many processes have been studied on cereal crops, including wheat, barley, and rice, as summarized below. Rice (*Oryza sativa*) is a model species of *Poaceae*, on which more advanced and mechanistic results have been reported.

1.3.1 Root uptake and short-distance transport to the stele

Cadmium is first bond to the root apoplast, which includes the cell walls of epidermis and cortex since there is no exodermis in roots of wheat species. The cation exchange capacity (CEC) of roots, which is dependent on the number of negatively charged sites of the root cell walls, is strongly linked to the capacity of root to bind Cd from the soil solution (Greger and Landberg, 2008). Then, Cd is transported radially towards the root stele across the cortex by the apoplastic or symplastic pathways (Figure 1-8). Apoplastic transport refers to the movement of Cd in cell walls and extracellular spaces whereas the symplastic transport requires that Cd in internalized into the cytoplasm of cells and is then transported from cell to cell through the plasmodesma or sequestrated into the vacuoles. Before reaching the stele, the apoplastic pathway is interrupted by barriers formed by the thickened walls of root endodermis cells, i.e. the Casparian strip. To be

further transported into the root stele, Cd must cross the plasma membrane into the root symplast. However, the Casparian strip is not mature and fully developed near the tip of the root, which explains the higher influx of Cd in this region (Laporte *et al.*, 2014; Laporte *et al.*, 2013; Lux *et al.*, 2011; Piñeros *et al.*, 1998). Hence, a root system with more root tips can tend to absorb more Cd (Berkelaar and Hale, 2000). Meanwhile, Cd exposure may accelerate the formation of the apoplastic barrier toward the root tip as a defense mechanisms against Cd toxicity (Lux *et al.*, 2011).

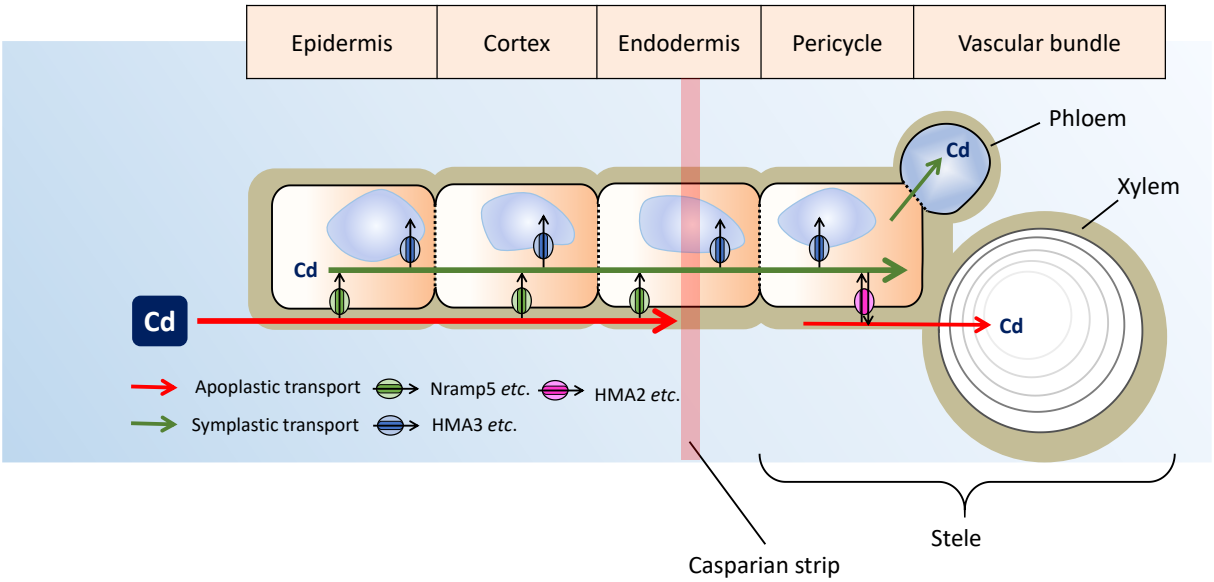

Figure 1-8 Schematic model of Cd uptake, radical transport and loading to the vascular bundle in roots of *Poaceae*.

Cadmium can enter root cells actively as an energy-depend influx mediated by transporters (Figure 1-8). Ion channels, such as the Ca^{2+} channel, allow the Cd influx, which is driven by the large negative electrochemical potential across the plasma membrane of root cells. The influx of Cd in roots is often modeled by a Michaelis-Menten function which reflects that the influx increases with increasing the external free Cd ion activity until the transporters are saturated (Tudoreanu and Phillips, 2004). The maximum rate of root influx (V_{max}) for Cd was reported to be between 0.87 and 38 nmol g^{-1} FW h^{-1}, and the affinity constant (i.e. K_m), which is the concentration of Cd in the solution corresponding to half of the V_{max}, was between 40 and 166 nM (Lux *et al.*, 2011). Given the low concentration of Cd (< 20 nM) in the pore water of agricultural soils, the root uptake of Cd is unlikely to be saturated in the field (Nolan *et al.*, 2003). This saturable relationship characterizes that Cd uptake is mainly transporter-mediated. The manganese (Mn) transporter natural resistance-associated macrophage protein 5 (Nramp5) plays a major role in Cd entry into the root cells of rice, barley and wheat (Peng *et al.*, 2018; Sasaki *et al.*, 2012; Wu *et al.*, 2016). Other

transporters, iron (Fe) regulated transporters 1 and 2 of rice (OsIRT1 and OsIRT2) were suggested to mediate the root uptake of Cd (Nakanishi *et al.*, 2006).

Once in the cytosol, Cd can subsequently move toward the stele through plasmodesmata, which forms a continuous symplastic pathway between cells (Figure 1-8). The radial movement of Cd can be restricted by retention in vacuoles, which limits the loading of Cd into the vascular bundle and therefore the long-distance transport of Cd within the plant (Clemens *et al.*, 2013). The vacuole-localized influx transporters of Cd HMA3 mediating Cd^{2+}/H^{+}-antiport transport and Cd sequestration in the vacuole is probably the most critical step of Cd sequestration in roots. In rice, the loss of function of OsHMA3 transporter significantly inhibits the sequestration of Cd in roots and results in a high Cd accumulation in shoots and grains (Yan *et al.*, 2016). In turn, overexpression of *OsHMA3* causes the opposite effects (Ueno *et al.*, 2010). In durum wheat, the low-Cd accumulating lines carry a major QTL, *Cdu1*, which promotes Cd retention in roots (Harris and Taylor, 2013). This *Cdu1* has been mapped and shown to be associated with the wheat orthologous of *OsHMA3* (Wiebe, 2012), but what this locus encodes for still need to be specified. Besides, Cd in root symplast can be complexed by ligands, such as phytochelatins (PCs) and metallothioneins (MTs), before being loaded into the vacuole (Lux *et al.*, 2011; Verbruggen *et al.*, 2009).

The apoplastic bypass is a pathway for Cd that allows to reach the root stele without the limitations of the apoplastic barriers at the endodermis of wheat. This low-restriction pathway exists at some root regions lacking apoplastic barriers, such as the root tip, the sites of emerging lateral roots or physically damaged root tissues. However, Van der Vliet *et al.* (2007) found that the contribution of this apoplastic bypass to the root-to-shoot translocation of Cd appears to be low in durum wheat grown in hydroponics at low-dose Cd exposure. The authors thus suggested that the symplastic pathway represented the major pathway for Cd radial transport into the root stele.

Roots possess a series of strategies to adapt to excessive Cd exposures. In the presence of excess Cd, roots can lower their exposure to Cd by reducing the phytoavailability of soil Cd, for instance through the exudation of organic acids that complex Cd. This is an example of how roots can contribute to regulate the rhizosphere chemical environment (Cieśliński *et al.*, 1998; Wang *et al.*, 2017). Roots can also limit the uptake and the transfer of Cd to the stele by reducing the production of root hairs, strengthening the apoplastic barrier, and regulating the expression of transporter genes involved in Cd uptake and vacuolization (Lux *et al.*, 2011; Ma *et al.*, 2015; Shahid *et al.*, 2016; Zhang *et al.*, 2012). Moreover, roots possess homeostatic mechanisms to limit the potential toxicity of free Cd ion present in root cells, which may include the biosynthesis of Cd ligands such as glutathione, PCs, and MTs (Kneer and Zenk, 1992; Lux *et al.*, 2011).

1.3.2 Long-distance transport and partitioning

Long-distance transport further allocates Cd among shoot organs, which starts from the loading of Cd into the xylem (Figure 1-8). In the root stele, Cd can be discharged from the cytosol to the apoplast and loaded by passive diffusion to the xylem (Clemens and Ma, 2016). A locus named Cd accumulation in leaf 1 (*CAL1*) has been recently identified in rice to be preferentially expressed in the xylem parenchyma cells of root vascular bundles. It drives the translocation of Cd from roots to shoots via the xylem by facilitating Cd efflux to the root apoplast in the stele before the xylem loading (Luo *et al.*, 2018). Apoplastic Cd can be loaded back to the symplast. In rice, a root pericycle-localized transporter OsHMA2 is hypothesized to drive the influx of Cd from the apoplast to the symplast, which facilitates the phloem transport towards root tips (Clemens and Ma, 2016; Yamaji *et al.*, 2013). Moreover, studies on the hyperaccumulator *Arabidopsis halleri* highlighted that the high expression of *HMA4*, which encodes a transporter loading root Cd into the xylem, is responsible for the large accumulation of Cd in shoots (Courbot *et al.*, 2007). HMA4 was also found to be important in the root-to-shoot translocation of Cd in the non-hyperaccumulator *Arabidopsis thaliana* (Wong and Cobbett, 2009).

Cadmium loaded into the root vascular bundle is translocated to shoots shortly thereafter. The xylem flow represents a major pathway of delivering Cd from roots to shoots and a major determinant of shoot and grain Cd accumulation (Uraguchi and Fujiwara, 2013; Uraguchi *et al.*, 2009). Cd in the xylem is mostly transported as free ion, and to a less extent, with organic acids (Tudoreanu and Phillips, 2004). In shoots, the movement of Cd in non-living xylem vessels follows the gradient in water potential resulting from high-transpiring parts. By contrast, Cd moves bidirectionally and more slowly in the living sieve tube cells of phloem. Owing to the alkaline environment and the elevated concentration of phosphates in the phloem sap, the efficiency of Cd transport in the phloem is dependent on the formation of soluble Cd-ligand complexes, which prevent precipitation of metals, and which include thiol-containing compounds such as PCs (Marschner, 2011; McLaughlin and Singh, 1999; Stolt *et al.*, 2003).

Transport of Cd in the xylem strongly depends on the transpiration stream, and therefore, if not reallocated by the phloem sap, Cd is strongly accumulated in high-transpiring tissues such as mature leaves and bracts (Clemens and Ma, 2016). Cd in the xylem sap is unloaded into apoplastic spaces of, for example, leaf mesophyll cells (McLaughlin and Singh, 1999). Taken up through the plasma membrane, Cd enters the leaf symplast and is stored in the vacuole (Figure 1-9) (Tudoreanu and Phillips, 2004).

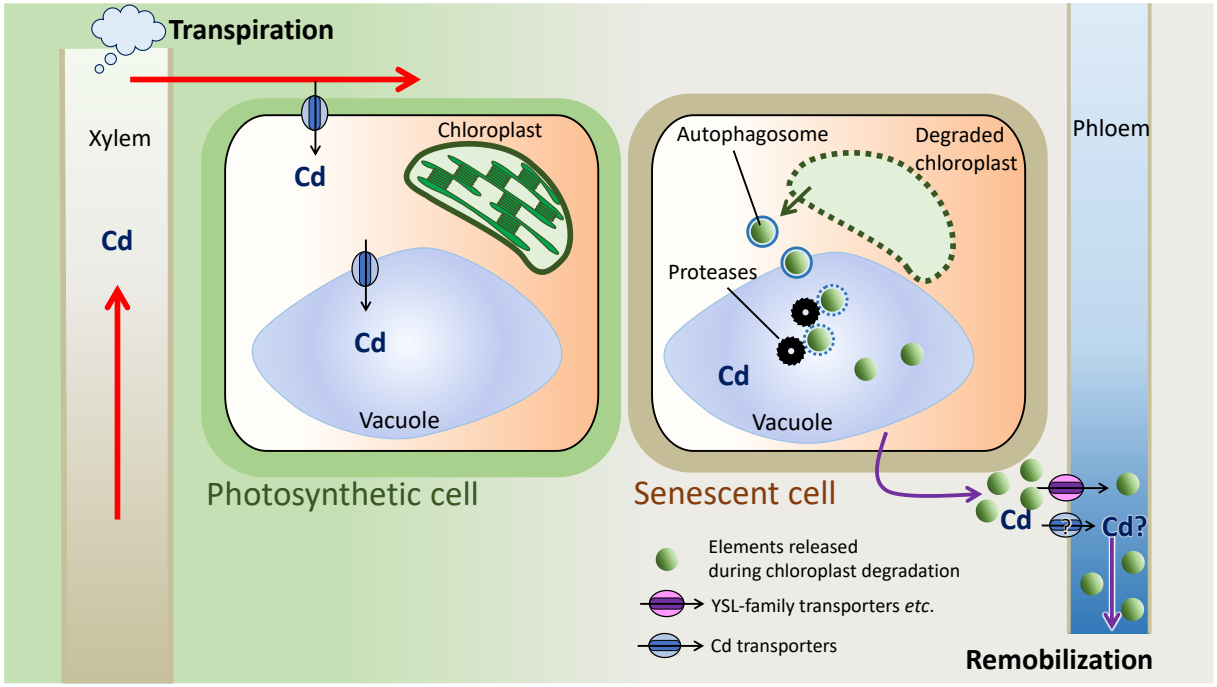

Figure 1-9 Loading of Cd in photosynthetic cells (left side); and minerals remobilized from the senescent cell (right side). Question mark indicate the unknown pathway of Cd transport.

The stem nodes are central in distributing elements to developing organs independently of the transpiration flow (Yamaji and Ma, 2017). Nodes joint stems, leaves and heads, possessing complex and well-developed vascular systems (Figure 1-10). In a simplified model of node in *Poaceae* (Yamaji and Ma, 2014a), elements coming in the xylem from roots or the lower node arrive in the node in an enlarged vascular bundles (EVBs). EVBs contains 10-time enlarged xylem vessels, where the transpiration flow and therefore that of elements are physically slowed down. This structure facilitates the xylem unloading consisting in uptake of the elements by the cells surrounding the xylem of the EVB. These cells are the companion cells that loads the phloem of the EVB (phloem parenchyma cells and companion cells; PPC&CC) and the xylem transfer cells (XTCs) and the parenchyma cell bridge (PCB). XTCs and PCB together transfer the elements to the xylem of the diffuse vascular bundles (DVBs), which itself connects the node to the upper parts of the plant. Elements in the DVBs xylem can move upwards or be withdrawn by the PPC&CC of the DVBs phloem. An apoplastic barrier in PCB can prevent the passive flow of elements via apoplastic pathway between EVBs and DVBs (Yamaji and Ma, 2017). Moreover, elements remobilized from leaves can be transferred from the phloem of EVBs to the phloem of DVBs through nodal vascular anastomosis (NVA) at the basal part of the node (Uraguchi and Fujiwara, 2013). Altogether, these mechanisms allow cross-links between the xylem and the phloem and the reallocation of elements both downwards back to the roots or upwards to the upper parts of the plant, including grains.

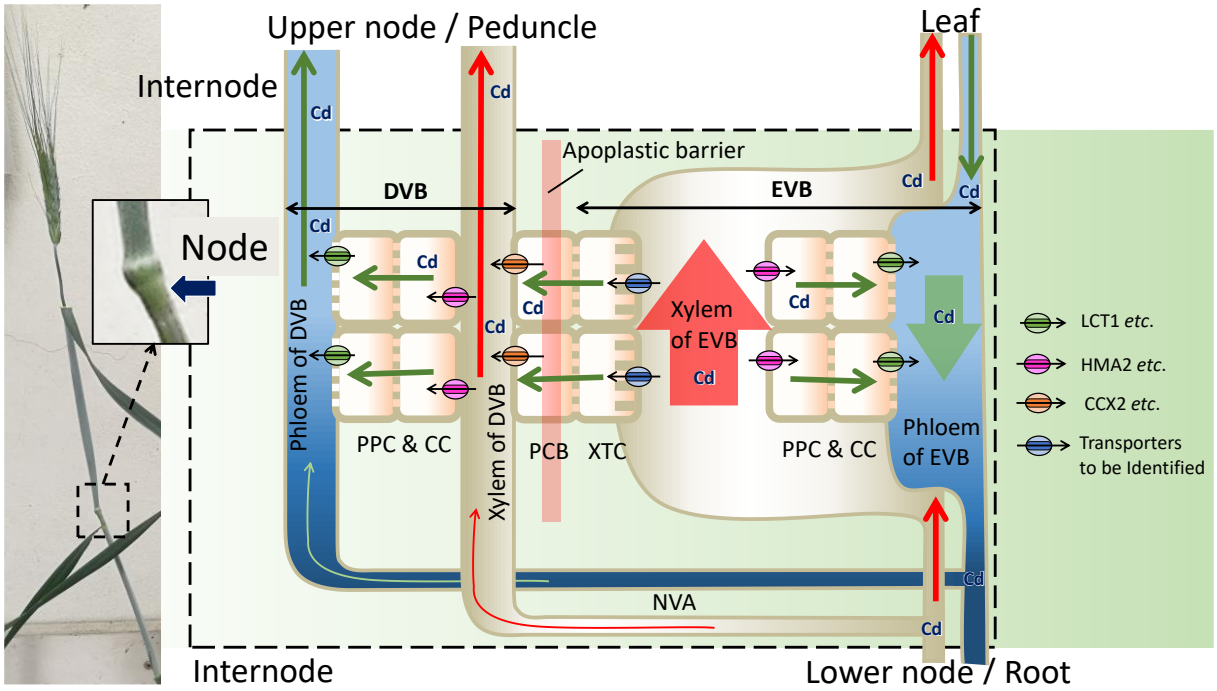

Figure 1-10 Schematic model of intervascular transfer of Cd in a *Poaceae* node from a longitudinal view. *DVB* diffuse vascular bundle; *EVB* enlarged vascular bundle; *NVA* nodal vascular anastomosis; *PCB* parenchyma cell bridge; *PPC&CC* phloem parenchyma cell and companion cell; *XTC* xylem transfer cell.

The node I is a node beneath the peduncle. It is connected to the stem, the flag leaf and the head, and plays a pivotal role in controlling the allocation of Cd to grains. First, phloem is the only pathway for Cd entry into grains, in particular because of the xylem discontinuity of grasses, which interrupts the xylem flow towards the head (Figure 1-11a) (O'Brien *et al.*, 1985), so the xylem-to-phloem transfer is a key step in grain Cd loading. Most of the Cd loaded in grains is first transferred from the xylem to the phloem in the node I (Clemens and Ma, 2016; Herren and Feller, 1997), probably in the DVBs which is connected directly to the head (Figure 1-10). The phloem parenchyma cells and companion cells in between of the phloem and the xylem mediate the xylem-to-phloem transfer of Cd. In rice, Cd transporters, OsHMA2 and OsLCT1, are involved in this process (Uraguchi *et al.*, 2011; Yamaji *et al.*, 2013). Second, many transporters are localized in the PCB and XTCs mediating the transfer of elements from EVBs to DVBs (Yamaji and Ma, 2017). It can be suspected that the transporter-mediated intervascular transfer of Cd could regulate the distribution of Cd between leaf and grains. Recently, a node-expressed transporter, cation/Ca exchangers 2 (OsCCX2) was found to contribute to the Cd loading from the PCB to the xylem of the DVB in rice (Hao *et al.*, 2018). Knockout of *OsCCX2* resulted in significantly lower concentrations of Cd in the grain. Third, Cd is more concentrated in nodes, especially in the node I, than in internodes (Fujimaki *et al.*, 2010). It underlines the potential ability of nodes to sequestrate and to filter Cd on its way to the grain. Cd in the node I is mainly found in the xylem of EVBs and

the PCB, associated with sulfur (S) and oxygen (O) containing compounds (Yamaguchi *et al.*, 2012). The nodal sequestration of Cd can be seen as a defense mechanism of plant to face excessive Cd exposure or as a collateral effect of micronutrient sequestration to adjust the delivery to growing tissues. As observed for Zn, storage of micronutrients in nodes allows a rapid delivery to the high demand of rapidly growing plant part (Suzui *et al.*, 2017). Significant accumulation in the node I was also found for arsenic (As) (Moore *et al.*, 2014), which is a toxic metal element with which Cd shares some specificity like its complexation by PCs. However, for mineral nutrients like N, there is no preferential accumulation in nodes (Harper and Lynch, 1981). Fourth, the efficiency of Cd transfer from the node I to grains is a function of the loading efficiency of DVBs. A smaller area of DVB can load relatively less Cd, which results in the higher sequestration of Cd in the node whereby less Cd can be transferred to the grain (Huang *et al.*, 2017).

Cadmium is toxic to plants, even at low levels (Shahid *et al.*, 2016). In wheat, the critical level of Cd toxicity is estimated as 5-10 μg Cd g^{-1} dry weight in leaves. Above, the plant growth is depressed (White and Brown, 2010). Excess Cd can disturb plant physiological and biochemical processes because Cd bind to S-containing enzymes due to its high affinity for thiols groups, also because it can compete with Zn for metalloproteins. For example, photosynthesis can be inhibited due to the down-regulation of photosynthesis-related gene, inactivation of photosynthetic carbon assimilation enzymes, chloroplast structure damage and decrease in stomatal conductance (Gallego *et al.*, 2012). Meanwhile, as reviewed by Rizwan *et al.* (2016), wheat plants possess several mechanisms to regulate Cd homeostasis to cope with Cd toxicity. For example, the complexation of Cd with sulphydryl-containing ligands in tissues could neutralize the potential toxicity of free Cd ion. Moreover, compartmentalization could limit the transfer of Cd to metabolically active tissues or cell compartments. Though the toxicology of Cd in plants has been widely studied, this point is not detailed here because it is out of the topic of the thesis, which mainly focuses on the response of plants to low-dose Cd.

1.3.3 Remobilization from vegetative organs to developing grains

Before the formation of grain, Cd is accumulated in vegetative tissues. After anthesis, the grain starts to fill and can accumulate both the Cd newly taken up by roots and the Cd remobilized from vegetative stores (

Figure 1-5). Thus, two sources potentially contribute to the loading of Cd in grains: direct root uptake and remobilization.

Remobilization is generally considered as a strategy of plants to provide enough nutrients to the grain when the uptake by roots is restricted – for instance in soils where the availability of a given nutrient is especially low. In other words, remobilization promotes the recycling of mineral nutrients and maximize the nutrient use efficiency. The remobilization of N is particularly obvious and is reported to account for 40-90% of the N in wheat grains (Kong *et al.*, 2016). To satisfy the sink demand of developing grains, the remobilization of Zn is strongly influenced by the plant nutrient status. It has been shown that almost all the grain Zn are from post-anthesis uptake when the supplies of Zn (and N) are high, whereas about 90% of the grain Zn can be attributed to the remobilization under Zn deprivation during grain filling (Kutman *et al.*, 2012).

Remobilization is often associated with plant senescence. Removal of reproductive head of wheat delays the leaf senescence (Patterson and Brun, 1980), whereas accelerating senescence increases the remobilization of N, Zn and Fe from leaves to grains (Uauy *et al.*, 2006). Autophagy is a cellular degradation process that makes the minerals stored in vegetative tissues available for the remobilization during senescence (Figure 1-9). A model on photosynthetic cells shows that decomposed organelles are encapsulated and transferred to the vacuole for degradation into minerals (Pottier *et al.*, 2014). Efflux transporters are thus needed to release minerals from the vacuole until the membranes becomes permeabilized at the final stage of cell death. It has been suggested that the link between remobilization and senescence depends on the cellular compartment and on the form in which elements are stored in source tissues (Maillard *et al.*, 2015). Chloroplasts are the first target of autophagy. Minerals such as N and Cu are highly concentrated in chloroplasts and their remobilization is frequently induced by senescence (Kong *et al.*, 2016; Maillard *et al.*, 2015). By contrast, vacuoles remain intact until late after the onset of autophagy. The remobilization of vacuole-stored elements such as S is less dependent on senescence (Abdallah *et al.*, 2011).

The phloem is the main pathway for the transport of remobilized elements. An element released by autophagy requires to be loaded into the phloem of source tissues in order to be used by growing sinks (Figure 1-9). As previously indicated, to avoid precipitation of metals in the phloem sap, they form soluble complexes. In rice leaves, the phloem loading is mainly mediated by nicotianamine (NA)-metal transporter such as copper (Cu)–NA transporter Yellow Stripe-like 16 (OsYSL16) (Khan *et al.*, 2014; Zheng *et al.*, 2012). However, the NA complex does not seem to be the binding form of Cd in cells (McLaughlin and Singh, 1999). The transporter mediating Cd remobilization from leaves blades, if there is one, is still unknown. Once loaded in the phloem, minerals can be transported toward the sinks. The phloem mobility appears to affect the efficiency of mineral remobilization (Riesen and Feller, 2005).

At least in nutrient solution, Cd can be remobilized from vegetative tissues to the developing grain (Figure 1-9). This has been evidenced by the recovery of ^{109}Cd in grains after labeling the flag leaf and stem (Harris and Taylor, 2001). However, the contribution of remobilization to the loading of Cd in durum wheat grains is still unclear. In other words, there is still a lack of conclusive evidence for the relative importance of pre- and post-anthesis origin of Cd in grain Cd accumulation. Harris and Tylor (2013) reported continuous Cd accumulation in all the organs of durum wheat grown in hydroponics and concluded that no net remobilization of Cd occurred in vegetative organs during grain filling. They suggested that the delayed senescence and unrestricted transpiration in hydroponics was suspected to favor the direct flow of Cd transport from the root to the grain. Similarly, Tavarez *et al.* (2015) observed a constant increase of Cd concentration in leaves of durum wheat after anthesis. They suggested that the lack of Cd remobilization from leaves was due to the storage of Cd in non-labile cellular compartments. By contrast, after a short-term (24 hours) feeding of ^{106}Cd to the nutrient solution at either anthesis or ripening stage of durum wheat, only little even no ^{106}Cd taken up by root can be found in the head (Chan and Hale, 2004). The authors thus concluded that remobilization represented the majority of the Cd loaded into the developing head. They explained that the low flux of post-anthesis uptake of Cd may be linked to the poor root activities in exporting Cd. In between these contradictory points of view, Rodda *et al.* (2011) showed on rice grown in hydroponics that the remobilization and post-anthesis uptake are of almost the same importance in grain Cd loading. The authors supplied Cd to plants at three different timing regimes: continuously throughout the life cycle, only before or only after anthesis. Cd concentrations in the mature grains after supplying Cd before and after anthesis are respectively about 60% and 44% of that with continuous Cd supply.

As shown by these studies, there are multiple approaches to assess element remobilization. The 'apparent remobilization' method is the simplest one, which is commonly used not only for Cd (Harris and Taylor, 2013; Tavarez *et al.*, 2015) but for other elements (Kutman *et al.*, 2011a; Maillard *et al.*, 2015; Masoni *et al.*, 2007; Xue *et al.*, 2012). This approach relies on the budget of the quantity of an element in plant organs at different growth stages. An organ is considered as a source of remobilization when its amount of an element decreases. Meanwhile, any plant part that shows a net accumulation of the element including the developing grains is considered as a sink receiving elements remobilized from vegetative organs. In this approach, only the net changes in contents are measured and an organ can both remobilize while absorbing Cd, and the net remobilization underestimates the true gross one in this case. By contrast, isotopic labeling is a more appropriate approach. Isotopes applied at a specific period allow to distinguish and to trace different sources of an element. The labeling can be performed as a short-term pulse that lasts for hours or days

(Chan and Hale, 2004; Hegelund *et al.*, 2012) or can extend throughout a longer period (Wu *et al.*, 2010). Short-term labeling results are strongly depending on the plant status at the time of labeling, such as the root uptake activity and the translocation rate transfer, and it may be an issue to extrapolate the instantaneous fluxes for a longer period. To assess more accurately the remobilization of Cd from pre-anthesis stored pool throughout the whole grain filling period and to know the contribution of remobilization to grain Cd, long-term labeling is more appropriate. This approach has been used for Zn (Wu *et al.*, 2010), but never for Cd.

1.3.4 Loading and storage in grains

Grains are both the offspring and the edible part of durum wheat. A mature grain is composed of germ and endosperm, which are filial tissues derived from fertilized cells, and the maternal tissues enveloping the filial tissues, which develops from the ovular integuments (Figure 1-11). The germ is composed of the embryo with the root and shoot primordia that will develop into the plant of next generation. The scutellum in the germ supplies nutrients from the endosperm to the embryo during grain germination (Lu *et al.*, 2013). The endosperm is the largest part of the grain in size and thus the part mainly consumed by humans. Most of the endosperm is starchy. The starchy endosperm is enclosed by the monocellular layer of aleurone. The maternal peripheral envelop contains multiple layers. From outside to inside, it can be separated into outer and inner pericarp, seed coat and nucellar epidermis. The wheat grain has a crease in the ventral side that extends deeply inward. In the crease region, the major vascular bundle is embedded into the pericarp and extended to the distal end of the grain. The mechanistic knowledge about Cd loading and distribution in wheat grains is extremely limited. By contrast, studies have been conducted broadly on the transfer of carbohydrate, and to a less extent, of mineral nutrients in the developing grain. The information available for nutrients are possibly relevant for the transport route of Cd inside grains (Figure 1-11).

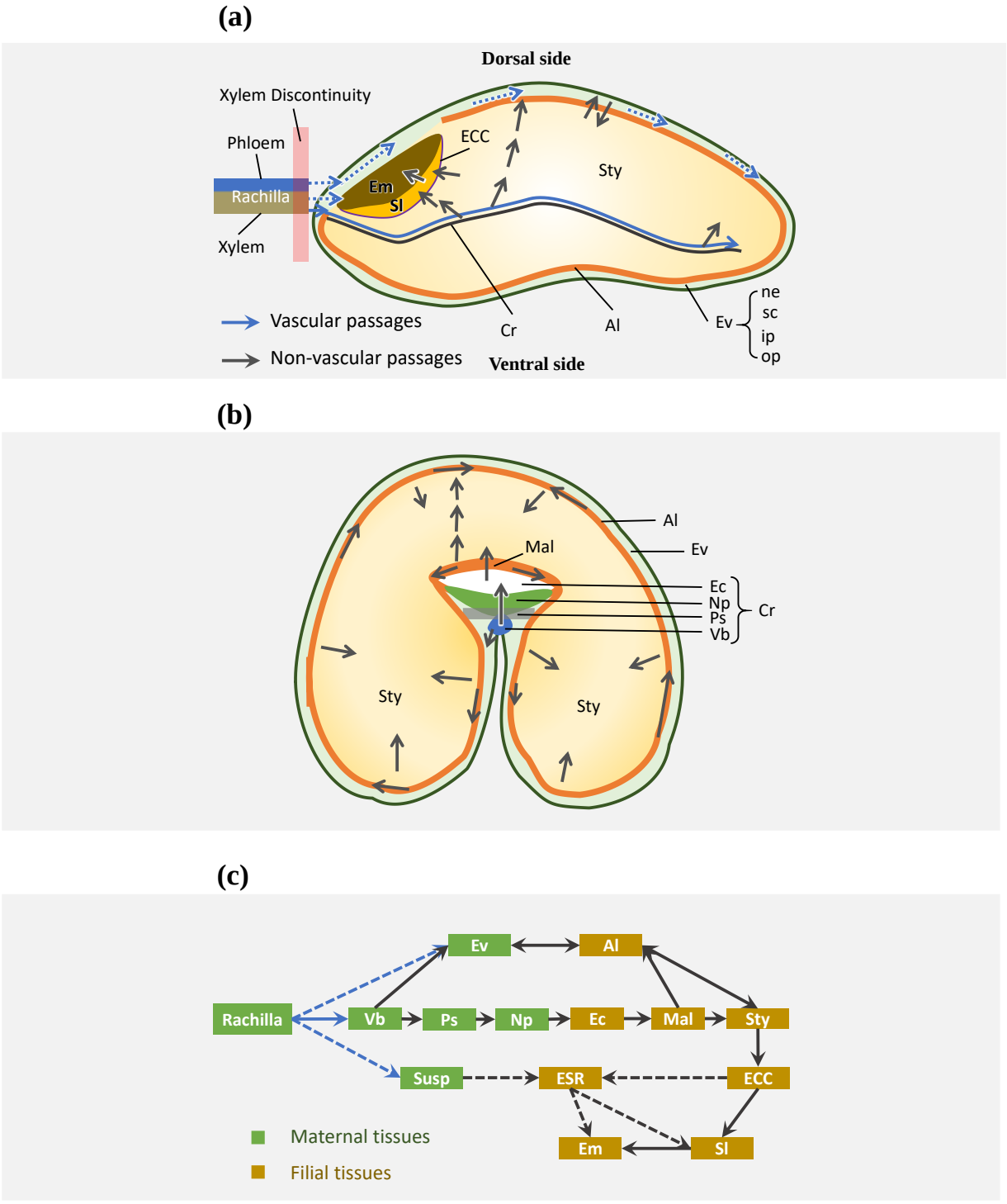

Figure 1-11 Schematic of nutrient transfer to the different parts of a developing wheat grain from a longitudinal (a) and a transversal (b) sectional view; and a summary of the pathways (c). The dashed lines indicate that the pathway persists partway through the grain filling period. *Al* aleurone layer, *Cr* crease, *Ec* endosperm cavity, *ECC* endosperm conducting cell, *Em* embryo, *ESR* embryo surrounding region, *Ev* envelop, *ip* inner pericarp, *Mal* modified aleurone, *ne* nucellar epidermis, *Np* nucellar projection, *op* outer pericarp, *Ps* pigment strand, *sc* seed coat, *Sl* scutellum, *Sty* starchy endosperm, *Susp* suspensor, *Vb* vascular bundle.

Nutrients can only reach the grain through the phloem because the xylem pathway is interrupted by thickened walls between the rachilla and the grain, also known as the xylem discontinuity (Figure 1-11a) (Zee and O'Brien, 1970b). The crease represents the major route for nutrients entry into the filial tissues (Figure 1-11c) (Fisher and Gifford, 1986). In the crease, nutrients unloaded from the vessel are absorbed by the nucellar projection, then transferred inward and discharged into the endosperm cavity (Figure 1-11b) (Wang *et al.*, 1995; Yu *et al.*, 2015a). This pathway is blocked gradually with the grain development by the maturation of pigment strand in between the vascular bundle and the nucellar projection (Zee and O'Brien, 1970a). The endosperm cavity is an apoplastic structure at the interface of grain maternal and filial tissues, where transporters are required for the efflux from nucellar projection and the influx to the modified aleurone layer (Patrick and Offler, 2001). Nutrients in the aleurone cells can be transferred through the extensive plasmodesmata circumferentially to the dorsal side of the grain or radially into the starchy endosperm (Morrison *et al.*, 1975; Patrick and Offler, 2001; Pearson *et al.*, 1998). Meanwhile, aleurone cells have myriad vacuoles that can intensively sequestrate nutrients (Morrison *et al.*, 1975).

Maternal peripheral tissues can obtain nutrients from the mean vessel within the crease as well as from the non-crease protophloem strands located in the pericarp (Figure 1-11a). There are three protophloem strands which can persist at least during the early period of grain development (Fisher, 1990). Two of them extends in the lateral part to the distal end of grain, while the third runs partially in the dorsal side opposite the crease (Fisher, 1990). Moreover, Pearson *et al.* (1998) suggested that the solution secreted from the aleurone may provide nutrients to the pericarp. With the grain maturation, the maternal peripheral tissues can be degraded and remobilize nutrients to the filial tissues (Morrison, 1976; Pearson *et al.*, 1998; Yu *et al.*, 2015b).

Nutrients can enter the germ with the help of embryo surrounding region (ESR) during about the first two weeks after anthesis (Figure 1-11c) (Yu *et al.*, 2015a; Yu *et al.*, 2015c). The ESR can absorb nutrients through the suspensor connecting directly to the rachilla or obtain nutrients from the starchy endosperm through the endosperm conducting cells (ECCs) (Sabelli and Larkins, 2009; Yu *et al.*, 2015c; Zheng *et al.*, 2014). The suspensor and ESR are degenerated after this period, and thereafter the endosperm is the only nutrient source for the germ (Figure 1-11a). Aleurone cells trigger the digestion of endosperm cell walls and mobilize nutrients stored in the endosperm (Sabelli and Larkins, 2009). The ECCs transfer nutrient from the endosperm to the scutellum of germ and further to the developing embryo (Yu *et al.*, 2015c; Zheng *et al.*, 2014).

It has been suggested that Cd is mainly associated with biological macromolecules in cereals (Günther and Kastenholz, 2005). In the mature grain of wheat, Cd is bound to proteins with

molecular weights of 54.5 kDa and 5.5 kDa (He *et al.*, 2002). In rice grains, Cd is associated with S-containing amino acids including cysteine and methionine (Wei *et al.*, 2017). It is known that Cd is not evenly distributed in mature wheat grains. The pearling of wheat grain (removal of grain envelopes) showed that Cd is more concentrated in the outer parts than in the inner part of the grain, but in quantity, around half of the Cd is retained in the residual pearled kernel (Figure 1-12) (Giordano and Blandino, 2018). A dissection experiment provided more detailed information about the gradient Cd concentration among grain tissues: germs > aleurones > maternal peripherals > starchy endosperms (Pieczonka and Rosopulo, 1985). Thanks to advances in imaging techniques it is possible to obtain *in situ* information about element spatial distribution in wheat grains (Lombi *et al.*, 2011a). For instance, X-ray fluorescence spectrometry can be used to map numerous micronutrients in the wheat grain (De Brier *et al.*, 2016). However, the grain map of Cd is still limited. This is partly due to the relative low abundance of Cd in tissues of wheat grains, which makes it more difficult to detect by imaging methods like synchrotron-based techniques (Clemens and Ma, 2016; Zhao *et al.*, 2014).

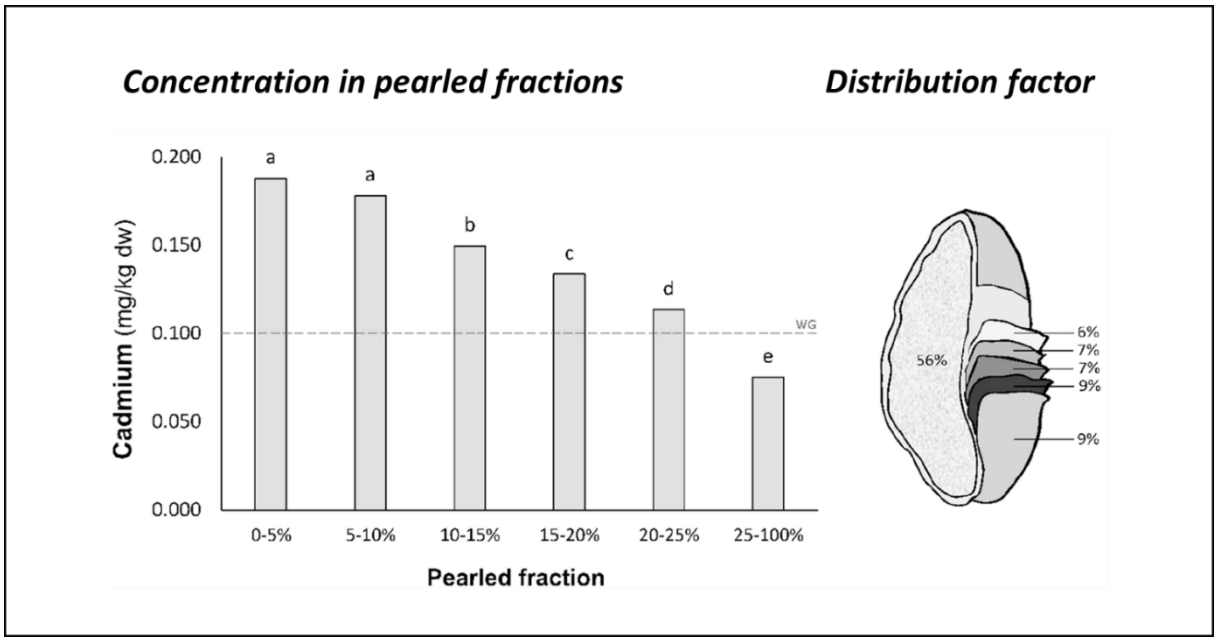

Figure 1-12 Average concentration and distribution factor of Cd in pearled wheat fractions. Bars indicate the mean values of six wheat cultivars harvested in 2014 and 2015 (n = 18). They are significantly (p < 0.001) different when labeled with different letters. The distribution factor was calculated considering both the Cd concentration and the relative weight of each fraction in comparison to the whole-grain flour (WG) (Giordano and Blandino 2018).

1.4 WAYS TO MINIMIZE THE LEVEL OF CADMIUM IN DURUM WHEAT-BASED FOODS

1.4.1 Agriculture practices

Theoretically, the level of Cd in durum wheat grains can be modified to some extend by agricultural practices that change the phytoavailability of soil Cd (McLaughlin and Singh, 1999). Reducing the input of Cd especially through P fertilization can limit or even decrease the total Cd present in the soil (Baize *et al.*, 2003). Maintaining a soil pH close to neutrality can limit the solubility of Cd^{2+} in soils, which can be achieved by adding soil amendments such as agricultural lime to acid soils and limiting the use of acidifying fertilizers such as ammonium and sulfur (Rizwan *et al.*, 2016). Applying organic matters with high sorption capacities, such as manure and biochar, can also decrease the abundance of soluble Cd in soils. In contrast, the phytoavailability of soil Cd can be promoted by increasing the formation of labile Cd complexes such as those with chloride (Cl) and soluble organic matters. These practices result in changes in the physiochemical conditions of soil, which may influence the availability of other nutrients as well. Mineral nutrient status may indirectly affect the level of Cd in grains by physiologically influencing root uptake and partitioning processes (Sarwar *et al.*, 2010).

Cultivar selection is another way to reduce the concentration of Cd in durum wheat grains. Canadian durum breeding programs considered grain Cd level as a selection criterion since 1990s and have released low-Cd cultivars such as *Strongfield* and *Arcola*, which can have around 3 times less Cd in grains than the normal high-Cd cultivars (Clarke *et al.*, 2010). Recent advances in the genetics of Cd uptake and allocation could be useful to guide the breeding efforts towards low-Cd varieties. For example, 80-90% of the phenotypic variation in grain Cd concentration between low- and high-Cd lines can be explained by a single locus, namely *Cdu1*, on chromosome 5B (Wiebe *et al.*, 2010). However, this breeding strategy has not yet been initiated in Europe. In France, it was found that 75% of the durum wheat cultivars do not possess the low-Cd accumulating allele at *Cdu1* (Zimmerl *et al.*, 2014). Breeding and assessing new cultivars is a long-term process because not only Cd accumulation but also agronomic performances such as the productivity and the nutritional quality have to be taken into account. Even so, the performance of low-Cd cultivars is often mainly adapted to local agricultural context, including the climate, soil characteristics, and management practices (Grant *et al.*, 2008). Therefore, combining low-Cd cultivars of durum wheat with proper field management would be more effective to limit the transfer of Cd from the soil to the grain.

1.4.2 Post-harvest practices

The uneven distribution of Cd in grains provides the possibility to reduce the level of Cd in the durum wheat-based foods by adapting milling processes to remove specific parts that are rich in Cd. In the milling of wheat, higher concentration of Cd can be found in the bran, which mainly contains the outer parts of the grain (Guttieri *et al.*, 2015). Meanwhile, high fractions of the mineral nutrients such as Zn can also be removed with the milling bran (De Brier *et al.*, 2015). It underlines that efforts to remove Cd enriched parts of the grain may lead to a strong loss of mineral nutrients at the same time. The development of fractionation techniques in wheat processing provides opportunities for removing more specifically the Cd-rich parts of the grain while retaining as much as nutrients in the fractions employed for food manufacture (Delcour *et al.*, 2012). However, there is still a lack of detailed picture on the spatial distribution of Cd and mineral nutrients in durum wheat grains.

1.5 STRUCTURE AND AIMS OF THE THESIS

Durum wheat contamination by Cd is a serious concern in terms of food safety. Therefore, understanding the mechanism of Cd transfer from soils to grains is of major importance. On the one hand, higher level of phytoavailable Cd in soils is the main cause of the elevated accumulation of Cd in the grain. Many works have been dedicated to understanding the processes governing the phytoavailability of Cd in agricultural soils (McLaughlin and Singh, 1999). They provided practical advices for agronomic actions to limit the level of free Cd in the rhizosphere, such as maintaining a neutral pH in the soil (Rizwan *et al.*, 2016). In parallel, building mechanistic models to predict the soil Cd phytoavailability helps to better understand it (Cornu *et al.*, 2017; Lin *et al.*, 2015; Schneider *et al.*, 2018). On the other hand, understanding the processes controlling the in planta allocation of Cd to the grain could help to lower the grain contamination in Cd (Clemens *et al.*, 2013). For instance, the efficient root sequestration of Cd limits the allocation of Cd toward the grain in Canadian low-Cd lines of durum wheat (Harris and Taylor, 2013), as well as in rice and in common wheat (Clemens *et al.*, 2013), which underlines the key role played by the root-to-shoot translocation in influencing the grain Cd level. However, huge variability was observed among low-Cd lines of durum wheat in their grain Cd concentrations across years and growing sites (Zimmerl *et al.*, 2014). There must be other processes other than root uptake and root-to-shoot translocation that influence the grain Cd accumulation.

The basis hypothesis of this thesis is that, in aboveground, there are mechanisms controlling the allocation of Cd to the grain that could be alternative ways to minimize the grain Cd content. These mechanisms include the sources of grain Cd, namely direct root uptake or remobilization during grain filling from vegetative parts, and their potential interactions with the nutrient status and level of Cd exposure. The ecophysiological approach was chosen to gain knowledge about the partitioning of Cd in plants, because the results can be more readily converted into agronomical practices. Additionally, studies that include the reproductive stage are scarce so that the knowledge regarding the ecophysiology of Cd allocation to the grain is limited. Hence, this thesis aimed at more deeply understanding the dynamics of Cd during the grain filling stage, in durum wheat plants exposed to low doses of Cd, in order to help reducing the concentration of Cd in its grains. The thesis is organized in four parts dedicated to specific aims:

In the first part (Chapter 2), we characterized the between-cultivar variability in grain Cd in durum wheat and tested the hypothesis that the partitioning of shoot biomass explains part of the variability in grain Cd observed among French cultivars.

In the second part (Chapter 3), we quantified to which extent Cd remobilization contributed to the amount of Cd accumulated in durum wheat and assessed how this contribution was affected by a change in post-anthesis nitrogen supply.

In the third part (Chapter 4), we examined how changes in the level of Cd exposure - at ranges comparable to that found in agriculture context - may affect the post-anthesis dynamics of Cd in durum wheat.

In the last part (Chapter 5), we enlightened the spatial distribution of Cd and mineral nutrients in durum wheat grains and tested how the removal of specific grain parts during the milling process may reduce the concentration of Cd in durum wheat products without strongly lowering their nutritional value.

Chapter 2

Variability in grain cadmium concentration among durum wheat cultivars: impact of aboveground biomass partitioning

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The level of Cd in durum wheat grains is the main concern of the thesis. As presented in this chapter, the first experiment was dedicated to investigating the variability in grain Cd concentrations among 8 cultivars that are commonly grown in France. Their different traits related to Cd uptake and Cd partitioning during the whole growth cycle were characterized to understand the ecophysiological process responsible for the intraspecific variability in grain Cd.

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Abstract

Aims: Varietal screening was conducted to characterize how French durum wheat lines (*Triticum turgidum* L. subsp. *durum*) differ in the concentration of cadmium (Cd) in their grains and to identify the main (eco) physiological processes behind these differences.

Methods: Eight French and two Canadian durum wheat lines were grown hydroponically in a nutrient solution with a low concentration of Cd (2 nM). At maturity, the partitioning of biomass and Cd among organs was analyzed and elemental profiles of the grain were obtained.

Results: Grain Cd concentration ranged from 0.03 to 0.08 μg g^{-1} and was thus in the same range as that measured in field trials. The level of Cd in the grain was correlated with levels of P, Mn and Zn. French lines behaved like high-Cd cultivars. A 2.4-fold variation in grain Cd was observed within French lines, which was not explained either by a difference in uptake or by a difference in the root sequestration of Cd. One important finding is that the leaf biomass was the most influential variable explaining the genotypic variation in grain Cd observed within French lines.

Conclusions The partitioning of aboveground biomass may influence the concentration of Cd in grain, in addition to the sequestration of Cd in roots.

Keywords

Low-dose cadmium, cereal, genotypic variation, shoot sequestration, stem height.

2.1 INTRODUCTION

Cadmium (Cd) is toxic for humans and damages, notably the kidney function, even at very low concentrations (Järup *et al.*, 1998). Many areas of arable soils in the world are contaminated with Cd through the use of sludges, phosphate fertilizers and/or irrigation water containing Cd (McGrath *et al.*, 2001). The soil-to-plant transfer of Cd is often more intense than that of trace elements like Cu and Pb, notably because of the relatively high phytoavailability of Cd in the soil (Adriano, 2001). Excess Cd accumulation in crops is thus a common problem around the world, especially in cereals, a major component of the human diet (McLaughlin and Singh, 1999). Durum wheat is the fourth most important cereal crop in France after bread wheat, grain corn and barley in terms of cultivated area (330 000 ha) and grain production (1.7 million tons per year) (source Arvalis-FranceAgriMer, data 2013). The problem of cadmium is particularly important in durum wheat since this species accumulates more Cd than other commonly grown cereals. Cd accumulation increases in the following order: rye < barley < oats < bread wheat < durum wheat (Jansson, 2002). It is thus very important to reduce the accumulation of Cd in durum wheat grains to below the maximum concentration of 0.2 μg Cd g^{-1} allowed in Europe (EC, 2006).

One way to minimize the Cd load in durum wheat grains is to adapt mineral nutrition (Sarwar *et al.*, 2010), for instance in nitrogen (Gao *et al.*, 2010; Gray *et al.*, 2002) or in silicon (Rizwan *et al.*, 2012). Another way is using low-Cd accumulating cultivars, i.e. cultivars with a low ability to accumulate Cd in the grain. This option first requires evaluating to what extent the concentration of Cd in the grain varies among durum wheat cultivars and identifying the main processes responsible for the differences. This approach was first used in Canada in the 1990s and revealed that the concentration of Cd in the grain can vary up to a factor 4 among Canadian lines (Clarke *et al.*, 2010). QTL analysis revealed that one locus named *Cdu1*, mapping to the long arm of chromosome 5B (Knox *et al.*, 2009), explained more than 80% of the phenotypic variation in the grain Cd concentration measured in a doubled haploid population (Wiebe *et al.*, 2010). As a consequence, Canadian breeders developed several low-Cd durum wheat cultivars (e.g. *Strongfiel*d, *Brigade*, *Eurostar*) harboring the low-Cd accumulating allele at locus *Cdu1*. Specific investigations conducted in near-isogenic lines revealed that allelic variation at locus *Cdu1* did not affect root uptake of Cd but did affect its root-to-shoot translocation (Chan and Hale, 2004; Harris and Taylor, 2004; Hart *et al.*, 2006). Root sequestration of Cd was thus considered to be the main driver of the intraspecific variability of grain Cd accumulation in Canadian lines.

The ability of widely used French durum wheat cultivars to accumulate Cd in the grain has not been as frequently characterized as that of their Canadian counterparts. Only recently, Zimmerl *et*

al. (2014) showed that 75% of "French durum wheat cultivars" do not possess the low-Cd accumulating allele at *Cdu1*, including *Miradoux*, *Sculptur*, *Pescadou* and *Karur*, the four most widely cultivated lines in France (source Arvalis-FranceAgriMer, data 2013). There is thus an urgent need to characterize the level of Cd accumulation of French lines and to assess the genotypic variation responsible for Cd concentration in their grains, which are the two first objectives of the varietal screening described here. In addition, this work aims at understanding the ecophysiological processes that drive the accumulation of Cd in durum wheat grains. The partitioning of Cd among aboveground organs is poorly described in the literature, even though durum wheat lines differ considerably in stem height and grain size, i.e. in their aboveground partitioning of biomass. Assuming that the leaves and stem are sinks for Cd in competition with grains during grain filling, one would expect that a shift in the aboveground partitioning of biomass would affect the concentration of Cd in the grain. The third objective of this work was thus to test this hypothesis in eight French durum wheat lines that do not have the low-Cd accumulating allele at locus *Cdu1* and that differ in their aboveground structure.

2.2 MATERIALS AND METHODS

2.2.1 Design of the experiment

To investigate the intraspecific variability of grain Cd accumulation in durum wheat (*Tritium turgidum* L. subsp. *durum* (Desf.) Husn.), eight French widely cultivated spring cultivars were grown in the presence of Cd, with five replicates per cultivar. The cultivars differed in their stem height (from very short to short) and in their grain size (from medium to very big), but also in precocity and in their grain protein content (Table 2-1). None possess the allele responsible for the low-Cd trait at *Cdu1* (Zimmerl *et al.*, 2014). Two Canadian lines (*Kyle* and *Strongfield*) with long stems (Table 2-1) were included in the experimental design. One has the low-Cd accumulating allele at *Cdu1* (i.e. *Strongfield*) and was used as a reference for the low-Cd accumulation trait. The plants were sampled at the early ripening stage (Zadoks Stage 87-89), which corresponds to 68 to 82 days of Cd exposure after transplanting. To ensure a constant level of exposure to Cd and to allow sampling of the entire root system, the plants were grown in hydroponics. The concentration of Cd^{2+} in the nutrient solution was fixed at 2 nM to reproduce the level of exposure to Cd found in low to moderately contaminated agricultural soils (Sauvé *et al.*, 2000).

Table 2-1 Characteristics of the 10 spring durum wheat cultivars screened in the study.

Cultivars	Code	Producer	Precocity at head emergence	Stem height	Grain size	Protein level
Clovis	CLO	Limagrain	Mid-late	Short	Big	Medium
Dakter	DAK	Limagrain	Mid-early to mid-late	Very short	Big	Mid-high
Isildur	ISI	RAGT Semences	Mid-late	Very short	Mid-big	Medium
Miradoux	MIR	Florimond Desprez	Mid-late	Short	Very big	Medium
Nefer	NEF	Limagrain	Mid-early to mid-late	Short	Big	Medium
Pescadou	PES	Florimond Desprez	Mid-early to mid-late	Short	Big	Mid-high
Pharaon	PHA	Limagrain	Early	Short	Big	Medium
Sculptur	SCU	RAGT Semences	Mid-early	Very short	Medium	Mid-low
Kyle	KYL	AAFC	na	Tall	Big	High
Strongfield	STR	AAFC	na	Tall	Big	High

Source: Arvalis-Institut du végétal (http://www.arvalisinstitutduvegetal.fr), except for *Kyle* and *Strongfield*.
na: not addressed

2.2.2 Plant culture

Grains were surface sterilized in 6% H_2O_2 for 10 min, rinsed and left on a wet paper for four days at room temperature in the dark. Germinated grains were then pre-grown for 10 days in a growth chamber (20 °C, 16-h photoperiod) in 50 mL Falcon® tubes filled with nutrient solution (see below). Thereafter, the seedlings were transferred to a greenhouse and transplanted to polypropylene containers filled with 5 L of modified Hoagland's nutrient solution (¼ strength for macronutrients, full strength for micronutrients) supplied with 2 nM $Cd(NO_3)_2$, at pH 6.0. The free ionic fraction of the Cd in nutrient solution was estimated at 83% (pH 6.0) by geochemical modelling (Visual MINTEQ 3.0, http://vminteq.lwr.kth.se/). As described in Laporte *et al.* (2015), the nutrient solution was continuously aerated and automatically renewed with complete solution on a daily basis using an overflow-type system to maintain the pH between 6 and 7, electrical conductivity above 400 μS cm^{-1}, dissolved oxygen above 8 mg L^{-1} and the total concentration of Cd at 2.0 ± 0.3 nM. This was successfully achieved as indicated by monitoring these parameters (Suppl. Table S2-1). The turnover rate gradually increased from 5% day^{-1} during the first week up to 60% day^{-1} during the last week of vegetative growth and remained constant at 60% day^{-1} during the grain filling period. The air temperature used to calculate the growing degree-days (GDD) was recorded together with air humidity and photosynthetic active radiation using a Campbell CR32X data logger. As usually observed in hydroponics (e.g. Harris and Taylor, 2013), high rates of tillering occurred in our culture. Consequently, only the first four tillers were allowed to develop. From the

start of stem elongation (ZS 30) until anthesis (ZS 65), additional tillers were removed every two to three days and discarded. Almost no tillers developed post-anthesis. The mean day of head emergence (ZS 59) was calculated for each cultivar by averaging the five replicates. It ranged from 36 to 50 days (544 to 793 GDD, 4 °C basis) after transplanting, in increasing order: *Pharaon*, *Isildur*, *Nefer*, *Strongfield*, *Sculptur, Kyle*, *Clovis*, *Miradoux*, *Dakter* and *Pescadou* (Suppl. Table S2-2). All cultivars were harvested 32 days (about 550 GDD) after head emergence. The kernels of all the cultivars were hard, i.e. difficult to divide with the thumbnail. Five replicates per cultivar were harvested on the same day.

2.2.3 Plant sampling and analyses

First, the root systems were immersed for 10 min at 4 °C in 5 L of 5 mM $CaCl_2$ to desorb root apoplastic Cd (Buckley *et al.*, 2010). Second, the plants were separated into roots, stems, leaves and heads. The leaves and stems were washed in two successive baths of deionized water and the heads were separated into grains and bracts+rachis (B+R). The grains were weighed fresh to determine kernel residual water (KRW) at harvest. All the plant organs were dried at 50 °C, weighed and milled (Retsch PM 400) before wet digestion, according to the procedure described in Laporte *et al.* (2015). The concentration of Cd in the plant organs was determined by HR-ICP-MS (Element XR, Thermo Scientific) at the GET laboratory in Toulouse (http://www.get.obs-mip.fr/). The detection limit of the Element XR in the plant digests was around 0.5 ppt for Cd. A grain ionomic profile was established based on the concentrations of K, P, Ca, Mg, Na determined by ICP-OES (ACTIVA, Horiba Jobin Yvon) and Mn, Fe, Co, Ni, Cu, Zn, Mo, Cr determined by ICP-MS (7700x, Agilent Technologies), by the central analytical service of the University of Basque Country (http://www.ehu.eus). The concentrations of N in the grain (multiplied by 5.7 to obtain the protein level) and C were assayed with an elemental analyzer (Flash EA1112, ThermoFisher), according to the Dumas method. The yield components TKW (thousand kernel weight) and KPH (kernels per head) were calculated for each plant from the weight and the number of grains collected on the heads of the four tillers.

2.2.4 Determination of the root cation exchange capacity

The cation exchange capacity (CEC) of the roots was determined in all durum wheat cultivars according to a procedure adapted from Guigues *et al.* (2014). Briefly, 50 mg of dried roots were shaken for 30 min on a roller in 15 mL of 10 mM $CuSO_4$. The suspension was centrifuged for 5

min at 10 000 g and the pellet was rinsed three times with 50 mL of 0.1 mM $CuSO_4$. The roots were then shaken for 20 min on a roller in 40 mL of 0.1 M HNO_3. The suspension was finally centrifuged for 5 min at 10 000 g before flame-AAS determination of the Cu concentration in the filtrate.

2.2.5 Modelling between-cultivar variability in grain cadmium

The concentration of Cd in the grain (*Grain Cd*, μg g^{-1}) depends on grain biomass (*Grain DW*, g) and on the amount of Cd allocated to the grains (QCd_{grain}, μg). Assuming that the amount of Cd reallocated from aboveground parts to the roots is negligible, grain Cd concentration can be expressed as a function of the amount of Cd allocated aboveground ($QCd_{aboveground}$, μg) and a grain allocation factor (*GAF*), but also as a function of the amount of Cd taken up by the roots (QCd_{tot}, μg), a root sequestration factor (*RSF*) and *GAF*, through the equation:

$$Grain\ Cd = \frac{QCd_{grain}}{Grain\ DW} = GAF \times \frac{QCd_{aboveground}}{Grain\ DW} = GAF \times \frac{QCd_{tot} \times (1 - RSF)}{Grain\ DW}$$

Equation 2-1

Leaves and B+R can sequestrate Cd in their tissues before the metal reaches the grain. Therefore, the more biomass there is in the stems, leaves and B+R, the less aboveground Cd is likely to be allocated to the grains. Hence, *GAF* can be assumed to depend on the biomass of leaves + stems + bracts + rachis relative to the biomass of grains. Because of strong correlations between the biomass of B+R and that of grains ($r = 0.83$) on one hand and between the biomass of stems and the biomass of leaves ($r = 0.83$) on the other hand (Suppl. Fig. S2-1), *GAF* was modeled as a function of the ratio between the biomass of leaves and that of grains, these two variables being little correlated (Suppl. Fig. S2-2)

$$GAF = \alpha \times \left(\frac{Leaves\ DW}{Grain\ DW}\right)^{-\beta}$$

Equation 2-2

When *GAF* is replaced by this formalism in Equation 2-1, this gives:

$$Grain\ Cd = \alpha \times \frac{QCd_{tot} \times (1 - RSF) \times Leaves\ DW^{-\beta}}{Grain\ DW^{(1-\beta)}}$$

Equation 2-3

This model was adjusted to the experimental data obtained for the French and for the French + Canadian cultivars.

2.2.6 Data processing

Data were processed using ©R 3.0.1 statistical software (R Development CoreTeam). Analyses of variance (ANOVA) with mean grouping using Tukey's test were performed to identify significant differences between cultivars. Principal component analysis (PCA) was used to study groups of correlations between the grain ionomic data (R package FactoMineR, version 1.25).

Table 2-2 Plant biomass, root biomass, ratio of aboveground to root biomass (AR), aboveground biomass partitioning and grain yield measured at maturity in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd

Cultivars	Plant biomass	Root biomass	AR	Aboveground biomass partitioning				Grain yield
				Stems	Leaves	B+R	Grains	
	g DW	g DW		%	%	%	%	g DW
CLO	23.3 d	3.57 b	5.6 bc	19 ± 1	14 ± 1	12 ± 1	55 ± 1	2.73 c
DAK	14.0 ab	3.64 b	3.0 a	19 ± 2	23 ± 3	12 ± 1	46 ± 5	1.23 a
ISI	19.9 cd	3.13 ab	5.4 abc	18 ± 1	13 ± 1	12 ± 1	58 ± 2	2.41 bc
MIR	29.8 e	6.15 c	4.0 ab	23 ± 2	18 ± 4	12 ± 2	47 ± 4	2.78 c
NEF	17.2 bc	2.99 ab	4.8 abc	17 ± 1	11 ± 1	12 ± 1	60 ± 2	2.15 b
PES	19.7 cd	3.29 ab	5.4 abc	20 ± 1	17 ± 4	12 ± 1	50 ± 5	2.07 b
PHA	20.7 cd	4.18 b	4.1 ab	17 ± 1	12 ± 2	12 ± 1	59 ± 2	2.45 bc
SCU	12.1 a	1.54 a	7.3 c	15 ± 1	13 ± 1	14 ± 1	59 ± 1	1.56 ab
KYL	16.1 abc	2.55 ab	5.8 bc	29 ± 3	15 ± 1	13 ± 2	43 ± 5	1.45 a
STR	18.5 c	2.25 ab	7.4 c	22 ± 2	13 ± 1	12 ± 1	52 ± 3	2.11 b

Different letters in the same column indicate significant differences ($P < 0.05$)
B+R: bracts+rachis
Grain yield is expressed as g DW per head

2.3 RESULTS

2.3.1 Plant growth, biomass partitioning and grain yield

No visual symptom of Cd toxicity and/or mineral deficiency was observed, whatever the cultivar considered. The cumulative thermal time recorded at head emergence ranged from 544 to 793 GDD depending on the cultivar (Suppl. Table S2-2). The kernel residual water (KRW) in all the cultivars at harvest was close to 40%, showing that the grains had reached their physiological maturity. The French lines differed significantly in growth, biomass partitioning and grain yield (Table 2-2). Plant dry weight (DW) ranged from 12.1 g in *Sculptur* up to 29.8 g in *Miradou*x. The

aboveground-root biomass ratio (AR) ranged from 3.0 in *Dakte*r to 7.3 in *Sculptur*. Aboveground, grains were the main biomass compartment in all cultivars. However, between-organ partitioning of aboveground dry matter (DM) varied from one cultivar to another. *Nefer*, *Pharaon* and *Sculptur* lines allocated the highest proportion of aboveground DM to the grain (about 60%) while *Dakter* and *Miradoux* allocated the highest proportion of aboveground DM to the leaves + stems (about 40%). The proportion of aboveground DM allocated to the bracts+rachis was very similar in all cultivars and averaged 12%. The grain yield ranged from 1.2 g per head in *Dakter* up to 2.8 g per head in *Miradoux*, as a result of a between-cultivar variation in both the number of kernels per head (KPH) and the thousand kernel weight (TKW). Indeed, KPH ranged from 27 to 47 and TKW from 44 to 71 g (Suppl. Table S2-2). As expected from their reported grain size (Table 2-1), TKW was lowest in *Sculptur* and highest in *Miradoux*.

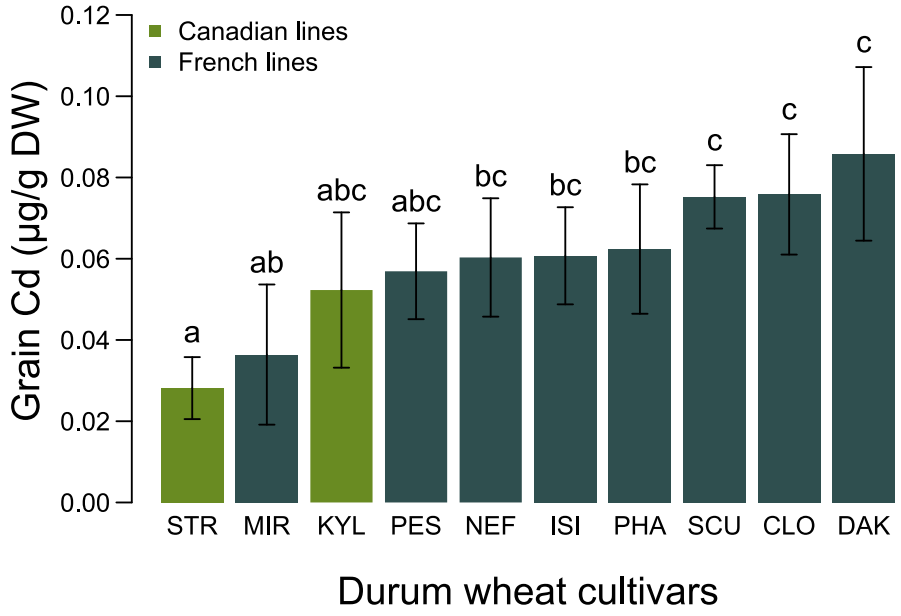


Figure 2-1 Grain Cd concentration measured at maturity in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd. Data are average values ± standard deviation (indicated as bars in the figures) calculated from 5 replicates per treatment. The error bars are standard deviations. Mean values with different letters are significantly different ($P < 0.05$) as measured by analysis of variance according to a Tukey's test.

2.3.2 Grain cadmium

The grain Cd concentration ranged on average from 0.028 to 0.086 µg g^{-1} DW and differed significantly ($P < 0.001$) among the durum wheat cultivars (Figure 2-1). As expected from its low-Cd trait, *Strongfield* had the lowest grain Cd concentration, 1.9 times lower than that of *Kyle*. Interestingly, a 2.4-fold variation in grain Cd was observed among the French cultivars, *Miradoux* having the lowest and *Dakter* the highest concentration of Cd in the grain. No French cultivar had

a significantly different concentration of Cd in the grain ($P > 0.05$) from that of *Kyle*, whereas all except *Miradoux* and *Pescadou* had a higher concentration of grain Cd ($P < 0.05$) than *Strongfield*. The variability of grain Cd between replicates was rather high in some cultivars (e.g. *Kyle*). This may be due to fluctuations in Cd availability caused by the low concentration of Cd in the nutrient solution and may explain why some differences in grain Cd, e.g. between *Kyle* and *Strongfield*, were unexpectedly not statistically different.

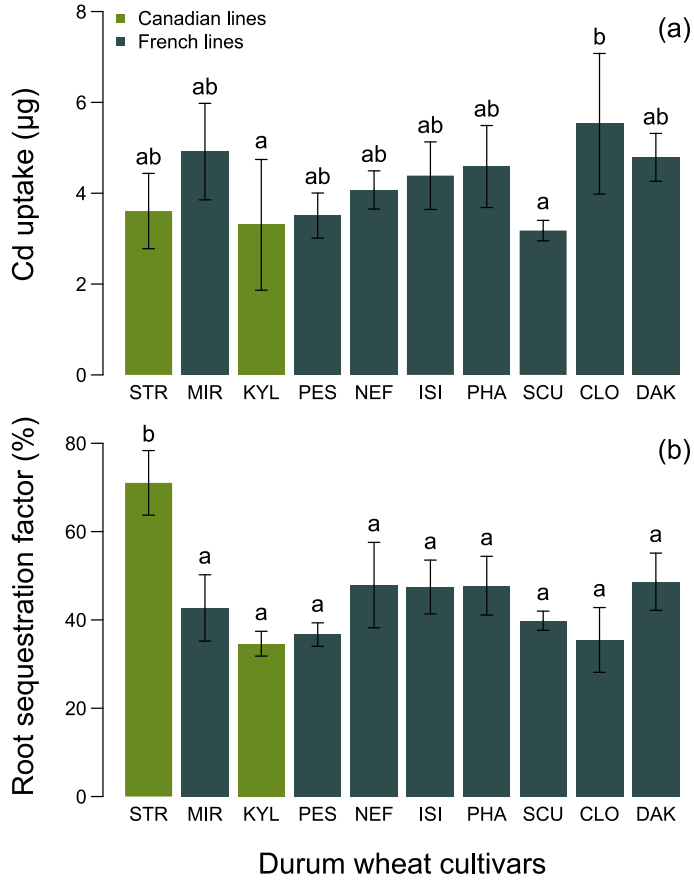


Figure 2-2 (a) Cadmium uptake and (b) root sequestration factor (*RSF*), measured at maturity in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd. *RSF* is expressed as a percentage of Cd uptake. Data are average values ± standard deviation (bars in the figures) calculated from 5 replicates per treatment. Mean values with different letters are significantly different ($P < 0.05$) as measured by analysis of variance according to a Tukey's test.

2.3.3 Plant cadmium

Plant uptake of Cd (Figure 2-2a) varied significantly among the French cultivars ($P < 0.01$). However, the variation was moderate (a factor of 1.7) and was thus less marked than the difference in the concentration of Cd in the grain. Tukey's test showed that *Sculptur* took up the least Cd and *Clovis* took up the most Cd, the other cultivars being between the two. Cd uptake was moderately but significantly correlated with root biomass ($r = 0.51$, Suppl. Fig. S2-3). Even though French lines with a high concentration of Cd in the grain (i.e. those on the right in Figure 2-2a) tended to take up more Cd, there were several exceptions. For instance, *Miradoux* and *Dakter* took up almost the same amount of Cd but their grain Cd concentration was highly contrasted. Despite sometimes

contrasted partitioning of biomass between roots and aboveground tissues (AR; Table 2-2), all the French lines sequestrated a statistically equivalent ($P > 0.05$) proportion of Cd in their roots (Figure 2-2b). The so-called root sequestration factor (*RSF*) averaged 40% in all French cultivars plus *Kyle* while it reached 70% in *Strongfield*. The related root concentration of Cd was thus significantly higher ($P < 0.05$) in *Strongfield* than in all other cultivars (Suppl. Table S2-3). The amount of Cd allocated to the shoots varied significantly among cultivars ($P < 0.001$) and was lowest in *Strongfield* (1.02 µg) and highest in *Clovis* (3.55 µg) (Table 2-3). Unlike biomass, leaves were the main sink for Cd aboveground rather than grains. On average, more than one third of aboveground Cd was allocated to the leaves and the proportion reached nearly half in *Miradoux* and *Dakter*. The percentage of aboveground Cd allocated to B+R was non-negligible (12-26%) and was close to that allocated to the grains (Table 2-3). The concentration of Cd in the aboveground tissues ranked in the decreasing order: leaves > B+R > stems > grains, and the ranking of the Cd concentration in the leaves, stems and B+R among cultivars was almost the same as that in the grains (Suppl. Table S2-3).

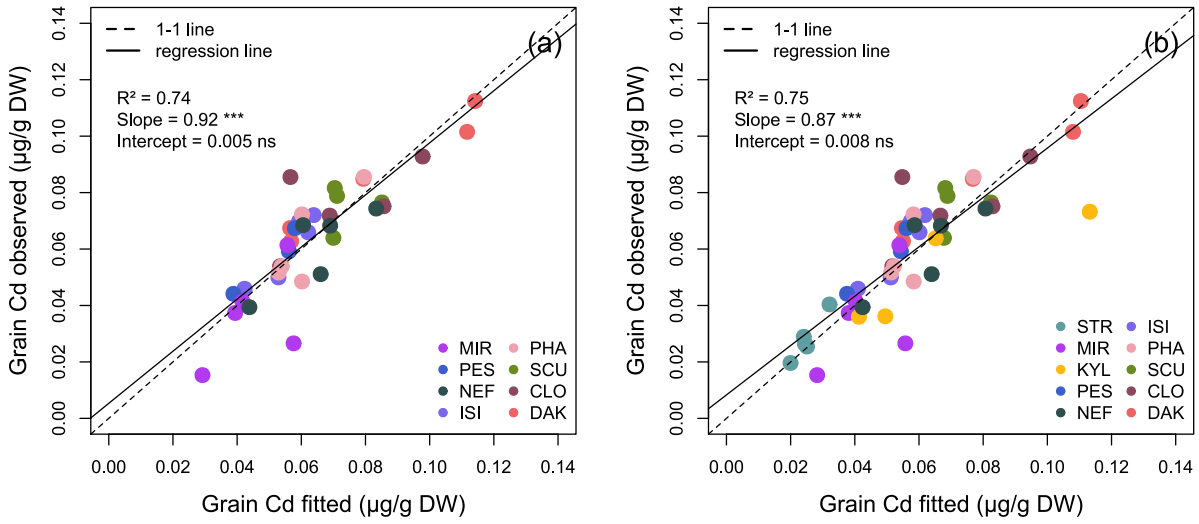


Figure 2-3 Relationships between the values of grain Cd concentration predicted by the model shown in Equation 2-3 and those measured experimentally in (a) the 8 French lines and (b) all the 10 durum wheat lines considered in the study. The statistics in both figures are relative to the correlation between predicted and experimental values of grain Cd.

2.3.4 Predictive model for grain cadmium concentration

Figure 2-3 shows the grain Cd concentration simulated by the model (Equation 2-3) as a function of the grain Cd concentration observed experimentally. The model satisfactorily explained the between-cultivar differences in grain Cd observed in the French cultivars (Figure 2-3a). Indeed,

the model explained 74% of the total variance observed in grain Cd and the slope (close to 1) + the non-significant intercept of the regression line suggest that there is no major bias in its predictions. In addition, the quality of the regression remained almost the same when the two Canadian lines were included (Figure 2-3b). The model coefficients introduced in Equation 2-3 and adjusted from the experimental data are shown in Table 2-4. Interestingly, the values of a and β did not change significantly when the two Canadian lines were included. As can be seen in Table 2-4, the fitted value for β was positive. We calculated the range of variation in all terms of the model (Table 2-5) to examine which variables among QCd_{tot}, *RSF*, *Leaf DW* and *Grain DW* had the strongest effect on *Grain Cd*. Within French cultivars, the greatest range of variation was in $Leaf\ DW^{-\beta}$ (Table 2-5), which means that the biomass of leaves had the strongest effect on grain Cd. In our experiment, the increase in leaf biomass across the French cultivars reduced the concentration of Cd in the grain by a factor of 2.17 (Table 2-5). When the two Canadian lines were included, the most influential variable was the root sequestration factor (*RSF*) followed by the root uptake of Cd (Table 2-5).

Table 2-3 Amount of Cd allocated to aboveground organs and aboveground partitioning of Cd, measured at maturity in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd

Cultivars	Aboveground Cd	Aboveground partitioning of Cd			
		Stems	Leaves	B+R	Grains
	µg	%	%	%	%
CLO	3.55 c	22 ± 2	31 ± 3	23 ± 3	24 ± 6
DAK	2.46 bc	19 ± 2	48 ± 3	16 ± 3	16 ± 3
ISI	2.30 b	21 ± 2	37 ± 1	16 ± 1	25 ± 2
MIR	2.84 bc	25 ± 4	48 ± 5	12 ± 3	15 ± 4
NEF	2.10 ab	18 ± 3	37 ± 5	21 ± 4	24 ± 3
PES	2.23 abc	21 ± 2	37 ± 5	21 ± 3	21 ± 2
PHA	2.37 bc	18 ± 3	34 ± 7	23 ± 5	25 ± 2
SCU	1.91 ab	17 ± 1	32 ± 5	26 ± 4	25 ± 3
KYL	2.14 ab	29 ± 4	31 ± 7	25 ± 11	15 ± 2
STR	1.02 a	27 ± 3	30 ± 3	20 ± 2	23 ± 2

Different letters in the same column indicate significant differences ($P < 0.05$)
B+R: bracts+rachis

2.3.5 Correlations between grain cadmium and other grain characteristics

The variables related to grain characteristics were analyzed by principal component analysis (PCA, Suppl. Fig. S2-4). The first two components accounted for 41.9% of total variance. The first dimension was associated with the concentrations in Mn (cos^2 = 0.70), P (cos^2 = 0.68) and Mg (cos^2 = 0.66) and the second dimension was associated with the concentration in K (cos^2 = 0.63), the level of protein (cos^2 = 0.58) and the TKW (cos^2 = 0.57). The bigger the grains, the lower their K content, as underlined by the negative correlation between TKW and K (r = -0.71). Grain Cd was associated with both the first (cos^2 = 0.31) and the second dimension (cos^2 = 0.32). In the French cultivars, grain Cd was positively correlated with the concentration of P < Mn < Zn in the grain (Table 2-6; Suppl. Fig. S2-5) and negatively correlated with TKW and KPH (Table 2-6). There was no significant correlation (P > 0.05) between Cd content in the grain and grain protein content and grain Fe and Cu content. When the two Canadian cultivars *Kyle* and *Strongfield* were included, almost the same relationships were obtained between the grain characteristics and the grain concentration of Cd (Table 2-6).

2.4 DISCUSSION

The grain concentration of Cd was below the European maximum limit set for durum wheat, which is 0.2 μg Cd g^{-1} (EC, 2006). It ranged from 0.03 to 0.08 μg g^{-1} (Figure 2-1) and was thus in line with the median concentration of Cd reported in French durum wheat grains, which is 0.07 μg g^{-1} (source Arvalis-FranceAgriMer, data 2009). We thus succeeded in obtaining durum wheat grains with a Cd contamination level comparable to that commonly measured in the field. It is worth noting that the concentration of Cd^{2+} in the porewater of some agricultural soils (e.g. acidic soils) can be up to 10 times higher than that tested in our study (Sauvé *et al.*, 2000). The concentration of Cd in the grain is thus probably close to or even higher than 0.2 μg g^{-1} in at least some French durum wheat lines, when grown on these soils. The number (KPH) and the size (TKW) of grains in our study (Suppl. Table S2-2) were similar to those observed in the field (source Arvalis, data 2013). The between-cultivar differences in stem height, grain size and grain protein level were roughly the same as the ranking provided by breeders (Suppl. Table S2-2; Table 2-1). Thus, the physiological characteristics of the 10 cultivars screened in this study were in line with the standards. Only the average grain protein level was a little high at 16% (Suppl. Table S2-2), in the field, the average is 13% (source Arvalis-FranceAgriMer, data 2013). This is probably the result of the continuous non-limiting supply of nitrogen provided during the plant growth in our study.

Our experiment confirmed the difference in the grain concentration of Cd between the two Canadian lines, underlining the prominent role played by the *Cdu1* locus in alleviating the accumulation of Cd in durum wheat grains. The difference in grain Cd between *Kyle* and *Strongfield* was however limited to a factor of 1.9. This is lower than values reported so far between lines harboring contrasted alleles at *Cdu1* (e.g. Harris and Taylor, 2013). One possible explanation is that the magnitude of variations in grain Cd between cultivars depends on the level of accumulation of Cd in the grain, the latter being lower in our study than, for instance, in Harris and Taylor (2013). All French lines had the same (or higher) concentration of Cd in their grain than *Kyle*, and therefore behaved like high-Cd cultivars. This is consistent with the fact that, like *Kyle*, they do not possess the allele at *Cdu1* responsible for the low-Cd trait (Zimmerl *et al.*, 2014).

Table 2-4 Coefficients (α, β) of the model introduced in Equation 2-3 and associated standard errors (in brackets), for the French cultivars (n = 8) and for all the cultivars (n = 10) considered in the study

	French cultivars	**All cultivars**
a	0.106 (0.008)	0.102 (0.009)
β	0.532 (0.058)	0.532 (0.065)

Table 2-5 Ranges of variation (in square brackets) and associated variation factor (in bold) of the different terms of the model (QCd_{tot}, RSF, $Leaf\ DW^{-\beta}$, $Grain\ DW^{(1-\beta)}$), for the French cultivars (n = 8) and for all the cultivars (n = 10) considered in the study

	French cultivars	**All cultivars**
QCd_{tot}	[3.046; 6.214] **× 2.04**	[2.529; 6.027] **× 2.38**
$1 - RSF$	[0.430; 0.699] **× 1.63**	[0.229; 0.695] **× 3.03**
$Leaf\ DW^{-\beta}$	[0.410; 0.890] **÷ 2.17**	[0.420; 0.880] **÷ 2.10**
$Grain\ DW^{(1-\beta)}$	[0.314; 0.567] **÷ 1.81**	[0.315; 0.549] **÷ 1.74**

Ranges of variation were calculated from 95% of the data
The multiplication sign (×) means that the terms QCd_{tot} and $1 - RSF$ have a positive effect on the concentration of Cd in the grain. The division sign (÷) means that the terms $Leaf\ DW^{-\beta}$ and $Grain\ DW^{(1-\beta)}$ have a negative effect on the concentration of Cd in the grain.

2.4.1 Variability in grain cadmium within French lines was not explained by uptake and root sequestration of cadmium

Interestingly, a 2.4-fold variation in grain Cd was observed within French cultivars. This variation was larger than that observed between *Kyle* and *Strongfield*. It may be due to different mechanisms including variability of Cd uptake, Cd sequestration in roots and partitioning of Cd between aboveground organs.

In the present study, the uptake of Cd differed among French cultivars, partly because of differences in root DW ($r = 0.51$). The moderate correlation between Cd uptake and root biomass suggests there may be differences in the capacity of cultivars to take up Cd per root mass unit. The cell wall is often seen as a sink for cations whose retention capacity may vary among plant species. According to the biotic ligand model (Slaveykova and Wilkinson, 2005), fixation of a metal to the root surface is a pre-requisite for its internalization. A root with a high CEC is thus assumed to mobilize and buffer Cd availability at the root membrane more efficiently. This hypothesis was formulated by Greger and Landberg (2008) to explain why wheat cultivars with a higher CEC accumulated more Cd in their grains. We measured root CEC on the 50 plants grown in this study. The root CEC ranged from 22 to 32 $cmol_c\ kg^{-1}$ and varied significantly among cultivars ($P = 0.011$) (Suppl. Fig. S2-6). However, root CEC was not correlated with QCd_{tot} ($P = 0.199$). Therefore, the low correlation between Cd uptake and root biomass suggests that French cultivars differed in the development of the apoplastic barriers and/or in the expression or the affinity of the transporters responsible for Cd internalization in the root cells.

The variables QCd_{tot} and *Grain Cd* were not correlated ($P = 0.161$), showing that the French cultivars that took up the most Cd were not those with the highest concentration of Cd in the grain. This is in agreement with results reported so far (e.g. Harris and Taylor, 2004). According to the literature, the intraspecific variability in the concentration of Cd in durum wheat grain does not originate from a variation in the uptake of Cd but from a variation in the root-to-shoot translocation of the metal (Chan and Hale, 2004; Harris and Taylor, 2004; Hart *et al.*, 2006). Indeed, the locus *Cdu1* explains 80-90% of the phenotypic variations in the grain Cd observed in durum wheat (Wiebe *et al.*, 2010). Thus, low-Cd cultivars do not take up less Cd than high-Cd cultivars but restrict its translocation aboveground more efficiently. By homology with the tonoplast-localized metal pumping *OsHMA3* detected in rice (Ueno *et al.*, 2010), the *Cdu1* locus could include a gene coding for a protein involved in the vacuolar sequestration of Cd in root cells. However, in our work, the root sequestration of Cd did not vary significantly among French lines. This probably results from the fact that all the French lines screened in this study possessed little variability at

Cdu1, since no specific selection was made with respect to the concentration of Cd in the grain. It is interesting to note that the root sequestration factor (*RSF*) remained constant even though the root-to-shoot partitioning of biomass varied considerably within French lines (Table 2-2). This is evidence that the coupling between the root-to-shoot partitioning of Cd and biomass differed among French lines.

To summarize, the 2.4-fold variation in grain Cd concentration observed among the French lines was not due either to a difference in Cd uptake or to a difference in their ability to sequestrate Cd in roots. As a result, all the French lines (except *Clovis*) allocated almost the same amount of Cd aboveground (Table 2-3). As discussed later, the variability of the concentration of Cd in the grain in the French cultivars originates in a differential pattern of Cd partitioning between aboveground organs. To our knowledge, this has never been reported so far.

2.4.2 Impact of the grain yield

There was a negative correlation between *Grain Cd* and *Grain DW* within French lines ($r = -0.59$, $P < 0.001$). This explains the difference in grain Cd observed between *Miradoux* and *Dakter*. These two cultivars allocated the same amount of Cd to the grains (0.4 ± 0.1 µg) as a result of similar allocation of Cd aboveground and a similar partitioning coefficient to the grains (Table 2-3). However, these two cultivars exhibited contrasted grain yields. Grain DW was 2.3 times larger in *Miradoux* than in *Dakter* as a result of a 1.4-times higher TKW and a 1.7-times higher KPH. Therefore, the lower concentration of Cd in *Miradoux* resulted from a more intense dilution of Cd in more and bigger grains. The negative correlation between *Grain Cd* and TKW was significant in the French cultivars and in all the cultivars (Table 2-6), which suggests that large grains could be a desirable trait to reduce their concentration of Cd. However, larger grains may also contain a lower concentration of nutrients and hence have a lower nutritional value than smaller grains. This was suggested for Zn in our study through the negative correlation observed between Grain Zn and TKW (Suppl. Fig. S2-7). The dilution of Cd and Zn might be linked to where they are stored in the grain. Indeed, one can suppose from the results reported for Zn on barley (Lombi *et al.*, 2011b) that, in durum wheat grains, Cd and Zn are preferentially stored in the peripheral aleuronic layer of the bran. The concentration of Cd and Zn in the grain would consequently decrease with a decrease in the surface-to-volume ratio of the grain.

2.4.3 Impact of the aboveground partitioning of biomass

The grain allocation factor was not constant within French lines (Table 2-3). We tested whether the allocation of Cd to the grain depends on the partitioning of biomass between grains and leaves. Under this hypothesis, the leaves are considered to be a Cd sink in competition with grains during grain filling. When *GAF* is expressed as a function of the biomass of grains and the biomass of leaves (Equation 2-2), the model provides a good prediction of the intraspecific variation in grain Cd (Figure 2-3). In the French lines, leaf biomass was the main factor explaining the variation in grain Cd (Table 2-5). This strongly suggests that the accumulation of Cd in the grain depends on the aboveground partitioning of biomass between grains and leaves, or grains and straw since *Leaf DW* and *Stem DW* were highly correlated. The positive value of β confirms that grains and straw are competing sinks for Cd. In other words, French cultivars that allocate a higher proportion of their aerial biomass to straw tended to have a lower concentration of Cd in the grains. The head (bracts+rachis) was also a significant sink for Cd since it contained an average of 21% of the Cd accumulated aboveground. The proportion of biomass in the head to that in the grains could thus theoretically modulate the accumulation of Cd in the grain. However, it was impossible to demonstrate from our experiment where the two biomasses were correlated. The accumulation of cadmium in the grain results from exchanges between xylem and phloem saps since in cereals, the grain is not directly connected to the xylem vessels (Clemens *et al.*, 2013; Waters and Sankaran, 2011). Through transpiration, leaves, stems and spikelets can mobilize part of the xylem sap containing the Cd translocated from the roots. The higher their biomass relative to the grain biomass, the less Cd is allocated to the grain. This is especially true when reallocation from high transpiration tissues is restricted, as is the case in hydroponics where the continuous supply of nitrogen after anthesis inhibits the senescence of photosynthetic organs (Harris and Taylor, 2013; Tavarez *et al.*, 2015). There is thus a physiological explanation for our results. One can suspect that the role played by high transpiration tissues in the aboveground partitioning of Cd would differ in the field, where remobilization occurs and where the phytoavailability of nutrients and contaminants (Cd included) decline considerably after anthesis. However, two other studies performed on soil are in agreement with our conclusions. By comparing two durum wheat lines grown in soil pots, Arduini *et al.* (2014) showed that the line that accumulated the least Cd in the grains was the line that produced the fewest grains and the most straw, i.e. the line with the longest stem. This hypothesis was also mentioned by Kubo *et al.* (2008) to explain the differences in grain Cd level observed among Japanese wheat cultivars grown in the field. So, it appears that the partitioning of aboveground biomass between grains and straws affects the grain concentration of Cd in the same way in hydroponics and in field conditions.

Table 2-6 Linear correlation coefficients (*r*) between grain Cd concentration and yield components (TKW, KPH), kernel residual water (KRW), protein level, concentration of C, K, P, Ca, Mg, Na, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo in the grain and root CEC, in the French cultivars (*n* = 8) and in all the cultivars (*n* = 10) considered in the study

	TKW	**KPH**	**KRW**	**Protein**	**C**	**K**	**P**	**Ca**	**Mg**	**Na**
French cultivars	-0.36*	-0.58***	0.03^{ns}	-0.18^{ns}	-0.28^{ns}	0.26^{ns}	0.50***	0.19^{ns}	0.38*	-0.04^{ns}
All cultivars	-0.32*	-0.33*	-0.07^{ns}	-0.27^{ns}	-0.32*	0.28^{ns}	0.40**	0.34*	0.26^{ns}	0.10^{ns}
	Cr	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**Mo**	**Root CEC**	
French cultivars	0.02^{ns}	0.61***	0.08^{ns}	0.07^{ns}	-0.35*	0.16^{ns}	0.81***	0.43**	0.24^{ns}	
All cultivars	0.04^{ns}	0.51***	0.16^{ns}	0.07^{ns}	-0.19^{ns}	0.30^{ns}	0.70***	0.27^{ns}	0.11^{ns}	

*, ** and *** indicate significant correlations at the probability level of $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
TKW: thousand kernels weight
KPH: kernels per head

2.4.4 Co-accumulation of cadmium with manganese, phosphorus and zinc in the grain

The concentration of Cd in the grain was positively correlated with that of Mn and Zn (Table 2-6), suggesting that high-Cd cultivars tended to accumulate more Mn and Zn in their grains than low-Cd cultivars. Similar correlations have already been reported for Cd and Mn in barley genotypes (Chen *et al.*, 2007) and for Cd and Zn in rice genotypes (Cao *et al.*, 2013). One can assume that the co-accumulation of Mn and Zn with Cd results from the fact that they share some allocation pathways to the grain with Cd. Indeed, Cd is not essential for plant growth and therefore no specific transporter has evolved for Cd. Its internalization and movement within the plant is mediated by transporters dedicated to micronutrients with similar chemical properties to Cd, such as Mn and Zn. Several plasma membrane transporters of Mn and Cd have been shown to mediate the transport of Cd in plants, especially in rice, for which the molecular understanding of Cd accumulation is more advanced (Clemens *et al.*, 2013). For instance, *OsNramp5* and *OsHMA2*, which are involved in the uptake of Mn and the xylem loading of Zn, respectively, were recently also found to mediate the transport of Cd (Sasaki *et al.*, 2012; Takahashi *et al.*, 2012). By homology with rice, Cd is thus suspected to use transporters of Mn and Zn on its way to the durum wheat grain. This shared transport system has often been cited to explain why supplemental Zn reduces the accumulation of Cd in plant tissues (Hart *et al.*, 2005) and vice-versa (Wu *et al.*, 2007; Wu and Zhang, 2002). In addition, we observed a positive correlation between the concentrations of Cd and P in the grain. This was previously reported by Shi *et al.* (2015) in wheat genotypes and suggests that high-Cd cultivars accumulate more phytate in the grain. Since phytate can bind Zn (Cakmak *et al.*, 2010), this could also explain why high-Cd cultivars tend to accumulate more Zn. Cd-phosphate

complexes have already been isolated in plants (Qiu *et al.*, 2011) but whether phytate can bind Cd efficiently and whether its concentration in the grain can impact that of Cd remains to be elucidated.

2.5 CONCLUSIONS

This study demonstrates that the intraspecific variability in the concentration of Cd in durum wheat grains is not linked to a differential ability to take up Cd. The comparison of French cultivars with the Canadian cultivar *Strongfield* confirmed that the absence of the low-Cd accumulating allele at locus *Cdu1* in French cultivars results in less efficient sequestration of Cd in root tissues, thereby contributing to the contamination of the grain. Once the Cd was translocated aboveground, we showed that the amount of Cd allocated to the grains depended on the biomass of the vegetative tissues relative to the biomass of the grains. From a physiological point of view, our results suggest that the allocation of Cd to the grain involves a two-step procedure: first a root-to-shoot translocation of Cd controlled by the roots and then partitioning of Cd between stem, leaves, bracts, rachis, and grains depending on their relative biomass. The contamination of durum wheat grains should thus be more pronounced when the proportion of biomass of the stem, leaves and of the head is less than the biomass of the grains. If this trend is confirmed in the field, there would thus be an advantage in growing long stem cultivars on Cd-contaminated soils. Breeding programs tend to reduce the stem height of cereals to improve their resistance to logging. The results of our study together with those obtained by Arduini *et al.* (2004) suggest that this selection may promote the accumulation of Cd in durum wheat grains.

2.6 ACKNOWLEDGMENTS

This work was supported by funding from Arvalis-Institut du végétal and from the French National Institute for Agricultural Research. The authors thank Agriculture and Agri-Food Canada for providing grains of the two Canadian durum wheat cultivars, Sylvie Milin (INRA) for technical help in determining the grain concentrations in N and C, PhD Juan Carlos Raposo from SGIker of UPV/EHU for technical and human support in ICP-MS and ICP-AES measurements and the China Scholarship Council (CSC) for the doctoral scholarship of Bofang Yan. Oleg Pokrovsky acknowledges partial support from the BIO-GEO-CLIM Grant No 14.B25.31.0001.

2.7 SUPPLEMENTARY MATERIAL

Suppl. Table S2-1 Changes in the total concentration of Cd, pH, conductivity and in the concentration of dissolved oxygen in nutrient solution over thermal time (expressed in GDD for growing day-degrees)

	Thermal time (GDD)											
	115	224	336	446	560	675	793	924	1050	1166	1289	Mean
Total Cd (nM)	1.88	2.17	2.40	2.27	2.38	2.00	1.68	1.69	1.72	1.92	2.18	2.03
pH	6.15	6.33	7.07	6.77	6.87	6.98	7.01	7.10	6.95	6.81	6.92	6.81
Conductivity (µS cm^{-1})	526	488	462	404	403	423	456	507	582	555	542	486
Dissolved O2 (mg L^{-1})	8.92	8.50	7.90	8.87	9.01	9.08	9.41	8.94	8.71	8.88	8.70	8.81

Every week, a 2.5-mL aliquot of nutrient solution was collected from one pot per cultivar and mixed with the nine others (collected from the nine other cultivars) to give a 25-mL composite solution, in which all these physico-parameters were measured.

Suppl. Table S2-2 Thermal time at head emergence (in GDD for growing degree-days), stem height and grain characteristics (thousand kernels weight, number of kernels per head, protein level) measured at maturity in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd. For every parameter except KPH, the cultivars ranking issued from the characteristics provided by breeders (Table 2-1) is shown in brackets

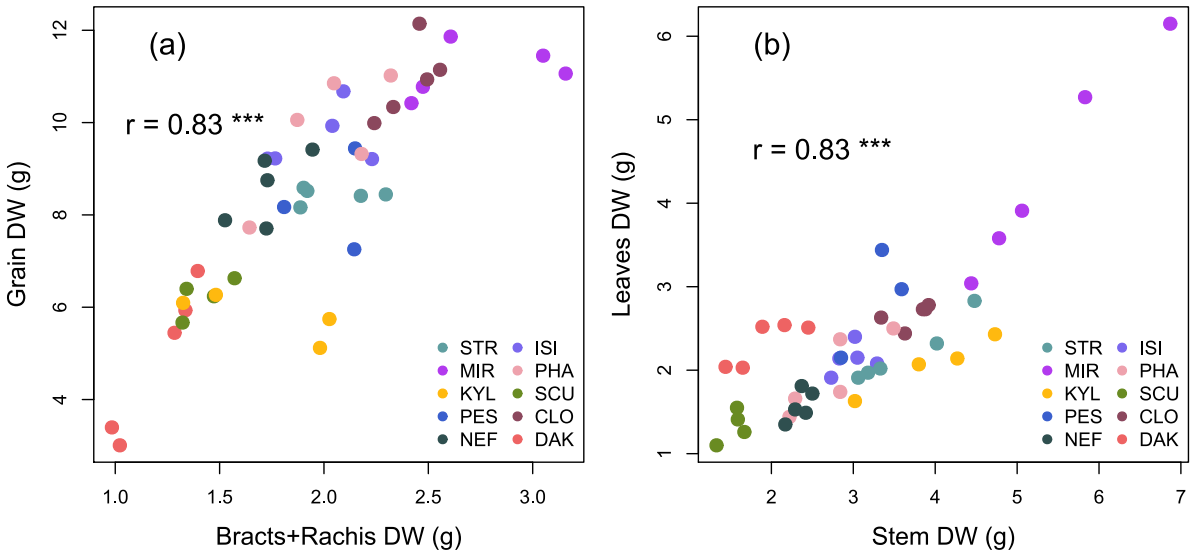
	Thermal time at head emergence	**Stem height**	**TKW [a]**	**KPH**	**Protein [a]**
	GDD	**cm**	**g**		**%**
CLO	643 (4)	66 c (2)	71 d (2)	45 b	15.6 abc (3)
DAK	775 (3)	51 ab (3)	51 ab (2)	27 a	15.4 ab (2)
ISI	560 (4)	55 b (3)	60 bc (3)	47 b	15.4 ab (3)
MIR	675 (4)	67 c (2)	70 d (1)	46 b	18.5 d (3)
NEF	576 (3)	56 b (2)	61 c (2)	40 b	13.7 a (3)
PES	793 (3)	62 bc (2)	62 cd (2)	38 ab	18.6 d (2)
PHA	544 (1)	50 ab (2)	60 bc (2)	47 b	13.8 a (3)
SCU	626 (2)	47 a (3)	44 a (4)	41 b	13.8 a (4)
KYL	626 (na)	90 e (1)	58 bc (2)	29 ab	18.1 cd (1)
STR	592 (na)	78 d (1)	62 cd (2)	39 b	17.1 bcd (1)

Different letters in the same column indicate significant difference ($P < 0.05$)
TKW: thousand kernel weight and KPH: kernels per head
[a] Values are based on 15% moisture basis
na: non-addressed

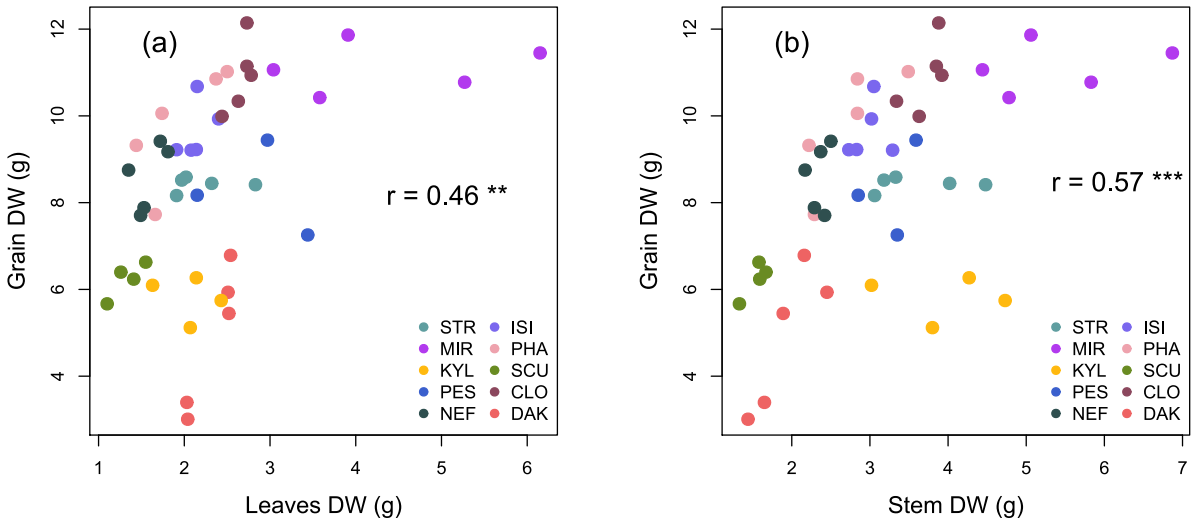
Suppl. Table S2-3 Cadmium concentration in roots, stems, leaves, bracts+rachis and grains measured at maturity in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd

	Roots	Stems	Leaves	B+R	Grains
			µg g^{-1} DW		
CLO	0.56 ab	0.21 cd	0.41 bc	0.35 b	0.076 c
DAK	0.65 ab	0.25 d	0.53 c	0.35 b	0.086 c
ISI	0.68 ab	0.16 bc	0.40 bc	0.19 ab	0.061 bc
MIR	0.35 a	0.12 ab	0.26 ab	0.12 a	0.036 ab
NEF	0.65 ab	0.16 bc	0.49 c	0.26 ab	0.060 bc
PES	0.41 ab	0.14 abc	0.29 abc	0.23 ab	0.057 abc
PHA	0.53 ab	0.15 bc	0.42 bc	0.27 ab	0.062 bc
SCU	0.86 bc	0.21 cd	0.46 bc	0.36 b	0.075 c
KYL	0.31 a	0.13 ab	0.28 abc	0.26 ab	0.052 abc
STR	1.17 c	0.08 a	0.14 a	0.10 a	0.028 a

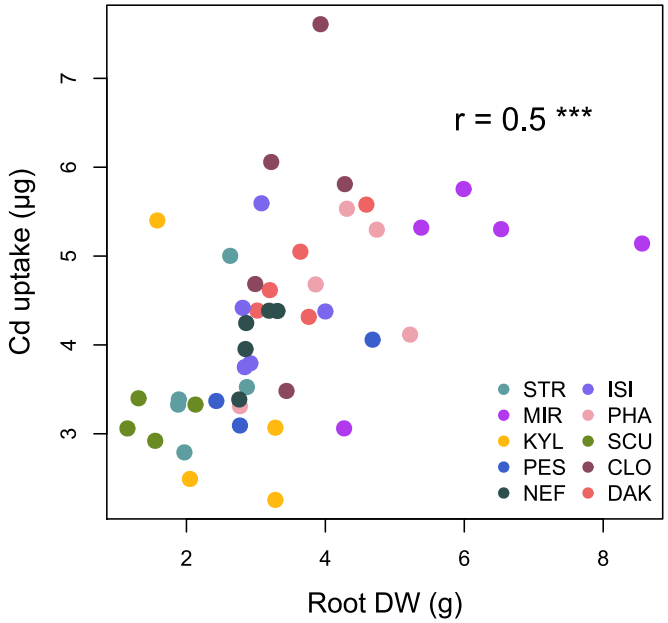
Different letters in the same column indicate significant difference ($P < 0.05$)
B+R: bracts+rachis



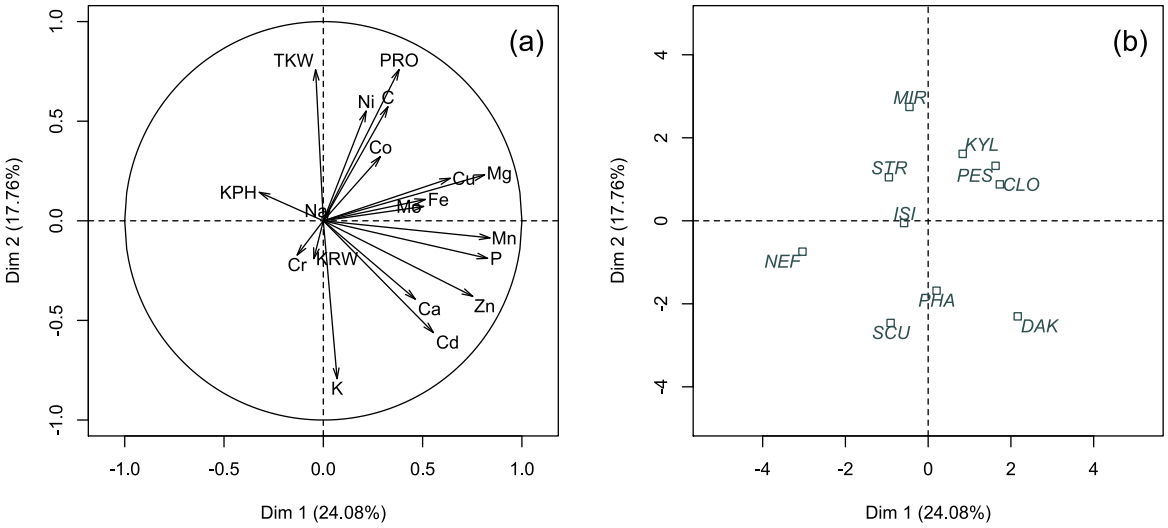
Suppl. Fig. S2-1 (a) Grain dry weight plotted against the dry weight of bracts+rachis (B+R in the manuscript) and (b) leaves dry weight plotted against the dry weight of stems in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd until maturity.



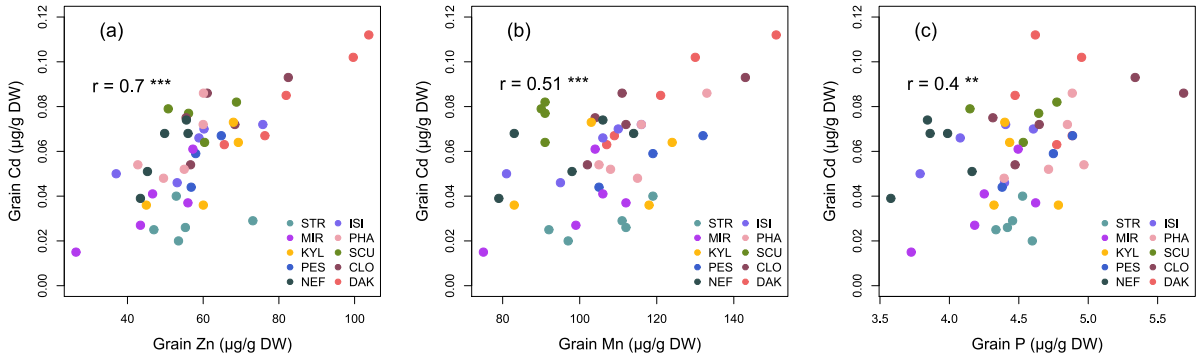
Suppl. Fig. S2-2 Grain dry weight plotted against (a) the dry weight of leaves and (b) the dry weight of stems in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd until maturity.



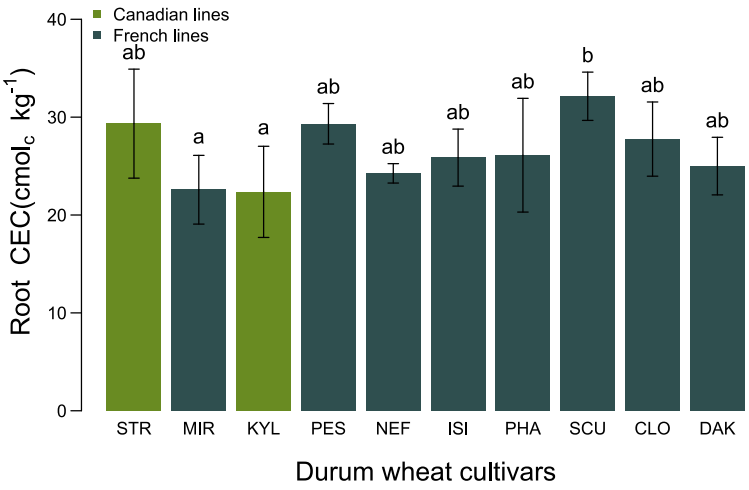
Suppl. Fig. S2-3 Cadmium uptake plotted against the dry weight of roots in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd until maturity.



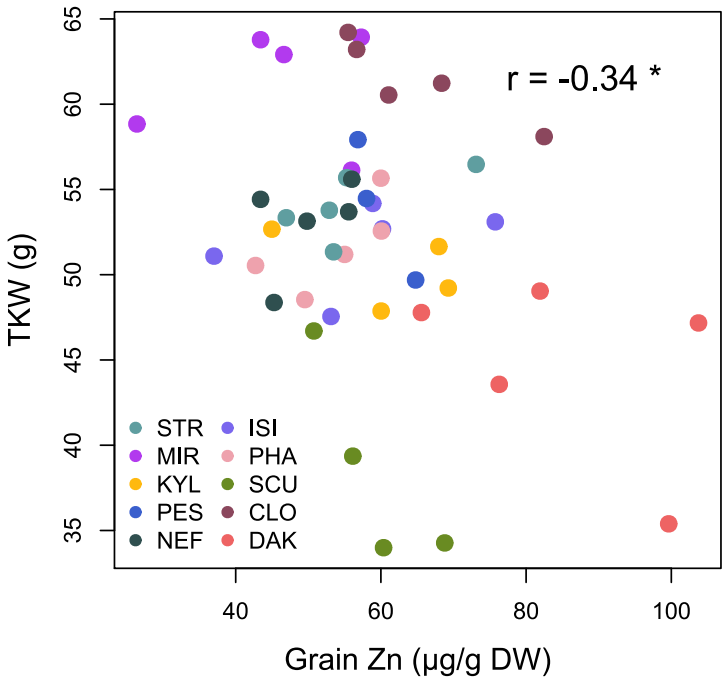
Suppl. Fig. S2-4 Principal component analysis on variables related to the elemental composition of grains collected from 10 durum wheat cultivars grown in hydroponics at 2 nM Cd until maturity. The variables are the thousand kernels weight (TKW), the number of kernels per head (KPH), the kernel residual water (KRW), the protein level (PRO) and the grain concentrations in C, K, P, Ca, Mg, Na, Mn, Fe, Co, Ni, Cu, Zn, Mo, Cr and Cd.



Suppl. Fig. S2-5 Grain Cd concentration plotted against (a) the grain concentration of Zn, (b) the grain concentration of Mn and (c) the grain concentration of P in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd until maturity.



Suppl. Fig. S2-6 Root CEC measured at maturity in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd. The error bars stand for standard deviations. Mean values with different letters are significantly different (P<0.05) as measured by analysis of variance according to a Tukey's test.



Suppl. Fig. S2-7 Grain Zn concentration plotted against thousand kernels weight (TKW) in 10 durum wheat cultivars grown in hydroponics at 2 nM Cd until maturity.

Chapter 3

Contribution of remobilization to the loading of cadmium in durum wheat grains: impact of post-anthesis nitrogen supply

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The results of the previous chapter (Chapter 2) highlighted that the shoot biomass partitioning between grains and straws was a possible cause for the between-cultivars differences in the concentration of Cd in durum wheat grains. Fluxes of Cd between plant parts need to be investigated further to better interpret this finding. Therefore, this chapter was dedicated to assessing the relative contribution of Cd taken up after anthesis and Cd remobilized from vegetative tissue to the grain. Long-term isotopic labeling was performed to trace these fluxes throughout the whole grain filling period. Remobilization of elements is linked to plant ontogeny which is modulated by nitrogen (N) status of plants. The impact of post-anthesis N supply on Cd remobilization was tested on two cultivars, *Sculptur* and *Miradoux*, with contrasted shoot biomass partitioning, as shown in Chapter 2 (Table 2-2).

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Abstract

Aims This study focuses on quantifying the contribution of remobilization to the amount of cadmium accumulated in durum wheat grains. The impact of post-anthesis N supply was tested in two cultivars that differ in their shoot biomass partitioning.

Methods Two French durum wheat cultivars were grown hydroponically and exposed to 100 nM Cd. After anthesis, the plants were fed with a solution enriched in the stable isotope ^{111}Cd to trace the Cd newly absorbed, and subjected or not to nitrogen deprivation. Plants were sampled at anthesis and grain maturity to assess the post-anthesis fluxes of Cd and N among organs.

Results Cadmium remobilized from pre-anthesis stores contributed to more than half of the Cd accumulated in mature grains. Cd was mainly remobilized from stem and poorly remobilized from leaves. Stopping N supply during grain filling enhanced N remobilization but had no impact on post-anthesis uptake and remobilization of Cd, and thereby, on Cd concentration in grains. No difference was observed between the two cultivars in the contribution of Cd remobilization and its dependence toward post-anthesis N supply.

Conclusions Cadmium remobilization significantly contributes to the accumulation of Cd in durum wheat grains. Cd remobilization is not tightly linked with N remobilization and behaves like a senescent-independent process in durum wheat.

Keywords

Low-dose cadmium, cereal, post-anthesis uptake, remobilization, senescence, stable isotope labeling.

3.1 INTRODUCTION

Cadmium (Cd) contamination of agricultural soils is a worldwide concern for food safety. Crop plants can readily accumulate Cd in their edible parts even though this trace metal is non-essential for them. For human beings, Cd can be easily accumulated over time in one's kidney, thus may threaten human health even at very low dose (Emanuelli *et al.*, 2014). Cereals and cereals-based products constitute the major part of human diet, and hence become a major source of human exposure to environmental Cd (EFSA, 2012). The problem of Cd contamination in durum wheat (*Triticum turgidum* L. subsp. *durum*) is more worthy of concern because this species accumulates more Cd in its grains than other commonly grown cereals (Welch and Norvell, 1999), up to levels close to the European regulatory limit of 0.2 mg kg^{-1} FW (≈ 0.23 mg kg^{-1} DW) fixed for wheat (EC, 2006). Reducing the level of Cd in durum wheat grains is thus a major issue. Some studies have been undertaken to find durum wheat cultivars with low potential for accumulating Cd in grains (Archambault *et al.*, 2001; Zimmerl *et al.*, 2014). Besides the genetic approach that generally takes several years before being transferred to the field (Clarke *et al.*, 2010), there is a need for gaining knowledge about the ecophysiology of Cd allocation to durum wheat grains to more readily adapt agronomical practices.

One pending question is whether remobilization contributes much or little to the amount of Cd accumulated in durum wheat grains, in other words, whether grain Cd mainly originates from endogenous Cd remobilized from pre-anthesis stores or from exogenous Cd taken up during grain filling. The extent of the contribution of remobilization is well documented for the most abundant nutrients such as N, S and P (Maillard *et al.*, 2015) with the aim to increase the nutrient use efficiency. For instance, 50 to 90% of grain N was estimated to be remobilized in rice, wheat or maize (Masclaux *et al.*, 2001). However, the contribution of remobilization is less documented for toxic elements such as Cd and the few studies carried out on this topic led to contrasted results. Studies assessing the weight of remobilization from the net difference in the budget of Cd in straws between anthesis and maturity (e.g. Harris and Taylor, 2013; Tavarez *et al.*, 2015) concluded that no Cd was apparently remobilized from vegetative organs in durum wheat. In contrast, short-term labeling experiments (e.g. Harris and Taylor, 2001) underlined that Cd remobilization to maturing grains occurred in durum wheat. By short-term root feeding with ^{106}Cd, Chan and Hale (2004) even stated that the post-anthesis uptake of Cd was much limited and, thereby, suggested that the contribution of remobilization was maximal for Cd in this species. In our view, long-term labeling is required to assess accurately the contribution of remobilization. This approach has been tested for Zn using the isotope ^{68}Zn (Wu *et al.*, 2010) but, to our knowledge, never for Cd.

The technological quality of durum wheat grains relies tightly on their protein content. The grain protein level in durum wheat should exceed 13% to limit the mitadinage (i.e. loss of vitreous aspect of grains) and to produce pasta with sufficient toughness. Nitrogen is the major component of protein and nitrogen supply to cereal crops is the principal factor influencing grain protein concentration (Henry and Kettlewell, 1996). Late N application in the early stage of grain growth is a commonly used strategy to maximize the level of protein in cereal crops because at this stage tillering and leaf expansion have ceased and a higher proportion of the N taken up is therefore allocated to the grains. The question of how this late supply of N affects the accumulation of Cd in durum wheat grains has sometimes been addressed (e.g. Gao *et al.*, 2010) but with focusing mainly on how it affects the availability of Cd in soil. However, there are several reasons to suspect that the (eco) physiological pathways of Cd loading in durum wheat grains would be sensitive to the late supply of N. First, the late supply of N could lower the remobilization of endogenous Cd by delaying the senescence of Cd source tissues. This reason is commonly invoked to explain why no Cd is apparently remobilized in hydroponics (Harris and Taylor, 2013; Tavarez *et al.*, 2015). This statement is based on the assumption that the remobilization of Cd in durum wheat is a senescent-dependent process, as observed for Zn by Kutman *et al.* (2012), and for Cu, Mn, Mo and Zn in bread wheat by Maillard *et al.* (2015). Second, the late supply of N could increase the amount of exogenous Cd taken up during grain filling. From the literature available for Zn (Erenoglu *et al.*, 2011) and Fe (Aciksoz *et al.*, 2011), with whom Cd shares transporters, one can assume that the rate of Cd uptake (and translocation) would also be increased by elevating the supply of N in the nitrate ($N\text{-}NO_3^-$) form. Therefore, it is reasonable to suspect that both the source and the level of Cd in grains would be affected by the late supply of N in durum wheat.

Through a long-term Cd isotope labeling approach, this study aimed (i) to quantify the contribution of remobilization to the amount of Cd accumulated in durum wheat grains, (ii) to assess how the contribution of remobilized Cd is affected by the post-anthesis supply of N, and (iii) to test if the contribution of remobilized Cd as well as its dependence towards the post-anthesis supply of N differs between two durum wheat cultivars showing contrasted shoot biomass partitioning.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

To investigate the impact of the post-anthesis supply of nitrogen on the allocation of Cd to the grain in durum wheat (*Tritium turgidum* L. subsp. *durum* (Desf.) Husn.), two French commonly cultivated spring cultivars, *Sculptur* and *Miradoux*, were grown hydroponically in the presence of Cd. According to Perrier *et al.* (2016), *Sculptur* allocates more shoot biomass to the grain and accumulates higher concentration of Cd in the grain than *Miradoux*. Starting from anthesis, plants were subjected to standard nitrogen supply (+N) or nitrogen deprivation (-N) and were continuously exposed to a source of Cd enriched in the stable isotope ^{111}Cd to label the newly accumulated Cd. At anthesis and at maturity, five independent replicates each consisting of one individual plant were harvested for each cultivar subjected to each N treatment.

3.2.2 Plant culture

Seeds were soaked in 6% H_2O_2 for 10 min, then rinsed three times in deionized water to have their surface sterilized and left on a wet paper in the dark for 4 days at room temperature to germinate. Germinated grains were then transferred to a greenhouse and pre-grown in 50 mL Falcon® tubes filled with the nutrient solution consisting of 0.25 mM KH_2PO_4, 1.25 mM KNO_3, 1.25 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 46.25 µM H_3BO_3, 1 µM $MnCl_2$, 10 µM $ZnSO_4$, 2 µM $CuSO_4$, 0.03 µM $(NH_4)_6MO_7O_{24}$, 50 µM KCl, and 71.63 µM FeNaEDTA. Ten days after, the seedlings were transplanted to polypropylene pots, one seedling per pot, filled with 5 L of the same nutrient solution supplemented with 100 nM $Cd(NO_3)_2$, 25 µM HEDTA, 4 mM MES buffer and 50 mg L^{-1} of SiO_2. The pH was adjusted to 6.0 with solid KOH. The supply of HEDTA buffered free ion metal activities (pM) to environmentally relevant values (McLaughlin *et al.*, 1997): 10.78 for Cd^{2+}, 17.15 for Fe^{3+}, 7.28 for Mn^{2+}, 12.88 for Cu^{2+} and 9.41 for Zn^{2+}, as calculated by the Visual MINTEQ geochemical model (Gustafsson, 2011). The nutrient solution was aerated continuously to maintain the dissolved oxygen above 7 mg L^{-1} and renewed to maintain the pH between 6 and 6.5, the electrical conductivity above 400 µS cm^{-1}, and the total concentration of Cd at 100 ± 20 nM. Before anthesis, the nutrient solution was automatically renewed on a daily basis using an overflow-type system. The turnover rate was gradually increased from 6% per day during the first two weeks up to 26% per day at the end of the vegetative growth. After anthesis, the whole nutrient solution of each pot was manually replaced twice a week. The air temperature used to calculate the growing degree-days (GDD) was recorded together with the air humidity and the photosynthetic

active radiation using a data logger (CR32X, Campbell). The daily mean temperature in the greenhouse ranged between 21 and 31 °C before anthesis (avg. 25 °C), and between 25 and 33 °C from anthesis to maturity (avg. 29 °C). From the start of stem elongation (Zadoks' Scale (ZS) 30), only the first four tillers were allowed to develop. Additional tillers were removed every 2–3 days and discarded over the whole growing period. Only few vegetative tillers (less than 3) developed during grain filling and were removed rapidly after emergence so that their contribution to the post-anthesis budget of Cd was neglected. The phenological stage was closely recorded for each single plant. Plants were considered to reach anthesis on the day their last ear was out of the sheath (ZS 59). *Sculptur* reached anthesis 40 days on average (793 GDD, 4 °C basis) after transplanting and one day earlier than *Miradoux*. For each cultivar, five plants were harvested at anthesis and the rest ten plants were grown until maturity under two contrasted nitrogen supplies: half of the plants was fed with a nitrogen-deprived nutrient solution (-N, $n = 5$) while the other half kept being fed with the standard solution used before anthesis (+N, $n = 5$). To avoid K^+ and Ca^{2+} depletion in -N solution, 0.625 mM K_2SO_4 and 1.25 mM $CaSO_4$ were added as a replacement for 1.25 mM KNO_3 and 1.25 mM $Ca(NO_3)_2$. The two cultivars were harvested 36 days (830 GDD) after ear emergence, when the kernels were hard, i.e. difficult to separate by fingernail (ZS 92).

3.2.3 Cadmium isotope labeling

Twenty milligrams of enriched stable ^{111}Cd isotope nugget obtained from Trace Sciences International (Ontario, Canada) was dissolved with 1 mL of 67.5% HNO_3 and diluted to 50 mL with ultrapure water to obtain a stock solution of 3.605 mM $Cd(NO_3)_2$. The isotopic abundance of standard Cd (Cd_s) and the enriched ^{111}Cd isotope (Cd_{ei}) is shown in Suppl. Table S3-1. The cadmium isotope labeling started at plant anthesis: root systems were drained, bathed for 10 min in ice-cold 5 mM $CaCl_2$ to remove the Cd (with standard isotopic composition) sorbed onto the apoplast, drained again and placed in contact with a fresh nutrient solution at 100 nM Cd where the ^{111}Cd abundance was 95.5% (Suppl. Table S3-1). The exposure to the nutrient solution enriched in ^{111}Cd lasted until harvest.

3.2.4 Plant sampling and analysis

At each harvest, the root systems were first bathed in 5 L of ice-cold 5 mM $CaCl_2$ solutions for 10 min to desorb root apoplastic Cd (Buckley *et al.*, 2010). Then, the plants were divided into heads, leaves, stems and roots. The leaves and stems were washed with deionized water for two times. All

the plant organs were oven-dried at 50 °C for 72 hours. For the plants harvested at maturity, the heads were weighed freshly to determine their kernel residual water (KRW) and separated into grains and bracts+rachis (B+R) after drying. The grains were counted to calculate for each plant the mean number of kernel per head (KPH) and thousand-kernel weight (TKW). All the dried organs were weighted to determine the dry weight (DW), ground into fine powders (Retsch PM 400 for roots; Retsch ZM 1 for grains and stems; Retsch MM 400 for the other organs) before further analysis. In this study, the term 'shoot' refers to all aboveground parts and the term 'straw' to aboveground parts excluding grains.

The digestion was performed in a graphite digestion block system (DigiPREP MS, SCP Science) according to the procedure described in Liñero *et al.* (2016). The total concentration of Cd in the plant organs was determined by graphite furnace atomic absorption spectroscopy (GF-AAS, PinAAcle 900T, Perkin Elmer). The concentrations of N (multiplied by 5.7 to obtain the protein level) and C were assayed with an elemental analyzer (Flash EA1112, ThermoFisher), according to the Dumas method. A grain ionomic profile was established by the central analytical service of the University of the Basque Country based on the concentrations of K, P, Ca, and Mg determined by inductively coupled plasma optical emission spectrometry (ICP-OES, ACTIVA, Horiba Jobin Yvon) and Mn, Fe, Cu, Zn, Mo determined by inductively coupled plasma mass spectrometry (ICP-MS, 7700x, Agilent Technologies).

The $^{114}Cd/^{111}Cd$ isotopic ratio was determined by high resolution (HR)-ICP-MS (Element XR, Thermo Scientific) at the GET laboratory in Toulouse. The detection limit of the Element XR in the plant digests was around 60 ng L^{-1} for ^{114}Cd and 6 ng L^{-1} for ^{111}Cd. To test the analytical accuracy in determining the changes of $^{114}Cd/^{111}Cd$ isotopic ratios, mixtures of Cd_s and Cd_{ei} were prepared with molar ratios of Cd_{ei}/Cd_s ranging from 1:1 to 1:512. The measured ratios against theoretical ones are plotted in Suppl. Fig. S3-1, obtaining a unique regression line with a slope of 1.027 and a determination coefficient of 0.995.

3.2.5 Data analysis

Total amount of Cd at maturity (Q_{Cd}^{tot}) is the sum of the amount of Cd that derives from pre-anthesis (Q_s) and post-anthesis (Q_{ei}) uptake. For each organ (roots, leaves, stems, bracts+rachis, grains) of each plant, the ratio between Q_s and Q_{ei} was calculated from the ^{114}Cd /^{111}Cd ratio of the digest (R_m) following the equation 1 (Rodríguez-Cea *et al.*, 2006):

$$\frac{Q_s}{Q_{ei}} = \left(\frac{M_s}{M_{ei}}\right) \cdot \left(\frac{^{111}A_{ei}}{^{114}A_s}\right) \cdot \left(\frac{R_m - R_{ei(114/111)}}{1 - R_m \cdot R_{s(111/114)}}\right)$$ Equation 3-1

where *M* designates molar weight (M_s = 112.412 g mol^{-1}, M_{ei} = 110.971 g mol^{-1}), *A* abundance of the isotope and *R* isotopic ratio of ^{114}Cd/^{111}Cd or ^{111}Cd/^{114}Cd. The subscript *s* and *ei* designate values derived from Cd with standard (*s*) or enriched ^{111}Cd isotope (*ei*) distributions.

From the Equation 3-1, the amount of Cd in one organ that derives from post-anthesis uptake was calculated as follows:

$$Q_{ei} = \frac{1}{1 + Q_s/Q_{ei}} \cdot Q_{Cd}^{tot}$$ Equation 3-2

Q_s was then obtained by subtracting Q_{ei} from Q_{Cd}^{tot}. The amount of Cd remobilized (Q_{Cd}^{rem}) from (<0) or to (>0) an organ during grain filling was calculated from the difference between Q_s and the total amount of Cd accumulated at anthesis ($\bar{Q}_{Cd\,(anthesis)}^{tot}$) in this organ (Equation 3-3):

$$Q_{Cd}^{rem} = Q_s - \bar{Q}_{Cd\,(anthesis)}^{tot}$$ Equation 3-3

For nitrogen, for which no isotope labeling was performed, the total amount (Q_N^{tot}) in each organ was calculated from the nitrogen concentration measured by the Dumas method, and the amount remobilized (Q_N^{rem}) from (<0) or to (>0) an organ was estimated from the difference in the total amount of N in this organ between anthesis and maturity (Equation 3-4):

$$Q_N^{rem} = Q_{N\,(maturity)}^{tot} - \bar{Q}_{N\,(anthesis)}^{tot}$$ Equation 3-4

All these calculations performed on every single plant allowed us to quantify for each cultivar and each nitrogen treatment, the mean inter-organ fluxes of Cd and N during grain filling and their associated uncertainty, assessed from the variability among the five replicates.

3.2.6 Statistical analysis

Data were processed using ©R 3.2.3 statistical software (R Development CoreTeam, 2015). Two-way ANOVA was performed on plant and grain characteristics measured at maturity to assess the significance of treatment effects (N supply, cultivar) and their interaction. Welch's t-test was performed to identify significant differences between harvest stages (anthesis vs maturity) and to highlight, within one cultivar, the parameters significantly affected by the post-anthesis N supply.

3.3 RESULTS

3.3.1 Plant growth

The plants grew healthily during the whole experimental period and did not show any symptom of Cd toxicity. No impact of N supply was found either on the biomass production or on the biomass partitioning between shoots and roots (SR ratio) and between grains and straws (GS ratio), regardless of the cultivar (Table 3-1). The dry weight (DW) of roots and leaves decreased between anthesis and maturity, in average by 42% and 16%, respectively, for the cultivar *Sculptur*. Leaves collected on mature plants were yellowing and exhibited a lower C concentration (Suppl. Table S3-2) than leaves collected on plants at anthesis. Taken together, these results suggest that mature plants (leaves at least) had initiated their senescence (chloroplast degradation at least) before they were harvested. However, no visible senescence delay was shown between the two N treatments. *Miradoux* produced more ($p < 0.05$) biomass than *Sculptur* for any of the organs and, at maturity, allocated a higher proportion of its shoot biomass to the straw (lower GS ratio) than *Sculptur*, but only under standard N supply.

Table 3-1 Plant biomass, root biomass, stem biomass, leaf biomass, bracts+rachis (B+R) biomass, grain yield, ratio of shoot to root biomass (SR) and ratio of grain to straw biomass (GS), measured at anthesis and maturity in two durum wheat cultivars grown in hydroponics in presence of Cd and subjected to standard nitrogen supply (+N) or nitrogen deprivation (-N) during grain filling. Values are means of five independent replicates

Cultivar	Treatment	Plant biomass	Root biomass	Stem biomass	Leaf biomass	B+R biomass	Grain yield	SR	GS
		g dry weight							
Sculptur	**Anthesis**	8.08	2.27	1.72	2.26	1.83		2.57	
	+N	11.80**	1.20***	1.89	1.88**	2.06	4.78	8.88***	0.82
	-N	11.06**	1.39**	1.72	1.92**	2.04	3.98	7.26**	0.70
Miradoux	**Anthesis**	15.59	4.62	3.58	4.64	2.76		2.39	
	+N	22.41*	2.89	4.14	4.02	3.47	7.89	6.96**	0.68
	-N	23.90*	3.26	4.38	4.28	3.83*	8.15	6.90*	0.66
ANOVA	**N**	ns	ns	ns	ns	ns	ns	ns	ns
	Cultivar	***	***	***	***	***	***	ns	ns
	N × cultivar	ns	ns	ns	ns	ns	ns	ns	ns

Grain yield is expressed as g dry weight per plant (n = 4 tillers).
Two-way ANOVA was performed to assess the effects of the N treatment and the cultivar on the plant growth parameters at maturity: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively.
For a given cultivar, *, ** and *** indicate that the mean value at maturity (+N or -N) significantly differed from that at anthesis, at the probability level of 0.05, 0.01 and 0.001, respectively.

Table 3-2 Kernel residual water (KRW), yield components (thousand kernel weight (TKW), kernel per head (KPH)), protein level and concentrations of Zn, Cu, Fe, Mn, Mo, Ca, K, Mg and P in grains of two durum wheat cultivars grown in presence of Cd and subjected to standard nitrogen supply (+N) or nitrogen deprivation (-N) during grain filling. Values are means of five independent replicates

Cultivar	Treatment	KRW	TKW	KPH	Protein
		%	g		%
Sculptur	**+N**	4.5	35.8	34	23.1
	-N	5.3	32.2	31	25.0
Miradoux	**+N**	4.6	40.1	50	22.9
	-N	4.9	41.0	50	21.3
ANOVA	**N**	ns	ns	ns	ns
	Cultivar	ns	*	***	ns
	N × cultivar	ns	ns	ns	ns

Cultivar	Treatment	Zn	Cu	Fe	Mn	Mo	Ca	K	Mg	P
		µg g^{-1} dry weight					mg g^{-1} dry weight			
Sculptur	**+N**	104	14.6	114	67.8	1.96	0.56	5.95	1.71	6.11
	-N	111	19.0	138	73.7	2.38	0.53	7.02	1.80	6.46
Miradoux	**+N**	122	14.8	116	70.8	3.51	0.78	5.84	1.86	6.40
	-N	130	15.4	113	70.5	3.60	0.71	5.21	1.76	5.82
ANOVA	**N**	ns	*	ns	ns	ns	**	ns	ns	ns
	Cultivar	*	ns	ns	ns	***	***	ns	ns	ns
	N × cultivar	ns	ns	ns	ns	ns	ns	ns	ns	ns

Two-way ANOVA was performed to assess the effects of the N treatment and the cultivar on the grain characteristics at maturity: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively.
For a given cultivar, mean values are underlined when they are significantly higher ($p < 0.05$) than that measured in the other N treatment.

3.3.2 Grain characteristics

The kernel residual water (KRW) was lower than 10% for all the plants indicating that plants were harvested at late maturity (Table 3-2). The thousand-kernel weight (TKW) averaged 34 g for *Sculptur* and 41 g for *Miradoux* while the number of kernel per head (KPH) averaged 32 for *Sculptur* and 50 for *Miradoux*. The grain size (TKW) was thus in line with field data (source Arvalis, data 2013) while the KPH was lower, at least for *Sculptur*. The post-anthesis N supply had no impact ($p > 0.05$) on these two yield components: *Miradoux* produced more and bigger grains than *Sculptur,* regardless of the N treatment. The grain protein level was high, being above 20% in both cultivars

when it rarely exceeds 16% in the field (source Arvalis, data 2017), and was almost not affected by the N treatment. Post-anthesis N-deprivation lowered the grain protein level of *Miradoux* ($p < 0.05$) by less than 2% while it did not affect that of *Sculptur*.

The grain concentration of Cd ranged from 0.56 to 1.57 μg g^{-1} DW and thereby exceeded the limit of 0.2 μg g^{-1} FW (0.23 μg g^{-1} DW) set by the EU for durum wheat (EC, 2006). Post-anthesis N deprivation had no effect ($p > 0.05$) on grain Cd level, which was significantly higher ($p < 0.05$) in *Sculptur* than in *Miradoux* (Table 3-4). The grain concentrations of micronutrients (Zn, Cu, Fe, Mn and Mo) and macronutrients (Ca, K, Mg and P) were higher than standards reported in the field (US Department of Agriculture, 2015), by 2- to 4-fold depending on the nutrient. Post-anthesis N-deprivation almost did not affect the nutrient composition of the grain. Little differences ($p < 0.05$) were observed between the two N treatments, for instance in the concentration of Mo, but these differences were not observed in both durum wheat lines. The grain concentration of Ca, Zn and Mo was respectively 27, 15 and 44% higher in *Miradoux* than in *Sculptur* while that of the other nutrients was similar in the two lines.

Table 3-3 Nitrogen concentration in roots, stems, leaves, bracts+rachis (B+R), and grains, measured at anthesis and maturity in two durum wheat cultivars grown in presence of Cd and subjected to standard nitrogen supply (+N) or nitrogen deprivation (-N) during grain filling. Values are means of five independent replicates

Cultivar	Treatment	Root N	Stem N	Leaf N	B+R N	Grain N
		%				
Sculptur	**Anthesis**	3.23	1.89	4.01	2.38	
	+N	1.22**	2.66*	2.51***	1.80**	4.05
	-N	0.87***	0.63***	1.85***	1.60***	4.38
Miradoux	**Anthesis**	3.64	1.89	4.02	2.60	
	+N	1.91**	1.28**	2.50***	2.65	4.02
	-N	1.23**	0.86**	2.10***	2.32	3.74
ANOVA	**N**	*	***	***	*	ns
	Cultivar	*	***	ns	***	ns
	N × cultivar	ns	***	ns	ns	ns

Two-way ANOVA was performed to assess the effects of the N treatment and the cultivar on the concentration of N in plant tissues at maturity: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively.
For a given cultivar, *, ** and *** indicate that the mean value at maturity (+N or -N) significantly differed from that at anthesis, at the probability level of 0.05, 0.01 and 0.001, respectively.
For a given cultivar, mean values at maturity are underlined when they are significantly higher ($p < 0.05$) than that measured in the other N treatment.

Table 3-4 Cadmium concentration in roots, stems, leaves, bracts+rachis (B+R), and grains, measured at anthesis and maturity in two durum wheat cultivars grown in presence of Cd and subjected either to standard nitrate supply (+N) or nitrate deprivation (-N) during grain filling. Values are means of five independent replicates

Cultivar	Treatment	Root Cd	Stem Cd	Leaf Cd	B+R Cd	Grain Cd
		µg g^{-1} DW				
Sculptur	**Anthesis**	10.16	5.31	3.37	0.45	
	+N	3.73**	3.02***	8.99***	4.07**	1.34
	-N	2.04***	2.34***	8.86***	4.59***	1.21
Miradoux	**Anthesis**	10.13	3.49	2.81	0.30	
	+N	9.34	2.81	7.25***	2.94***	0.75
	-N	6.42	2.82	6.96**	3.17**	0.72
ANOVA	**N**	ns	ns	ns	ns	ns
	Cultivar	*	ns	**	***	***
	N × cultivar	ns	ns	ns	ns	ns

Two-way ANOVA was performed to assess the effects of the N treatment and the cultivar on the concentration of Cd in plant tissues at maturity: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively.
For a given cultivar, *, ** and *** indicate that the mean value at maturity (+N or -N) significantly differed from that at anthesis, at the probability level of 0.05, 0.01 and 0.001, respectively.

3.3.3 Concentrations of nitrogen and cadmium in vegetative organs

As compared to vegetative organs, grain showed the highest concentration of N (Table 3-3) but the lowest concentration of Cd (Table 3-4). In mature plants, the concentration of Cd in shoot organs followed the ranking: leaves > B+R > stem > grains, while the ranking for N varied between cultivars and with the level of N supplied post-anthesis. In *Sculptur*, the N concentration in roots, leaves and B+R decreased significantly ($p < 0.05$) between anthesis and maturity, on average by 68%, 46% and 29%, respectively. In leaves, the decrease in N concentration was stronger under N deprivation (-54%) than under standard N supply (-37%). In stem, the change of N concentration varied between the two N treatments, increasing (+41%) under standard N supply while decreasing (-67%) when N was deprived. Regardless of the N treatment, the concentration of Cd decreased ($p < 0.05$) in roots (-73%) and stem (-50%) and increased in leaves (+164%) and B+R (+874%) over grain filling. As for grains, the concentration of Cd in leaves and B+R was lower in *Miradoux* than in *Sculptur*, regardless of the N treatment. The changes over grain filling in the concentrations of N and Cd in vegetative organs were fairly the same in the two cultivars. The only two differences are that in *Miradoux* (i) the decrease in N concentration was not greater under N deprivation and (ii) the N concentration in stem decreased over grain filling under the two N treatments.

3.3.4 Post-anthesis uptake and apparent remobilization of nitrogen

Figure 3-1 shows nitrogen uptake and apparent remobilization fluxes during grain filling in the durum wheat cultivar *Sculptur*. Under standard N supply (Figure 3-1a), the whole plant amount of N increased from 240 to 342 mg between anthesis and maturity due to post-anthesis N uptake. Over the same period, the amount of N in roots, leaves and B+R decreased by 59, 44 and 7 mg per plant, respectively, which highlights that these three organs behaved like apparent sources of N (i.e. organs from which N was remobilized with a net loss) during grain filling. Conversely, the amount of N in stem increased by 18 mg between anthesis and maturity, which underlines that stem behaved like an apparent sink of N (i.e. organ in which N was net accumulated) during grain filling. The apparent N remobilization efficiency (NRE), defined as the percentage of N remobilized from pre-anthesis stores, averaged 45 ± 5 %. Assuming that all the N remobilized from roots, leaves and B+R was allocated to the grain, the apparent contribution of N remobilization to the grain N averaged 57 ± 4 %.

Under post-anthesis N-deprivation (Figure 3-1b), the whole plant amount of N did not differ significantly ($p > 0.05$) between anthesis and maturity, which indicates that no N was taken up or lost during grain filling. Hence, all N accumulated in the grain was assumed to be remobilized from vegetative organs. As observed under standard N supply, N was remobilized from roots, leaves and B+R but the amounts remobilized were larger, averaging 62 mg for roots, 55 mg for leaves and 11 mg for B+R. Interestingly, stem behaved like a source of nitrogen under post-anthesis N-deprivation, from which 22 mg of N was remobilized, while it behaved like a sink of nitrogen under standard N supply. As a result, the apparent NRE increased to 71 ± 9 % when N was deprived.

In *Miradoux* (Suppl. Fig. S3-2), the rather high variability in plant growth observed between replicates limits the interpretation that can be made of the results. As in *Sculptur*, leaves and roots behaved like apparent sources of N during grain filling. The apparent NRE averaged 40 ± 6 % under standard N supply and was 45 ± 11 % when N was deprived post anthesis.

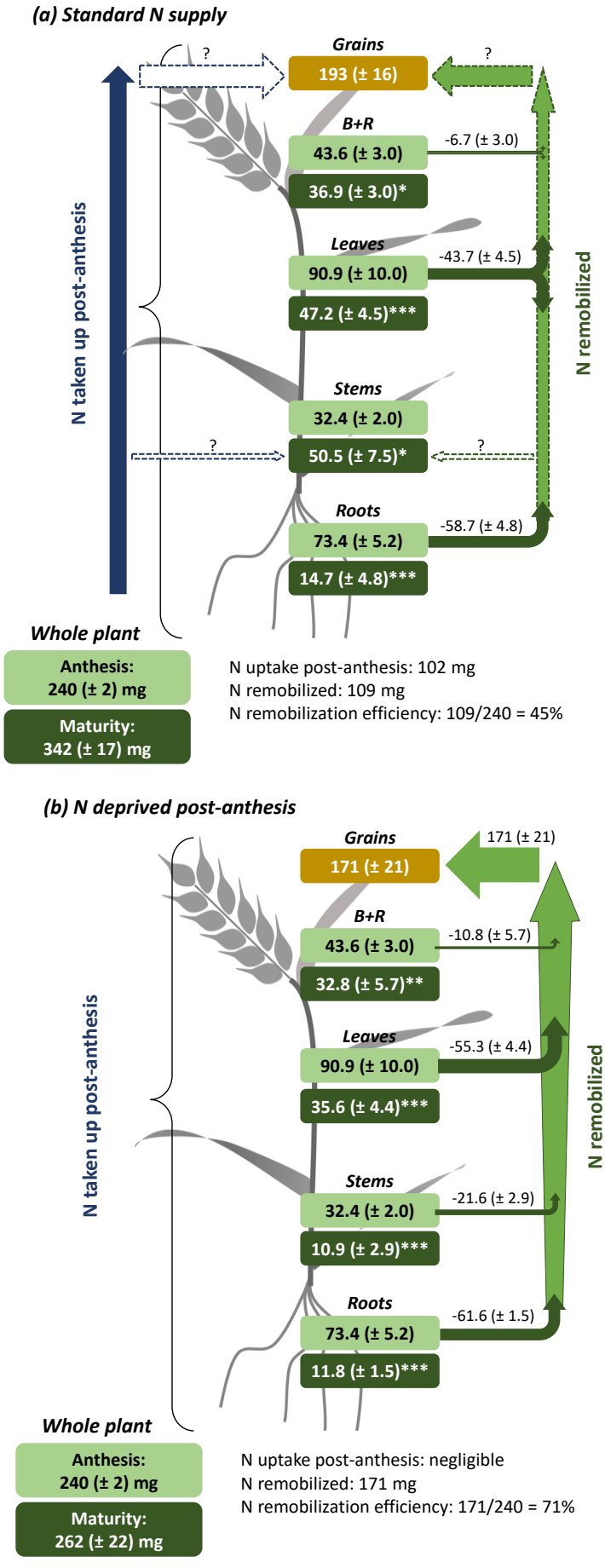

Figure 3-1 Nitrogen contents per plant in hydroponically grown durum wheat (cv. *Sculptur*) subjected to (a) standard nitrogen supply or (b) nitrogen deprivation during grain filling. Black and white values in boxes indicate nitrogen content for each organ at anthesis and maturity, respectively, and are given as mean values (± one standard deviation) for five replicates. Level of significance are indicated by *, ** or *** for $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, between plants at anthesis and maturity. Apparent nitrogen remobilization from (negative values) or to (positive values) an organ was calculated from Equation 3-4 and is given as mean ± one standard deviation. Question marks indicate potential fluxes of nitrogen, which could not be evidenced or accurately assessed.

3.3.5 Post-anthesis uptake and remobilization of cadmium

Figure 3-2 shows Cd uptake and remobilization fluxes during grain filling in the durum wheat cultivar *Sculptur*. Contrary to the case of nitrogen, an isotope labeling approach was used to quantitatively discriminate between Cd accumulated before anthesis (Cd_v) and Cd absorbed during grain filling (Cd_{ei}) in plant organs. Under standard N supply (Figure 3-2a), the amount of Cd taken up post-anthesis was 21 µg when it was about 41 µg pre-anthesis. At maturity, the labeled Cd, which corresponds to the Cd absorbed post anthesis, was recovered in all organs. Most of the newly absorbed Cd was found in vegetative organs: 16% in roots, 15% in stem, 35% in leaves and 19% in B+R, and a similar proportion (15%) as the roots, stem and B+R was allocated to the grains. This means that every organ (and not only grains) behaved like gross sinks for newly absorbed Cd. The amount of Cd accumulated before anthesis decreased over grain filling ($p < 0.05$) in stem (-6.5 µg) and roots (-22.0 µg) while it increased ($p < 0.05$) in B+R (+3.6 µg). This underlines that stem was a net source of Cd, whereas B+R was a net sink, and remobilized Cd during grain filling. Despite the fact that a substantial amount of Cd was taken up post-anthesis, the total amount of Cd in plant did not increase ($p > 0.05$) between anthesis and maturity. In our view, this resulted from a loss of Cd from roots during grain filling. Because of this loss, the flux of Cd remobilization from roots to shoots could not be assessed accurately. The fact that the amount of Cd_v in shoot organs did not increase between anthesis and maturity indicates that the flux of Cd remobilization from roots to shoots, if any, was counterbalanced by a basipetal remobilization of Cd from shoots to roots. Hence, whether roots were a source of Cd for the grain in *Sculptur* remains an open question, as highlighted by the question marks shown in Figure 3-2.

One major result is that Cd remobilized from pre-anthesis stores contributed to more than half (51 ± 7 %) of the Cd accumulated in mature grains under standard N supply. Very similar trends in terms of Cd uptake and remobilization were observed under post-anthesis N-deprivation (Figure 3-2b). The amount of newly absorbed Cd did not decrease ($p > 0.05$) and the relative contribution of remobilized Cd did not increase ($p > 0.05$) when N was deprived. Stem was still the only source of Cd for the grain while B+R was the main sink of remobilized Cd, before grains.

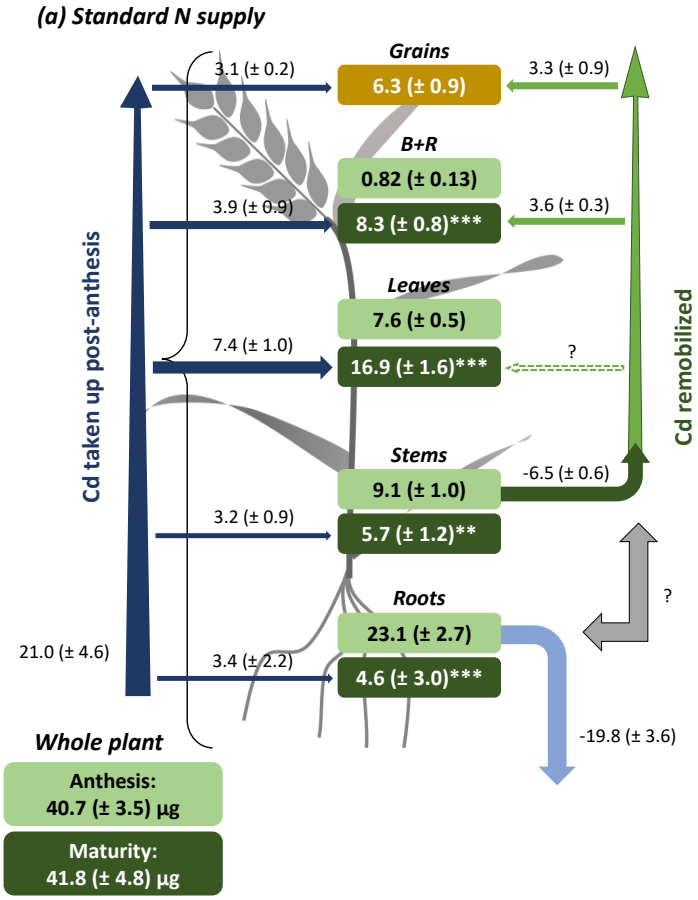

Figure 3-2 Cadmium contents per plant in hydroponically grown durum wheat (cv. *Sculptur*) subjected to (a) standard nitrogen supply or (b) nitrogen deprivation during grain filling. Black and white values in boxes indicate Cd content for each organ at anthesis and maturity, respectively, and are given as mean values (± one standard deviation) for five replicates. Level of significance are indicated by *, ** or *** for $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, between plants at anthesis and maturity. Cd remobilization from (negative values) or to (positive values) an organ as well as allocation of newly absorbed Cd taken was calculated from Equation 3-1, Equation 3-2 and Equation 3-3 and is given as mean ± one standard deviation. Question marks indicate potential fluxes of cadmium, which could not be evidenced or accurately assessed.

In *Miradoux* (Suppl. Fig. S3-3), again, the rather high variability in plant growth observed between replicates limits the interpretation that can be made of the results. However, the main results observed in *Sculptur* also apply to *Miradoux*. For instance, in both cultivars Cd remobilized from pre-anthesis stores contributed to more than half of the Cd accumulated in mature grains. The relative contribution of remobilized Cd was on average even higher in *Miradoux* than in *Sculptur*, regardless of the N treatment (64 ± 10% under standard N supply ($p < 0.05$); 75 ± 12 under N deprivation ($p > 0.05$)). As in *Sculptur*, the amount of newly absorbed Cd and the relative contribution of remobilized Cd were not significantly ($p > 0.05$) altered in *Miradoux* when N was deprived.

3.4 DISCUSSION

Plants were cultivated in hydroponics to make possible the isotopic labeling and to simplify the collection of the root system, in HEDTA-buffered nutrient solution to maintain a constant level of Cd exposure. By root feeding of a source of Cd enriched in ^{111}Cd throughout the grain-filling period, we quantified the contribution of remobilization to the amount of Cd accumulated in durum wheat grains. For the calculations, we assumed that no labeled Cd was remobilized during the time of grain filling, which supposes that Cd was remobilized exclusively from pre-anthesis stores.

3.4.1 Contribution of cadmium remobilization under standard nitrogen supply

About half of the Cd accumulated in durum wheat grains arose from remobilization under standard N supply, i.e. when the supply of nitrate was maintained during grain filling. This rather high contribution of remobilized Cd was observed in the two tested durum wheat lines. It agrees with the results of Rodda *et al.* (2011) who showed through a hydroponic approach in which Cd was supplied to the roots at three different timing regimes (pre-anthesis, post-anthesis and continuously) that 60% of the final amount of Cd in rice grains was remobilized from that accumulated prior to anthesis. It agrees also with the results of the long-term labeling approach conducted by Wu *et al.* (2010) on Zn, with which Cd shares transporters along its way to the grain (Cakmak and Kutman, 2017), which showed that more than half of Zn found in mature rice grains originated from Zn taken up during vegetative growth. However, this rather high contribution of remobilized Cd contrasts with several studies conducted specifically in durum wheat in which little (Tavarez *et al.*, 2015) or no (Harris and Taylor, 2013) net remobilization of shoot Cd pools was

observed. Unlike the Cd labeling we performed, the apparent remobilization approach used in these two studies relies on the budget of Cd amount in plant parts at different growing stages. Therefore, the lack of net remobilization of Cd reported in these two studies does not imply that Cd remobilization did not contribute to Cd accumulation in the grain. It suggests instead, that the rate of Cd remobilization from vegetative organs was lower than or equal to the rate of Cd import to vegetative organs during grain filling.

Because of the xylem discontinuity in the grain pedicel of wheat (O'Brien *et al.*, 1985), phloem is the only pathway for grain Cd loading. Therefore, the large contribution of Cd remobilization observed in this study suggests a rather high phloem mobility of Cd in durum wheat. This finding contrasts with the idea commonly accepted that the limited transport of Cd in the phloem is the main reason for the low rate of Cd remobilization observed from leaves (Harris and Taylor, 2013). Given the alkaline pH (> 7.5) and the elevated concentration of phosphate (> 10 mM) in the phloem (Marschner, 2011), metals must be bound to soluble ligands to be transported efficiently to developing grains. We speculate that the high phloem mobility of Cd results from the abundance of thiol-peptides in the phloem of durum wheat, including phytochelatins, which have been detected in the phloem sap of plants such as *Brassica napus* (Mendoza-Cózatl *et al.*, 2008) and have a strong affinity for Cd.

3.4.2 Source of cadmium remobilization

Uptake and translocation of Cd continued during grain filling and the majority of newly absorbed Cd was delivered to high transpiring organs (Figure 3-2a), in agreement with Harris and Taylor (2013). Most if not all Cd remobilized from pre-anthesis stores was exported from stems. We know from short-term ^{109}Cd labeling experiments that Cd can be remobilized from stems (Harris and Taylor, 2001). The present study highlights that stem Cd pools are major contributors to grain Cd accumulation in durum wheat. Conversely, it confirms that Cd is poorly remobilized from leaves during grain filling. Given the high phloem mobility of Cd observed in this study, we suspect the low rate of Cd remobilization from leaves to result from a low availability for remobilization of Cd stored in leaf cells. Tavarez *et al.* (2015) suggested that Cd is stored in non-labile subcellular compartments to explain why leaves of durum wheat did not show apparent remobilization of Cd. In rice, most of the transporters involved in micronutrient (Cu, Fe, Mn, Zn) mobilization out of leaves are nicotianamine-metal transporters of the YSL family (Khan *et al.*, 2014), which therefore do not likely play a role in the mobilization of Cd. We speculate that the low availability for remobilization of Cd stored in leaves of durum wheat is due to a lack of

transporter able to mobilize Cd. We are aware that the pooling of all the leaves in our experiment may have masked a potential remobilization of Cd from the upper leaves, notably from the flag leaf from which mineral elements are suspected to be remobilized the most (Sperotto *et al.*, 2009). However, we do not think this potential bias was too important since it implies that Cd moved from upper leaves to aging lower leaves, which has almost never been observed for nutrients. Harris and Taylor (2001) showed that the majority of ^{109}Cd exported from the flag leaf accumulated in the grains, while negligible amounts of ^{109}Cd accumulated in lower leaves. In graminaceous plants, nodes play a pivotal role in the accumulation and the distribution of mineral elements to developing grains and the concentration of mineral elements (Zn and Cd included) is usually several times higher in nodes than in other tissues (Yamaji and Ma, 2014b). We suspect that most of the stem Cd pool taken up pre-anthesis was stored in nodes, maybe in the parenchyma cell bridge surrounding enlarged vascular bundles (Yamaguchi *et al.*, 2012), until being remobilized. Under this hypothesis, the high contribution of stem Cd pools to grain Cd would rely on the existence of transporters specifically expressed in nodes and able to mediate the phloem loading of Cd from parenchyma cells, such as OsLCT1 (Uraguchi *et al.*, 2011) and OsHMA2 (Yamaji *et al.*, 2013) in rice.

Taken together, these results are in good agreement with the model proposed by Harris and Taylor (2013), which says that most of Cd accumulated in durum wheat grains is directly transported from the roots through the stem to the grain, with nodes playing a pivotal role in the intervascular transfer of Cd. In contrast with previous investigations (e.g. Harris and Taylor, 2001), half of Cd exported from stems was not directed to the grains but to the bracts+rachis, which suggests that bracts+rachis are phloem sinks in competition with grains during grain filling.

One pending question concerns the contribution of root Cd pools to grain Cd in durum wheat. Given the strong decrease in root Cd observed between anthesis and maturity, and the known ability of durum wheat to remobilize micronutrient such as Zn from root pre-anthesis stores (Kutman *et al.*, 2012), it can reasonably be assumed that part of the Cd exported from roots during grain filling was allocated to the grains. This would imply a basipetal remobilization of Cd (see above), which agrees with the observations of Chan and Hale (2004) in durum wheat. However, we suspect that most of the Cd exported from roots during grain filling was lost from the plant to the nutrient solution. We speculate that the warm conditions during the post-anthesis period increased the root turnover and provoked the decomposition of root cell integrity leading to Cd leakage from the cytoplasm and vacuole to the nutrient solution. Hence, roots are not considered as major contributors to grain Cd in durum wheat, contrary to stems.

3.4.3 Cadmium remobilization as a senescent-independent process?

Mineral element remobilization frequently occurs with vegetative senescence whereby cellular degradation makes nutrients available for filling the grain (Pottier *et al.*, 2014). This is the case for nitrogen. High sink demand of developing grains triggers N remobilization, which begins simultaneously with the start of leaf senescence (Maillard *et al.*, 2015) as a result of the senescent-induced proteolysis of chloroplast proteins, mainly Rubisco, which represent the major fraction of total cellular N of C_3-plants (Hörtensteiner and Feller, 2002). In the present study, N and Cd were not remobilized from the same organs under standard N supply. The changes between anthesis and maturity in the color, the dry weight and in the C concentration of straw tissues suggest that leaves, contrary to stems, had senesced at maturity. This means that Cd was not remobilized from senescing leaves but from non-senescing stems in our experiment. Therefore, we suspect that Cd remobilization is not linked with senescence in durum wheat.

Whether the remobilization of an element is coupled with senescence depends on its storage location in source organs. Given that chloroplasts are first degraded during the senescing process (Pottier *et al.*, 2014), it is reasonable to assume that the remobilization of elements mainly stored in chloroplasts such as N, Fe, Mg and Zn is more tightly linked to senescence than that of elements stored in vacuole (like Cd and S), which remains intact until late after senescence onset. This would explain why the remobilization of Cd is a senescent-independent process. Similarly, the time lag between the remobilization of N and Cd from roots may explain why only Cd was lost from the plant to the nutrient solution, while almost all N was remobilized to the shoots. One pending question is which process drives the remobilization of Cd out of stem in durum wheat. In cereals, the high sink demand of developing grains can trigger the loading of micronutrients from nodes (Yamaji and Ma, 2014b). Since nodal transporters required for loading micronutrients to the phloem might also transport Cd, such as OsHMA2 in rice that shows a transport activity for Zn and Cd (Yamaji *et al.*, 2013), one possibility is that Cd is remobilized out of stem together with micronutrients in durum wheat.

3.4.4 Impact of post-anthesis nitrogen-deprivation

We investigated how the N nutrition at post-anthesis stage affects Cd and micronutrient remobilization by stopping the supply of N during grain filling. It was hypothesized that N deprivation post-anthesis would increase Cd and micronutrient remobilization by inducing N redistribution from vegetative organs. As expected, N remobilization increased under N

deprivation to meet the demand of developing grains. The rate of N export increased, from leaves principally, while the role of stems turned from a net sink to a net source of nitrogen when N was deprived. This agrees with the known affinity of plants to more efficiently remobilize N to the grains under N starvation (Juraniec *et al.*, 2017) and underlines the key role played by stems for increasing the efficiency of N remobilization in wheat (Ben Slimane *et al.*, 2013).

However, there are several reasons to think that the impact of the N nutrition regime on the plant development was less marked in this study than in similar works conducted in durum wheat. N-deprivation did not alter the grain yield, i.e. the sink strength of developing grains, which contrasts with the effect of N nutrition reported, for instance, in Kutman *et al.* (2012). This result relies on the fact the N nutrition level was changed only after anthesis, after N had been supplied (probably in excess but non-toxically) during the whole vegetative stage. After anthesis, N is required for both grain filling and root uptake of nutrients. If the pre-anthesis N reserve is low, which is the case in some studies where N supply was stricter than in the present one, the uptake of nutrients is strongly reduced under post-anthesis N deprivation because the N reserve is dedicated to the grain. In this case, the contribution of nutrient remobilization is expected to be high under low N supply. In the present study, the N reserve was abundant so that there was enough N to be allocated to the grains and to the functioning of roots under post-anthesis N deprivation. Therefore, the uptake of nutrients was less impacted, so was their remobilization and the senescence of vegetative tissues, when N was deprived. We assume this is the main reason why almost no change in grain elemental composition was observed in the two cultivars between the two N treatments, even for micronutrients such as Zn and Fe whose remobilization is correlated with N recycling in durum wheat (Kutman *et al.*, 2012; Kutman *et al.*, 2011a). For Cd, the fact that the remobilization rate did not increase under N-deprivation, together with the increase in NRE, can be interpreted also as an evidence that Cd remobilization is not tightly linked with N remobilization in durum wheat. Further investigations should be done to test this last point in conditions where post-anthesis N-deprivation triggers the senescence of Cd source tissues.

3.4.5 Between-cultivar differences

Two French widely cultivated durum wheat cultivars with contrasted shoot biomass partitioning were compared in this study: *Sculptur* and *Miradoux*. According to our former work (Perrier *et al.*, 2016), *Sculptur* was expected to exhibit a higher grain Cd concentration than *Miradoux*. As a first objective, this varietal comparison aimed to confirm this point at a level of Cd exposure different from that previously tested. In this study, grain Cd was more than 10 times higher than in Perrier

et al. (2016) and exceeded the European maximum limit set for durum wheat. As expected, grain Cd was higher in *Sculptur* than in *Miradoux* and the difference between the two cultivars (1.8-fold variation under standard N supply) was in line with that observed in Perrier *et al.* (2016). This result supports the hypothesis that durum wheat cultivars such as *Miradoux*, which allocate a higher proportion of their aerial biomass to straw, tend to have a lower concentration of Cd in grains. Assuming that the source strength of one organ relies on its biomass, one can expect that the extent of the contribution of Cd remobilization to grain Cd would depend on the partitioning of shoot biomass between sinks and sources, i.e. between grains and straw. In other words, the contribution of Cd remobilization to grain Cd was expected to be higher in *Miradoux*. The second goal of this varietal comparison was precisely to test this hypothesis. Unexpectedly, the contribution of remobilization to grain Cd did not vary between the two cultivars. On one hand, this result strengthens the idea that part of the Cd accumulated in durum wheat grains originates from endogenous Cd taken up before anthesis. On the other hand, it suggests that the contribution of Cd remobilization to grain Cd is not closely linked to the relative biomass of Cd sources. One explanation could be that remobilization is governed by nodes and does not concern the whole straw. This last point has to be considered with caution since the two cultivars were less differing than expected in their GS ratio because of the low number of kernel per head (KPH) observed in *Sculptur*. The last goal of this varietal comparison was to test if differences in shoot biomass partitioning affect how the rate of Cd remobilization and, thereby, the concentration of Cd in durum wheat grains depends on the level of post-anthesis N supply. It was expected that, by triggering the senescence of Cd source tissues, post-anthesis N-deprivation would increase the contribution of Cd remobilization, especially in cultivars with a low GS ratio because of their higher source strength. The fact that both in *Sculptur* and in *Miradoux* the rate of Cd remobilization was not enhanced when N was deprived, even though the rate of N remobilization was, strengthens the idea that Cd remobilization and N remobilization are not tightly linked in durum wheat.

3.5 CONCLUSION

Whether Cd uptake during grain development or Cd remobilization from pre-anthesis stores contributes more to grain Cd accumulation is critical to develop agronomic strategies to minimize grain Cd concentrations in durum wheat. A hydroponic long-term labeling approach was used to quantify the contribution of both remobilization and post-anthesis uptake to grain Cd in two durum wheat cultivars differing in their shoot biomass partitioning and subjected (or not) to N deprivation after anthesis. First, this study showed that 50-60% of Cd in mature grains was

endogenous Cd remobilized from pre-anthesis stores, and the rest 40-50% was exogenous Cd taken up during grain filling. This result suggests that the phloem mobility of Cd in durum wheat is higher than usually described in the literature and highlights the need to control the availability of Cd in soil during the whole lifecycle of durum wheat. Second, this study confirmed that Cd is poorly remobilized from leaves, even when they are senescing. Thereby, leaves can be seen as irreversible sinks for Cd in competition with grains during grain filling. As suggested by Perrier *et al.* (2016), there would thus be an advantage in growing cultivars allocating a high proportion of their aerial biomass to the leaves on Cd-contaminated soils. Further research is needed to better know if this poor contribution of leaf Cd pools to grain Cd relies on the poor availability of Cd for remobilization in leaf tissues. If so, biofortification strategies, which aim at increasing the availability of micronutrients in source tissues for instance through the up-regulation of autophagy (Pottier *et al.*, 2014), should specifically target a metal to avoid increasing the grain concentration in contaminants like Cd at the same time. Third, this study highlighted that stem Cd pools are major contributors to grain Cd in durum wheat. In order to minimize the accumulation of Cd in durum wheat grains, there is thus a need to identify which process drive(s) the remobilization of Cd out of nodes and to understand why their efficiency in mobilizing Cd is higher than that of Cd remobilization processes prevailing in leaves. In last, this study suggests that the post-anthesis N supply does not much affect how Cd is loaded in durum wheat grains. Taken together with the fact that N fertilization rather tends to increase the availability of Cd in soil (Perilli *et al.*, 2010), this result suggests that lowering post-flowering N application could be a strategy to avoid excessive N fertilization (Juraniec *et al.*, 2017) and to decrease the Cd contamination level of durum wheat grains.

3.6 ACKNOWLEDGMENTS

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3.7 SUPPLEMENTARY MATERIAL

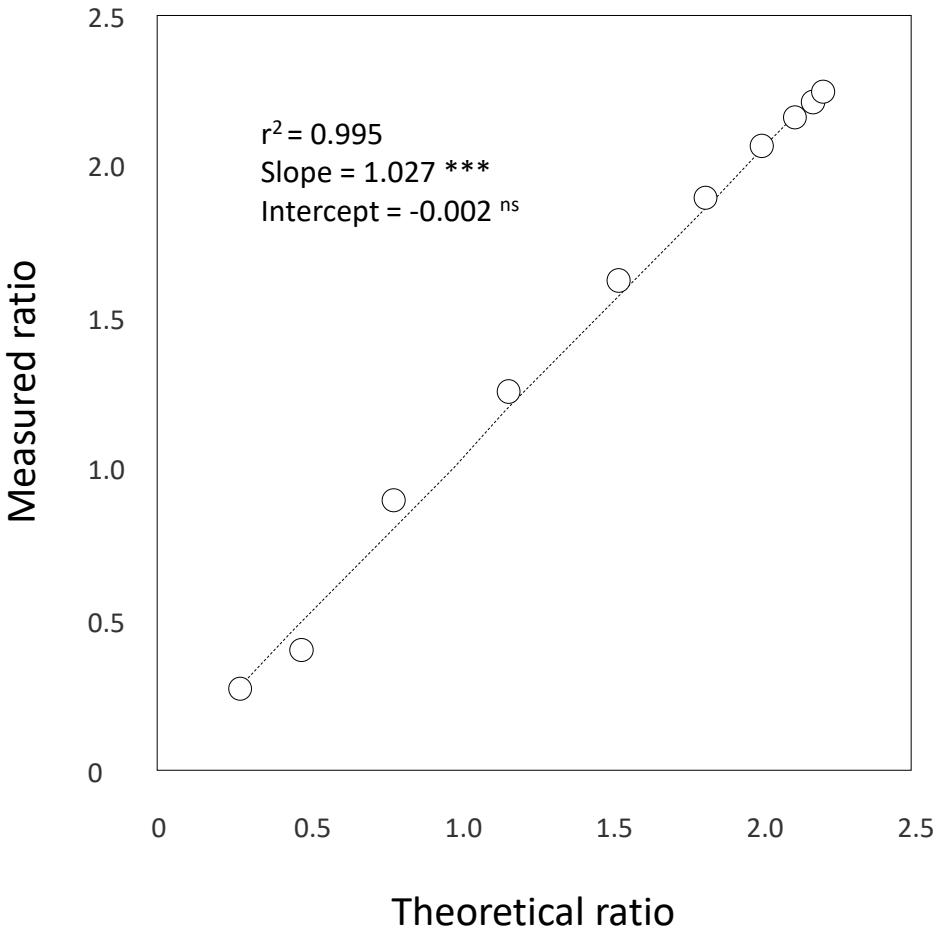
Suppl. Table S3-1 Isotopic abundances (%) of the different sources of Cd

	^{106}Cd	^{108}Cd	^{110}Cd	^{111}Cd	^{112}Cd	^{113}Cd	^{114}Cd	^{116}Cd
Standard (A_s %)	1.25	0.89	12.49	12.80	24.13	12.22	28.73	7.49
Enriched (A_{ei} %)	0.02	0.02	0.50	95.50	2.12	0.59	1.10	0.15

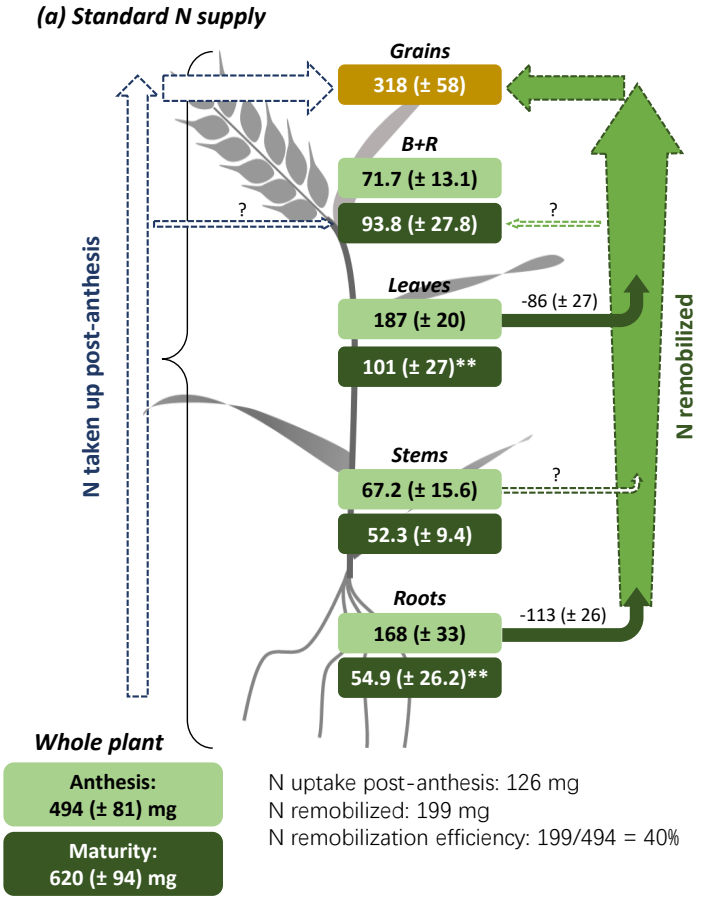
Suppl. Table S3-2 Carbon (C) concentration in roots, stems, leaves, bracts+rachis (B+R) and grains, measured at anthesis and maturity in two durum wheat cultivars grown in presence of Cd and subjected to standard nitrogen supply (+N) or nitrogen deprivation (-N) during grain filling. Values are means of five independent replicates

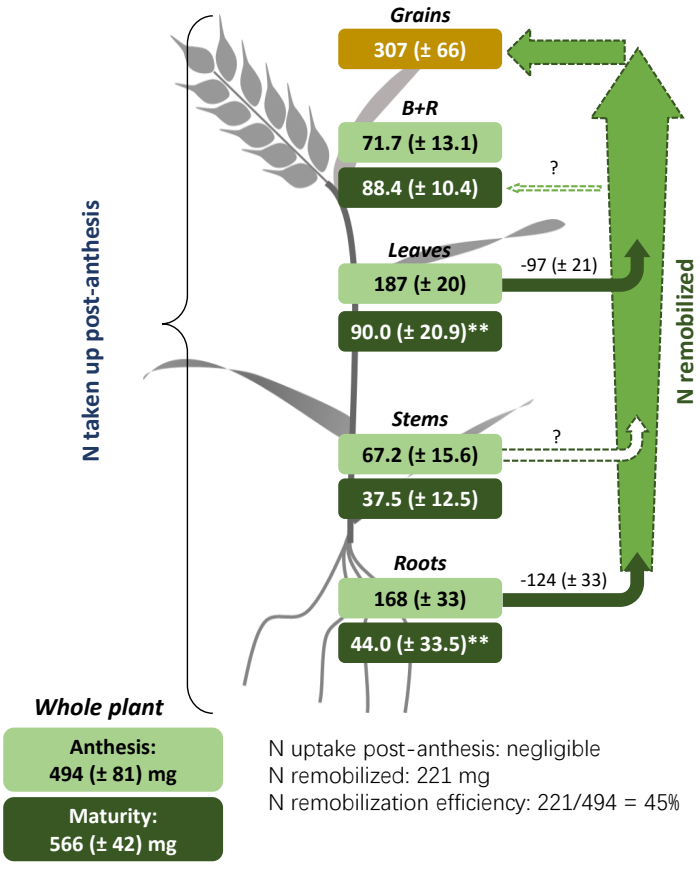
Cultivar	Treatment	Root C	Stem C	Leaf C	B+R C	Grain C
				%		
Sculptur	**Anthesis**	39.54	41.70	42.64	43.95	
	+N	45.86**	40.46	38.21***	41.29***	43.85
	-N	46.23***	<u>43.66</u>**	37.69***	40.36***	43.75
Miradoux	**Anthesis**	38.63	41.65	41.74	43.84	
	+N	43.64***	42.46	37.79***	40.93***	43.55
	-N	44.64**	43.35	37.49*	40.53***	43.62
ANOVA	**N**	ns	**	ns	*	ns
	Cultivar	**	ns	ns	ns	*
	N × cultivar	ns	ns	ns	ns	ns

Two-way ANOVA was performed to assess the effects of the N treatment and the cultivar on the concentration of C in plant tissues at maturity: ns stands for no significance difference while *, ** and *** indicate significant differences at the probability level of 0.05, 0.01 and 0.001, respectively.
For a given cultivar, *, ** and *** indicate that the mean value at maturity (+N or -N) significantly differed from that at anthesis, at the probability level of 0.05, 0.01 and 0.001, respectively.
For a given cultivar, mean values at maturity are underlined when they are significantly higher ($p < 0.05$) than that measured in the other N treatment.

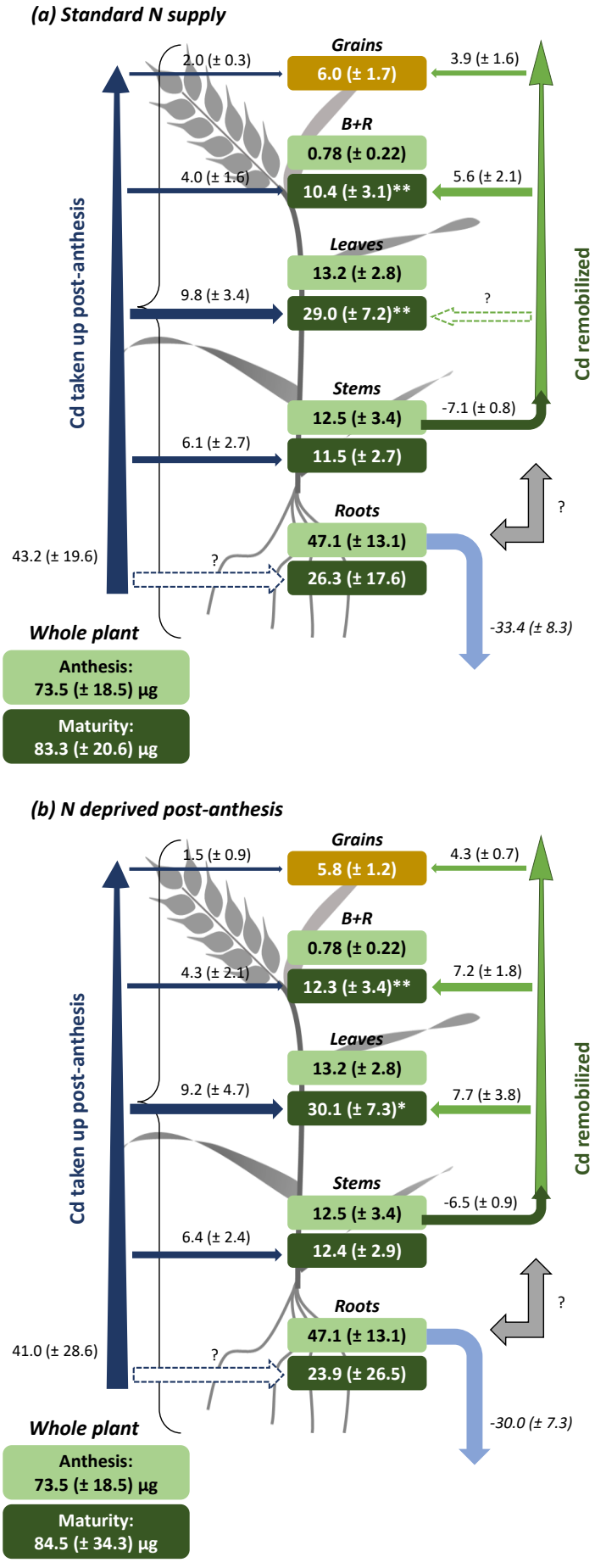


Suppl. Fig. S3-1 Relationships between the theoretical values of $^{114}Cd/^{111}Cd$ isotopic ratio and those measured by HR-ICP-MS, in mixtures of standard (Cd_s) and enriched (Cd_{ei}) sources of Cd with molar ratios ranging from 1:4 to 1:512.





Suppl. Fig. S3-2 Nitrogen contents per plant in hydroponically grown durum wheat (cv. *Miradoux*) subjected to (a) standard nitrogen supply or (b) nitrogen deprivation during grain filling. Black and white values in boxes indicate nitrogen content for each organ at anthesis and maturity, respectively, and are given as mean values (± one standard deviation) for five replicates. Level of significance are indicated by *, ** or *** for $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, between plants at anthesis and maturity. Apparent nitrogen remobilization from (negative values) or to (positive values) an organ was calculated from Equation 3-4 and is given as mean ± one standard deviation. Question marks indicate potential fluxes of nitrogen, which could not be evidenced or accurately assessed



Suppl. Fig. S3-3 Cadmium contents per plant in hydroponically grown durum wheat (cv. *Miradoux*) subjected to (a) standard nitrogen supply or (b) nitrogen deprivation during grain filling. Black and white values in boxes indicate Cd content for each organ at anthesis and maturity, respectively, and are given as mean values (± one standard deviation) for five replicates. Level of significance are indicated by *, ** or *** for $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, between plants at anthesis and maturity. Cd remobilization from (negative value) or to (positive value) an organ as well as allocation of newly absorbed Cd taken was calculated from Equation 3-1, Equation 3-2, and Equation 3-3 and is given as mean ± one standard deviation. Question marks indicate potential fluxes of cadmium, which could not be evidenced or accurately assessed.

Chapter 4
Allocation of cadmium to grains of durum wheat exposed to 5 or 100 nM cadmium in hydroponics

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In the previous chapters (Chapter 2 and Chapter 3), the contribution of remobilization to the loading of Cd in durum wheat grains was assessed at 100 nM Cd. The nanomolar range (low-dose) exposure of plants to Cd made the results relevant to the agricultural context. However, the level of phytoavailable Cd in soils may vary from site to site (Figure 1-2), which would be an ecophysiological factor affecting the allocation of Cd to durum wheat grains. While the response of plants to Cd has been widely investigated at micro- even milli- molar range, which can be rarely encountered in the field, little is known at low Cd exposure. Therefore, this chapter focuses on understanding the impact of Cd exposure at nanomolar range on the growth of durum wheat and on the uptake and internal partitioning of Cd. Isotopic labeling was conducted, as that in Chapter 2, to differentiate the post-anthesis Cd flow from the flow of Cd remobilized, so as to test if their relative contribution to grain Cd varied with the level of Cd exposure.

The chapter will soon be submitted to *Agriculture, Ecosystems & Environment*.

Abstract

This study focuses on characterizing the effect of cadmium (Cd) at low dose (nM range) on the growth and the dynamics of Cd in durum wheat during the grain filling stage. One French durum wheat cultivar *Sculptur* was grown hydroponically and exposed to 5 nM or 100 nM Cd. After anthesis, the plants were fed with a solution enriched in the stable isotope ^{111}Cd to trace Cd newly taken up and Cd remobilized from pre-anthesis pools during the grain filling stage. Plants were sampled at anthesis and at grain maturity to assess the post-anthesis fluxes of Cd among organs. Plant growth and grain yield decreased at 100 nM Cd due to the accumulation of Cd in leaves up to levels above the toxic threshold. The leaves and stem nodes behaved as vegetative sinks sequestrating higher yet insufficient fractions of Cd in plants at the higher exposure. Consequently, the direct flow of Cd from the root to the grain was promoted at the higher exposure, which resulted in a more than proportional increase in the grain Cd. By contrast, Cd remobilization was less affected. Together with an earlier decreased root uptake at 100 nM Cd, the relative importance of post-anthesis uptake and remobilization was not alter by Cd exposures.

Keywords

Low-dose cadmium; toxicity; remobilization; post-anthesis uptake; grain maturation rate; isotope labeling

4.1 INTRODUCTION

Cadmium (Cd) is a toxic element for both plants and humans. Human activities such as the repeated application of phosphate fertilizers have resulted in Cd pollution in a wide range of agricultural soils. Food crops can significantly transfer Cd from the soil to their edible parts. With the crops being meals, Cd can enter human bodies and stay for decades, owing to its long biological half-life (Järup and Åkesson, 2009). Hence, even trace amount of Cd taken up through daily diet could result in considerably body burden of Cd, and thereby, cause adverse health effects. Cereals account for a large fraction in the source of Cd dietary exposure (EFSA, 2012), so their accumulation of Cd needs to be reduced to improve food safety. Durum wheat is one of the four main cereals in France, the first ranking cereal producer and exporter in Europe (source FranceAgriMer, data 2015). The level of Cd in durum wheat is of special concern because this species often concentrates more Cd in its grains than other cereals, such as common wheat and maize (Grant *et al.*, 2008). Limiting the transfer of Cd to the grains is a way to reduce their Cd accumulation level but this requires better understanding the processes of Cd allocation to durum wheat grains.

Key processes controlling the allocation of Cd to the grains are root uptake, root-to-shoot translocation, partitioning among shoot organs, and remobilization from vegetative tissues to the grain (Uraguchi and Fujiwara, 2013). The root uptake of Cd depends on the concentration of free ionic Cd in the growth medium and can be characterized by a Michaelis-Menten equation within a range of Cd exposure (Lux *et al.*, 2011). In durum wheat, the Cd influx in roots increases linearly then reached a plateau along with the gradient increase of Cd concentration in the nutrient solution from 0 to 1.25 μM Cd (Harris and Taylor, 2004; Hart *et al.*, 1998; Lux *et al.*, 2011). Cd translocation from roots to shoot is deemed as an important mechanism in avoiding Cd toxicity to photosynthetic apparatus and a major determinant of grain Cd accumulation (Harris and Taylor, 2013; Shahid *et al.*, 2016). In response to Cd, retention of Cd in roots can be favored by developing apoplastic barriers, by vacuolar storage, and by limiting the xylem loading (Lux *et al.*, 2011). Once translocated to shoots, Cd can be distributed among different organs including the grain. Perrier *et al.* (2016) suggested that, in addition to root uptake and root-to-shoot translocation, Cd partitioning in shoots also plays an important role in influencing the concentration of Cd in mature grains. Shoots possess well-organized vascular and transporter system to regulate the partitioning of Cd among organs (Yamaji and Ma, 2014a). It can be expected that the allocation of Cd in shoots could respond to the change in Cd status.

The impact of Cd exposure on plants is often assessed in presence of micromolar, even millimolar, range of Cd which is rarely encountered in agricultural soils (Nolan *et al.*, 2003; Shahid *et al.*, 2016). By contrast, less is known about the responses at nanomolar range of Cd which is more relevant to the agriculture context (Sauvé *et al.*, 2000). To date, the impact of low-dose exposure to Cd has been tested for some species at vegetative stage. For example, by elevating the background level of Cd to 20 nM, neither the root uptake nor the root-to-shoot translocation of sunflower are restricted (Cornu *et al.*, 2016). In contrast, study on maize showed that even though there is no impact on root uptake, the translocation of Cd to shoots decreases at 20 nM Cd (Nguyen *et al.*, 2015). However, to our knowledge, the plant response at low dose of Cd has never been studied for durum wheat and never on the period of grain filling.

A previous study showed that about half of the grain Cd is remobilized from vegetative tissues (Yan *et al.*, 2018). One pending question is whether this contribution of remobilization would be affected by the level of Cd exposure in durum wheat. The link between remobilization and external availability has been assessed for some elements like zinc (Zn), with whom Cd shares allocation pathways. Low Zn supply results in the higher contribution of remobilization to the amount of Zn in grains (Kutman *et al.*, 2012). However, whether this observed for Zn may also apply for Cd is questionable. On the one hand, the allocation process of Zn was shown to be lack of selectively (Clemens *et al.*, 2013), so, at the lower level of Cd exposure, as the response of Zn remobilization at low Zn supply, the remobilization of Cd would be promoted. Relatively to Zn, on the other hand, Cd is non-essential to higher life. It is thus not necessary for plants to adjust the rate of remobilization of Cd to adapt the Cd status like that for Zn, and to reach a minimum level of Cd in grains. This question is also of importance in developing agronomic strategy to manage the level of Cd in grains. The result of Yan *et al.* (2018) highlights the necessity to control the availability of Cd in soils during vegetative stage of durum wheat because of the significant contribution of remobilization to the grain Cd loading. However, this conclusion still need to be checked at different levels of Cd exposure.

A hydroponic experiment was carried out on durum wheat exposed to 5 or 100 nM Cd which represent respectively the background and the upper limit of Cd in agricultural soils. Stable Cd isotope labeling was applied after anthesis to trace Cd uptake and remobilization fluxes throughout the grain filling period. The study aimed (i) to check if plant growth and grain yield changes between 5 and 100 nM Cd, (ii) to compare the uptake and the between-organ partitioning of Cd at the two levels of Cd exposure, (iii) to examine whether the contribution of remobilization to the amount of Cd in grains depends on the level of Cd exposure.

4.2 METHODS AND MATERIALS

4.2.1 Experimental design

To investigate the impact of the level of exposure to Cd on the uptake and the allocation of Cd to the grain in durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.*)*, *Sculptur*, a French commonly cultivated spring cultivar, was grown hydroponically in the presence of two concentrations of Cd: 5 or 100 nM. Starting from anthesis, plants were continuously exposed to a source of Cd enriched in the stable isotope ^{111}Cd to label the newly accumulated Cd. At anthesis and at maturity, five (or six) independent replicates, each consisting of one individual plant, were harvested for each Cd treatment.

4.2.2 Plant culture

The conditions of hydroponics were almost the same as those described in Yan *et al.* (2018). Briefly, germinated grains were pre-grown for ten days in 50-mL Falcon® tubes before being transferred to polypropylene pots, one seedling per pot, filled with a nutrient solution consisting of 0.25 mM KH_2PO_4, 1.25 mM KNO_3, 1.25 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 46.25 µM H_3BO_3, 1 µM $MnCl_2$, 10 µM $ZnSO_4$, 2 µM $CuSO_4$, 0.03 µM $(NH_4)_6MO_7O_{24}$, 100 µM KCl, 71.63 µM FeNaEDTA, 25 µM HEDTA, 50 mg L^{-1} SiO_2 and 2 mM MES buffer. The solution pH was adjusted to 6.0 with solid KOH and supplemented with either 5 (± 0.7) or 100 (± 10) nM $Cd(NO_3)_2$ according to the Cd treatment. The supply of HEDTA buffered free ion metal activities (pM) to environmentally relevant values: 17.15 for Fe^{3+}, 7.30 for Mn^{2+}, 12.89 for Cu^{2+}, 9.41 for Zn^{2+} and 12.09 (at 5 nM) or 10.78 (at 100 nM) for Cd^{2+}, as calculated by the Visual MINTEQ geochemical model (Gustafsson, 2011). From the start of stem elongation, only the first four tillers were allowed to develop. For each treatment, five plants were harvested at anthesis, i.e. on the day their last head was out of the sheath, and five (or six) other plants were harvested 29 days (550 growing degree-days) later, which corresponded to grain physiological maturity (Perrier et al. 2016). Each harvest was carried out on the same day for both treatments. The daily mean temperature in the greenhouse ranged between 16 and 25 °C before anthesis (avg. 21 °C) and between 19 and 28 °C from anthesis to maturity (avg. 24 °C).

4.2.3 Cadmium isotope labeling

Cadmium isotope labeling was performed according to the procedure described in Yan *et al.* (2018). Briefly, the stock solution enriched in the stable ^{111}Cd isotope was prepared from a nugget obtained from Trace Sciences International (Ontario, Canada). Cadmium isotope labeling started at anthesis by washing the root systems from the Cd (with standard isotopic composition) sorbed onto the apoplast and placing the plants in contact with a fresh nutrient solution with the same Cd concentration, where the ^{111}Cd abundance was 95.5%. The exposure to the nutrient solution enriched in ^{111}Cd lasted until harvest. The isotopic abundance of standard Cd (Cd_s) and the enriched ^{111}Cd isotope (Cd_{ci}) is shown in Suppl. Table S4-1.

4.2.4 Plant sampling and analysis

The procedure of plant sampling was almost the same as the one described in Yan *et al.* (2018). Briefly, root systems were bathed in ice-cold 5 mM $CaCl_2$ solutions to desorb root apoplastic Cd (Buckley et al. 2010) before plant organs (i.e. roots, stems, leaves, bracts, rachis and, if so, grains) were separated and oven-dried at 50 °C for 72 h. For the plants harvested at maturity, the ears were weighed freshly to determine their kernel residual water (KRW) and separated into bracts, rachis and grains after drying. All the dried organs were weighed, ground and digested before further analysis. For all organs except the rachis, the digestion was carried out in a graphite digestion block system (DigiPREP MS, SCP Science), according to the procedure described in Liñero *et al.* (2018). Considering their low mass and their possible low Cd concentration, the rachis was digested following a dedicated protocol. Around 50 mg of ungrounded tissue was added to 1 mL of a H_2O_2/HNO_3 mix (4:1, v/v) in a 5 mL polyethylene tube. After an overnight pre-digestion at room temperature, the rachis was digested in a ventilated oven with the following heating program: 60 °C for 30 min, 80 °C for 45 min and 100 °C for 90 min. After cooling, the digests were then filtered on paper disks and adjusted to 10 mL with ultrapure water before being analyzed. The recovery rate of this digestion procedure was checked using a certified sample of tomato leaves (NIST-SRM 1573a) and was 99.5% for Cd.

As described in Yan *et al.* (2018), the concentration of total Cd in plant organs was determined by GF-AAS (PinAAcle 900 T, Perkin Elmer), the concentration of N in grains (multiplied by 5.7 to obtain the protein level) and leaves was assayed by an elemental analyzer (Flash EA1112, ThermoFisher) and the grain elemental profiling was established based on the concentrations of K, P, Ca, Mg measured by ICP-OES (ACTIVA, Horiba Jobin Yvon) and Mn, Fe, Cu, Zn, Mo

measured by ICP-MS (7700x, Agilent Technologies) by the central analytical service of the University of the Basque Country. The $^{114}Cd/^{111}Cd$ isotopic ratio was determined by high resolution (HR)-ICP-MS (Element 2, Thermo Scientific) at the IPGP laboratory in Paris. The detection limit of the Element 2 in the plant digests was around 33 ng L^{-1} for ^{114}Cd and 2 ng L^{-1} for ^{111}Cd.

4.2.5 Cadmium accumulation in the first node and in the peduncle

This measurement was made on extra plants cultivated at the same time and in the same conditions as the plants dedicated to the Cd isotope labeling approach. The first node (i.e. the uppermost node) and the peduncle (i.e. the stem section between the first node and the ear) were cut on tillers (n = 25) randomly picked from plants exposed to the same concentration of Cd, and pooled five by five to get five replicates per Cd treatment. Tissues were cut into small pieces with a ceramic scalpel and oven-dried at 50 °C to a constant weight, then digested following the same procedure as the one described for the rachis (see section 4.2.4).

4.2.6 Data processing

For each organ of each plant, the ratio between the amount of unlabeled Cd taken up pre-anthesis (Q_s) and the amount of labeled Cd taken up post-anthesis (Q_{ei}) was calculated from the $^{114}Cd/^{111}Cd$ ratio (R_m) measured in the corresponding digest following the Equation 4-1 (Rodríguez-Cea *et al.*, 2006):

$$\frac{Q_s}{Q_{ei}} = \left(\frac{M_s}{M_{ei}}\right) \cdot \left(\frac{^{111}A_{ei}}{^{114}A_s}\right) \cdot \left(\frac{R_m - R_{ei(114/111)}}{1 - R_m \cdot R_{s(111/114)}}\right) \qquad \text{Equation 4-1}$$

where M designates molar weight (M_s = 112.412 g mol^{-1}, M_{ei} = 110.971 g mol^{-1}), A abundance of the isotope and R isotopic ratio of $^{114}Cd/^{111}Cd$ or $^{111}Cd/^{114}Cd$. The subscript s and ei designate values derived from Cd with standard (s) or enriched ^{111}Cd isotope (ei) distributions.

From Q_s/Q_{ei}, the amount of Cd in one organ that derives from post-anthesis uptake was calculated as follows:

$$Q_{ei} = \frac{1}{1 + Q_s/Q_{ei}} \cdot Q_{Cd}^{tot} \qquad \text{Equation 4-2}$$

where Q_{Cd}^{tot} is the total amount of Cd at maturity measured by GF-AAS. Q_s was then obtained by subtracting Q_{ei} from Q_{Cd}^{tot}.

As detailed in Yan *et al.* (2018), the amount of Cd remobilized from (< 0) or to (> 0) an organ was calculated from the difference between Q_s and the total amount of Cd present in this organ at anthesis. For nitrogen, for which no isotopic labeling was carried out, the amount remobilized from or to an organ was estimated from the net difference in the amount of N in this organ between anthesis and maturity.

4.2.7 Statistical analysis

Data were processed using ©R 3.4.2 statistical software. Student's t-test was used to identify the significant between two groups at $p < 0.05$.

Table 4-1 Grain yield, rachis biomass, bracts biomass, leaf biomass, stem biomass, root biomass, plant biomass, ratio of shoot to root biomass (SR) and ratio of grain to straw biomass (GS), measured at anthesis and maturity in durum wheat (cv. *Sculptur*) grown in hydroponics in presence of Cd at 5 or 100 nM. Values are means of five or six independent replicates

Treatment	Stage	Grain yield	Rachis biomass	Bract biomass	Leaf biomass	Stem biomass	Root biomass	Plant biomass	SR	GS
		g dry weight								
5 nM Cd	***Anthesis***	-	0.10 a	1.63 a	1.56 a	1.37 a	2.02 b	6.67 a	2.31 a	-
	Maturity	10.36	0.20 b	2.24 b	1.74 b	2.03 b	1.68 a	18.25 b	9.91 b	1.67
100 nM Cd	***Anthesis***	-	0.10 a'	1.71 a'	1.70 a'	1.52 a'	2.16 b'	7.18 a'	2.33 a'	-
	Maturity	9.22	0.16 b'	1.79 a'	1.58 a'	1.74 b'	1.25 a'	15.74 b'	11.56 b'	1.75

Grain yield is expressed as g dry weight per plant (n = 4 tillers).
For a given treatment, different letters in a column indicate significant differences ($p < 0.05$) between growth stages.
For each growth stage, values are underlined when they are significantly higher ($p < 0.05$) than that measured under the other treatment.

4.3 RESULTS

4.3.1 Plant growth

No visual symptom of Cd toxicity such as leaf rolling, chlorosis, and necrosis was observed at both Cd exposures throughout the culture period. However, some plants showed an accelerated yellowing of their leaves when exposed to 100 nM Cd (Suppl. Fig. S4-1). At anthesis, the dry weight (DW) of every organ was the same at both Cd exposures (Table 4-1), which means that plant growth was not affected ($p > 0.05$) by the presence of Cd during the vegetative stage. Conversely,

at maturity the dry weight of every organ was lower ($p < 0.05$) in plants exposed to 100 nM Cd. In average, the plant DW decreased by 14% between the two Cd exposures. The decrease in root DW (26%) was larger than in shoot DW (13%), which suggests that roots were more sensitive than shoots to the increase in Cd exposure.

Table 4-2 Kernel residual water (KRW), yield components (thousand kernel weight (TKW), kernel per head (KPH)), protein level and concentrations of Cd, Zn, Cu, Fe, Mn, and Mo in grains of durum wheat (cv. *Sculptur*) grown in hydroponics in presence of Cd at 5 or 100 nM. Values are means of five or six independent replicates

Treatment	KRW	TKW	KPH	Protein	Zn	Cu	Fe	Mn	Mo
	%	g		%	μg g^{-1} dry weight				
5 nM Cd	46.3	43.0	<u>60</u>	15.6	65.5	9.4	48.0	<u>65.8</u>	2.20
100 nM Cd	36.7	42.7	54	<u>17.8</u>	<u>78.2</u>	11.1	66.7	55.0	<u>2.73</u>

Values are underlined when they are significantly higher ($p < 0.05$) than that measured under the other treatment.

4.3.2 Grain characteristics

The grain size assessed from the thousand-kernel weight (TKW) and the number of kernel per head (KPH) were in line with field data reported for *Sculptur* (source Arvalis, data 2017) (Table 4-2). The grain size was not affected ($p > 0.05$) by the level of exposure to Cd while the KPH was slightly lower ($p < 0.05$) in plants exposed to 100 nM Cd. As a result, the grain yield was less (8% lower) in durum wheat plants exposed to 100 nM Cd. In average, the grain concentration of nitrogen (which makes the protein level) and of most micronutrients (Zn, Mo, Cu, Fe) was 14 to 39% higher in plants grown at 100 nM than at 5 nM Cd. Manganese (Mn) was the only micronutrient, which concentration in grain was less at 100 nM Cd. The kernel residual water (KRW) was in average lower at 100 nM than at 5 nM Cd. However, this difference was not significant ($p > 0.05$) since the KRW greatly varied (from 21 to 48%) between replicates at 100 nM Cd. The lower the KRW, the faster the grain maturation. It seems that grains matured faster at 100 nM Cd, but this trend was not observed on every replicate.

4.3.3 Cadmium accumulation level in plant tissues

The concentration of Cd in grains ranged from 0.039 to 0.052 μg g^{-1} DW at 5 nM Cd, which is the level commonly encountered in the field (Mench and Baize, 2004), and from 1.00 to 1.67 μg g^{-1} DW at 100 nM Cd, which is 4- to 7-time higher than the regulatory limit set by the European

Union for durum wheat (EC, 2006) (Table 4-3). Cadmium was not uniformly distributed within the plant. At anthesis, Cd concentration in plant tissues decreased in the order: roots > stems > leaves > rachis > bracts, while at maturity the ranking was leaves > bracts, roots > rachis > stems > grains, regardless of the level of exposure to Cd. Cd concentration increased between anthesis and maturity in high-transpiring organs such as bracts and leaves. In bracts, the level of Cd increased 17-fold at 5 nM and 14-fold at 100 nM between the two stages. In leaves, the concentration of Cd at maturity reached 15 μg g^{-1} DW in plants exposed to 100 nM Cd, which is above the Cd critical toxicity level reported for wheat (White and Brown, 2010). Conversely, the concentration of Cd remained constant in roots while decreased in stems during grain filling, at both Cd exposures. Within stems, the concentration of Cd was higher in the first node than in the peduncle at maturity (Figure 4-1), the difference between the two being larger at 100 nM than at 5 nM Cd.

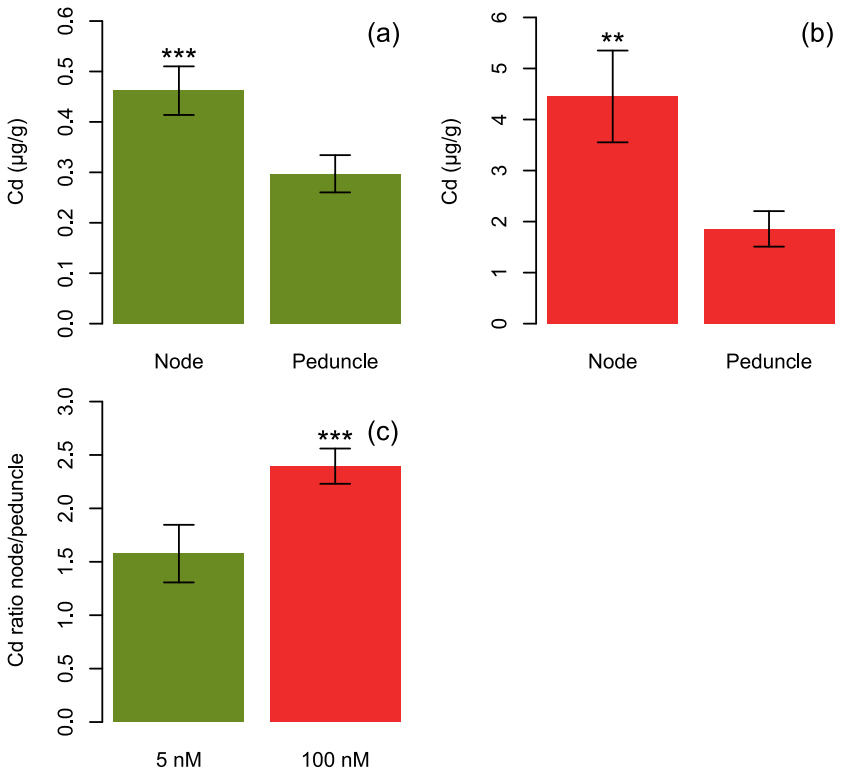

Figure 4-1 Concentration of Cd in the first node and the peduncle of hydroponically grown durum wheat (cv. Sculptur) exposed to (a) 5 or (b) 100 nM Cd; and (c) the ratio of Cd concentrations between the first node and the peduncle at 5 or 100 nM Cd. Data are mean values ± standard deviation (bars) calculated from 5 replicates. Levels of significance are indicated by *, ** or *** for $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, between bars in each plot.

4.3.4 Pre-anthesis uptake and partitioning of cadmium

At anthesis, the overall amount of Cd taken up by plant roots (Figure 4-2) was 20-fold higher at 100 nM than at 5 nM Cd. This means that the uptake of Cd increased in direct proportion to the concentration of Cd in the nutrient solution during the vegetative stage. The partitioning of Cd between organs varied between the two Cd exposures (Figure 4-3a). Most of the Cd taken up before anthesis was sequestrated in roots but this fraction was surprisingly lower ($p < 0.05$) at 100 nM than at 5 nM Cd. Aboveground, the fraction of Cd allocated to high-transpiring organs, namely leaves and bracts, increased between 5 and 100 nM to the detriment of the fraction that remained in stem. All these differences in Cd partitioning were moderate (< 5 points) and were not linked to differences in the partitioning of biomass, which was rigorously the same at the two Cd exposures (Table 4-1).

Table 4-3 Cadmium concentrations in plant organs at anthesis and maturity in durum wheat (cv. *Sculptur*) grown in hydroponics at 5 and 100 nM Cd, and their ratios between treatments at each growth stage. Values are means of five independent replicates. Ratios are between mean values ± standard deviation of five or six independent replicates calculated using Taylor expansions

Treatment	Stage	Grain Cd	Rachis Cd	Bract Cd	Leaf Cd	Stem Cd	Root Cd	Shoot Cd	Plant Cd
		µg/g dry weight							
5 nM Cd	*Anthesis*	-	0.07 a	0.02 a	0.17 a	0.29 b	0.54 a	0.15 a	0.27 a
	Maturity	0.04	0.27 b	0.34 b	0.72 b	0.20 a	0.66 a	0.18 a	0.22 a
100 nM Cd	*Anthesis*	-	1.53 a'	0.57 a'	4.31 a'	4.97 b'	9.29 a'	3.19 a'	5.01 b'
	Maturity	1.40	3.66 b'	8.05 b'	15.00 b'	2.49 a'	7.46 a'	3.85 b'	4.15 a'
Ratio (100/5 nM)	*Anthesis*	-	22 ± 7	<u>29 ± 5</u>	<u>26 ± 6</u>	17 ± 4	17 ± 5	21 ± 4	19 ± 4
	Maturity	<u>31 ± 6</u>	14 ± 4	<u>24 ± 3</u>	21 ± 3	<u>13 ± 5</u>	<u>11 ± 8</u>	22 ± 2	19 ± 3

For a given treatment, different letters in a column indicate significant differences ($p < 0.05$) between growth stages. Ratios are underlined when they are significantly different ($p < 0.05$) from 20.

4.3.5 Post-anthesis uptake and partitioning of cadmium

An isotope labeling approach was used to quantitatively discriminate between Cd absorbed before anthesis (Cd_s) and Cd absorbed after anthesis (Cd_{ei}) in plant organs. In average, the amount of Cd taken up post-anthesis was 2.5 µg at 5 nM when it was 39 µg (i.e. 16-fold higher) at 100 nM (Figure 4-2). This means that the uptake of Cd did not increase in direct proportion to the concentration of Cd in the nutrient solution during the grain filling stage. It is worth noting that the amount of Cd_{ei} absorbed at 100 nM varied greatly among replicates, which was not the case at 5 nM. At maturity, the labeled Cd was found in all organs, which means that every organ (and not only grains) behaved as gross sinks for the Cd absorbed after anthesis. Most of the newly absorbed

Cd was recovered in vegetative organs, especially in high transpiring organs such as leaves and bracts, to which almost half of the Cd_{ei} was allocated at both Cd exposures. Several differences in Cd_{ei} partitioning were observed at maturity between the two levels of Cd exposure (Figure 4-3b). They were substantially more pronounced than the differences in Cd_s partitioning observed at anthesis (and described above). Notably, the fraction of Cd_{ei} retained by roots and stem decreased ($p < 0.05$) with the increase in the level of Cd exposure, by a factor of 1.8 for roots and 1.5 for stem. Conversely, the fraction of Cd_{ei} allocated to the grains was almost twice higher ($p < 0.05$) at 100 nM than at 5 nM Cd. Part of these changes in the partitioning of Cd_{ei} were linked to differences in the partitioning of plant biomass. For instance, the lower fraction of Cd_{ei} retained by roots at 100 nM is related to the lower root DW measured at this level of Cd exposure ($r = 0.83$, $p = 0.001$). In contrast, the fraction of Cd_{ei} allocated to the grains has nothing to do with the grain DW, the first increasing when the second decreased between 5 and 100 nM Cd.

4.3.6 Cadmium remobilization

The direction of the net fluxes of Cd remobilized within the plant was not altered by the increase in the level of Cd exposure (Figure 4-2). At both Cd exposures, the amount of Cd accumulated before anthesis (Cd_s) decreased over grain filling in stem and roots, while it increased in all other organs. This highlights that stem and roots were net sources of Cd, from which Cd was remobilized during grain filling, whereas leaves, bracts, rachis and grains were net sinks receiving the Cd remobilized (Figure 4-2). At 100 nM, the overall amount of Cd_s recovered in plant tissues decreased ($p < 0.05$) between anthesis and maturity (Figure 4-2), which suggests a net loss of Cd from roots during grain filling. Because of this loss, the flux of Cd remobilization from roots to shoots could not be assessed accurately at this level of Cd exposure. However, the fact that the amount of Cd_s recovered in shoots was higher at maturity than at anthesis, even at 100 nM, strongly suggests that Cd was net remobilized from roots at both Cd exposures. The "efficiency" of Cd remobilization was calculated as the amount of Cd remobilized from the two net Cd sources (i.e. stem and roots) divided by the amount of Cd present at anthesis in all vegetative organs. Cd remobilization "efficiency" was not affected ($p > 0.05$) by the level of Cd exposure. However, the fraction of Cd remobilized delivered to the grains averaged 14 ± 1% at 5 nM Cd and 19 ± 4% at 100 nM Cd, so increased ($p = 0.019$) with the increase in the level of Cd exposure. Overall, Cd remobilized from pre-anthesis stores contributed to nearly half of the Cd accumulated in mature grains. This contribution was in average the same ($p > 0.05$) at 5 nM (45 ± 3%) and 100 nM Cd (42 ± 8%) but showed a higher variability in the latter case.

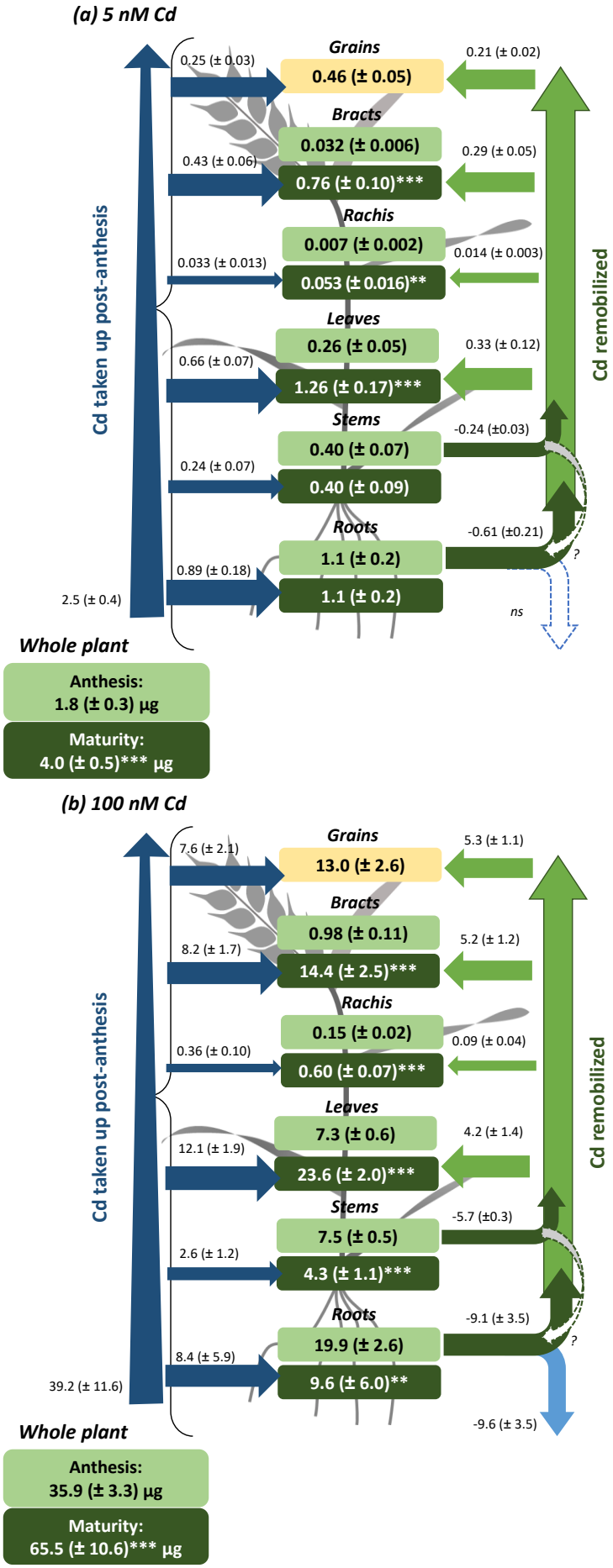

Figure 4-2 Cadmium contents per plant in hydroponically grown durum wheat (cv. *Sculptur*) exposed to (a) 5 or (b) 100 nM Cd. Black and white values in boxes indicate Cd content for each organ at anthesis and maturity, respectively, and are given as mean values (± one standard deviation) for five or six replicates. Levels of significance are indicated by *, ** or *** for $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, between plants at anthesis and maturity. Cd remobilization from (negative values) or to (positive values) an organ as well as allocation of newly absorbed Cd taken is given as mean ± one standard deviation. Question marks indicate potential fluxes of cadmium, which could not be evidenced or accurately assessed. *ns*, not significant.

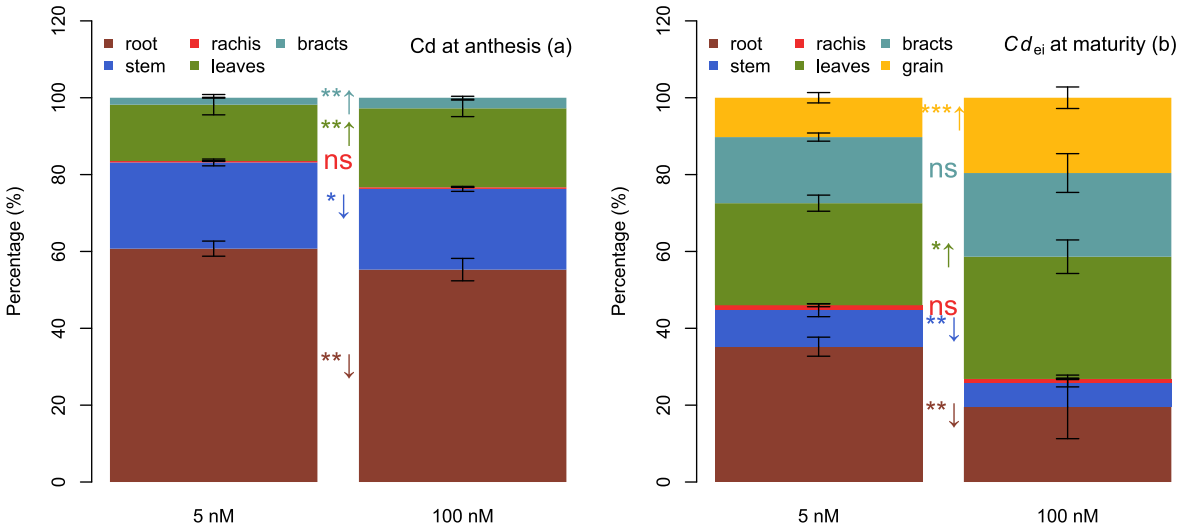

Figure 4-3 The partitioning of (a) Cd at anthesis and (b) Cd newly taken up during grain filling (Cd_{ei}) among plant organs of hydroponically grown durum wheat (cv. *Sculptur*) exposed to 5 or 100 nM Cd. Different plant organs are indicated by colors. Data are mean values ± standard deviation of five or six replicates. Levels of significance are indicated by *, ** or *** for $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, between each organ at 5 and 100 nM Cd. Arrows refer to the higher (↑) or lower (↓) fraction at 100 nM Cd compare to that at 5 nM Cd. *ns*, not significant.

4.3.7 Relationships between the grain maturation rate and the dynamics of cadmium post-anthesis

Plants grown at 100 nM were characterized by a large variability in the post-anthesis dynamics of Cd. They were also characterized by a large variability in their grain maturation rate. Figure 4-4 shows the relationships between the dynamics of Cd post-anthesis and the rate of grain maturation, using the KRW of grains measured at harvest as an indicator of the grain maturation rate. At 100 nM, no significant relationship ($p > 0.05$) was observed between KRW and the amount of Cd remobilized (Figure 4-4b), KRW and the fraction of Cd remobilized allocated to the grains (Figure 4-4c), and KRW and the fraction of Cd_{ei} allocated to the grains (Figure 4-4e). Conversely, the amount of Cd taken up after anthesis decreased ($p < 0.05$) with the decrease in KRW (Figure 4-4c). Hence, the lower the KRW was, the higher the Cd remobilization contributed to grain Cd (Figure 4-4a). This figure also suggests that for a given rate of grain maturation, for instance for a KRW comprised between 40 and 50%, the contribution of Cd remobilization tended to be lower at 100 nM than at 5 nM Cd. This point has to be related to the higher fraction of Cd_{ei} allocated to the grains observed at 100 nM (Figure 4-4e).

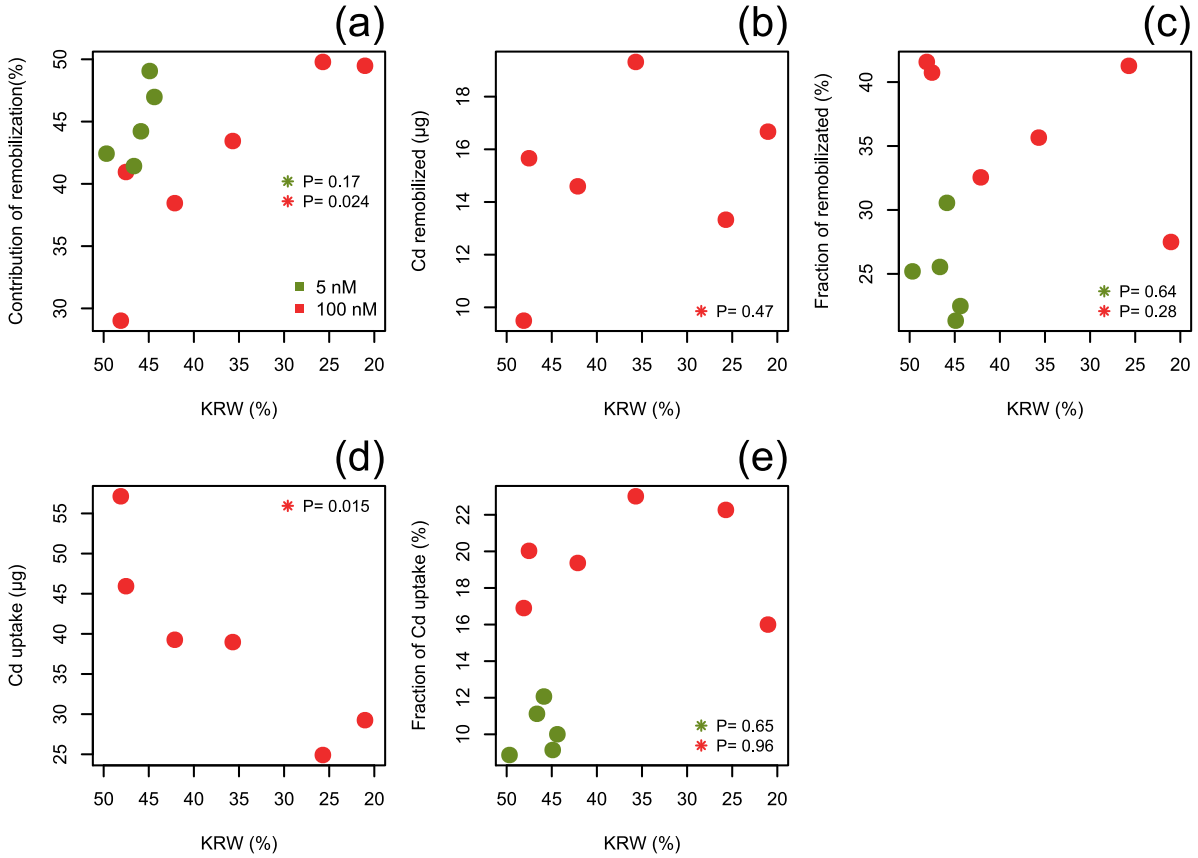

Figure 4-4 Relationships between the kernel residual water (KRW) and (a) the relative contribution of remobilization to the amount of Cd in mature grains; (b) the amount of Cd remobilized from pre-anthesis stores; (c) the fraction of Cd remobilized to the grain; (d) the amount of Cd taken up after anthesis; (e) the fraction of Cd taken up after anthesis to the grain, for hydroponically grown durum wheat (cv. *Sculptur*) exposed to 5 (●) or 100 nM Cd (●). *P* values following green (✳) or red (✳) asterisk in plots indicate significance of the correlation at 5 or 100 nM Cd, respectively.

4.4 DISCUSSION

4.4.1 Effect of cadmium exposure on plant growth

The increase in the level of Cd depressed the vegetative growth and grain yield at maturity but not at anthesis (Table 4-1). It may either result from a direct Cd toxicity or from the metabolic cost associated with Cd homeostatic mechanisms. The critical level of Cd toxicity is reported to be around 5-10 µg Cd g^{-1} leaves dry weight, above which plant growth is depressed (White and Brown, 2010). At anthesis, the concentration of Cd in leaves was below the critical level of Cd toxicity (Table 4-3), even at 100 nM Cd, which indicates that plant growth was unlikely affected by the presence of Cd during the vegetative stage. However, at maturity the leaf Cd reached 15 µg g^{-1} at 100 nM Cd, while still far lower than the critical level at 5 nM Cd. It suggests a direct toxicity of Cd at 100 nM Cd during grain filling. The excessive accumulation of Cd can affect a series of

physiological processes in plants including nutrient uptake, hormones release, reactive oxygen species (ROS) accumulation, cell growth and death, and photosynthesis (Shahid *et al.*, 2016). In plants, Cd toxicity is especially attributed to the inhibition of photosynthesis by down-regulating the expression of photosynthesis-related gene, inactivating of photosynthetic carbon assimilation enzymes, damaging the structure of chloroplasts and reducing stomatal conductance (Gallego *et al.*, 2012). In the present study, the impact of Cd on the photosynthetic apparatus was not strong enough to generate visual symptoms, for instance of depigmentation, onto the leaf blades. An alternative explanation is that the energy required to face Cd toxicity at 100 nM was substantial and was consumed to the detriment of biomass production (Verbruggen *et al.*, 2009). Indeed, both the biosynthesis of Cd ligands and that of transmembrane transporters involved in Cd detoxification and compartmentalization are energy-consuming activities.

At 100 nM Cd, plant replicates showed great variation on their KRWs. The lower KRW suggests that the plants may be earlier in maturing their grains than those with higher KRWs. This variability may result from the plant responses to Cd toxicity such as hormone releasing and signal transduction to the rapid maturation (Gallego *et al.*, 2012). However, it remains unclear why the effect was not observed constantly while the leaf concentration of Cd was constantly above the critical level of Cd toxicity at 100 nM Cd and not varied ($p = 0.91$) with the KRWs.

4.4.2 Buffer ability of durum wheat in the change of exposure level to cadmium

For both levels of Cd exposure, the concentration of Cd among organs was the lowest in the head (bracts and rachis) at anthesis and the lowest in the grain at maturity (Table 4-3). Unlike for Cd, durum wheat tends to maximize the level of some nutrients such as nitrogen in the grain (Yan *et al.*, 2018). It reveals that durum wheat plants naturally possess the ability to filter the allocation of Cd toward reproductive parts. However, the ability of plant in Cd filtering could be lower for the cultivar used in the present study than for those harboring the *Cdu1* locus that can sequestrate Cd in roots more efficiently (Harris and Taylor, 2013; Perrier *et al.*, 2016). Before anthesis, the roots and stems are major parts filtering Cd, where Cd was the most concentrated and accumulated in quantity (Table 4-3; Figure 4-3). During grain filling, in contrast, the leaves and bracts played the most important role in sequestrating Cd. The decrease in the ability of roots and stems to sequestrate Cd after anthesis may result from the degradation of roots, as evidenced by the decrease of root dry weight (Table 4-1), or from the remobilization of Cd from these organs during grain filling (Figure 4-2). Along with that, the continuous transpiration pull favored the flow of Cd toward leaves and bracts and progressively led their accumulations of Cd to the toxic level. In

addition to leaves and bracts, the concentration of Cd was higher in the first node than in the peduncle of stems, regardless of the level of Cd exposure, which reveals the role played by nodes in Cd sequestration (Figure 4-1). The result is in line with that reported in the study of Perrier *et al.* (2016) where the shoot vegetative tissues were deemed as a sink for Cd in competition with the grain.

When the level of Cd exposure increased, before anthesis, durum wheat didn't restrict the influx of Cd in roots. The 20-fold increase in the level of Cd exposure equivalently resulted in about 20-fold higher amount of Cd present in the plant at anthesis (Figure 4-2). These results reveal that 100 nM Cd may not yet saturate root influx transporters or trigger root mechanisms to limit Cd influx, such as the change of root architectures (Hart *et al.*, 2006; Lux *et al.*, 2011). It is also possible that the root exudes organic compounds to regulate the mobility and the phytoavailability of Cd in the rhizosphere, although this response, if any, cannot be decrypted owing to the frequent solution refreshment in hydroponics.

It was expected that durum wheat could buffer the allocation of Cd to grains from the increase of Cd exposure from 5 to 100 nM Cd. Indeed, the ability of Cd buffering has been reported on some plant species. For example, *Miscanthus* enhances the sequestration of Cd in roots to defend Cd toxicity in shoot tissues when the concentration of Cd increased in the nutrient solution (Arduini *et al.*, 2004); in sunflower, more Cd is allocated to old leaves under Cd stress to protect the physiological active tissues (De Maria *et al.*, 2013). In the present study, however, the concentration of Cd in mature grains increased 31-fold which is more than the 20-fold increase in the influx of Cd in roots and in the Cd concentration of nutrient solutions between 5 and 100 nM Cd (Table 4-3). It underlines that the durum wheat plant failed to buffer the accumulation of Cd in grains from the increase of exposure to Cd. The poor buffer ability at such a low dose, namely nanomolar range, of Cd exposure, at least from 5 to 100 nM, may partly explain why durum wheat plants can more easily accumulate Cd in their grains than other crops (Grant *et al.*, 2008).

The buffer abilities of roots and stems were obviously limited at 100 nM Cd during grain filling. The 20-fold increase in the level of Cd exposure led to a significant lower rate of increase in the concentration of Cd in these organs at maturity (Table 4-3). For the roots, the result indicates that the durum wheat may not accelerate the maturation of root endodermis to form apoplastic barrier that limits the radical transport of Cd toward the stele at 100 nM Cd (Geldner, 2013). Meanwhile, the root vacuoles where Cd mainly stored may be overloaded at 100 nM Cd that cannot efficiently prevent the loading of Cd to the vascular bundle (Clemens and Ma, 2016). In stems, the node is a structure connecting leaves, stems and head, and plays an important role in the partitioning of

metals between these organs (Yamaji and Ma, 2017). Cd unloaded in a node could be distributed from the node to other organs, including heads, or sequestrated in the node (Yamaguchi *et al.*, 2012). Therefore, the node sequestration of Cd is supposed to be critical in influencing the Cd allocation to grains. A specific investigation was carried out on the first node, where Cd was stored or transferred to the head, and on the peduncle just above the first node, which represents the Cd exported from the node to the head. The increased ratio of concentration between the node and the peduncle with the increase of Cd exposure may reflect the greater sequestration of Cd in nodes at 100 nM Cd than at 5 nM Cd (Figure 4-1). This result implies the role played by nodes in buffering Cd transferred to grains between Cd exposures. The parenchyma cell bridge is probably the sites of Cd sequestration in nodes, which receives metals unloaded from the xylem connected to the lower plant parts (Yamaguchi *et al.*, 2012; Yamaji and Ma, 2017). Even though, the accumulation of Cd in grains was more than proportional to the increase of the exposure, suggesting the unloading of Cd from the xylem in the node or the nodal sequestration of Cd seemed to be limited.

In contrast to the roots and stems, high-transpiring leaves and bracts were more efficient in sequestrating Cd when the level of Cd exposure increased. Their concentrations of Cd were more than or close to 20 times higher as the exposure increased from 5 to 100 nM Cd (Table 4-3). This high ratio of increase may due to the low efficiency of roots and stems in limiting Cd transport when the level of Cd exposure increased, while the leaves and bracts continuously absorbed Cd through the transpiration stream during grain filling. However, the ratios of Cd concentration between Cd exposures decreased for leaves and bracts at maturity compared to that at anthesis (Table 4-3), which indicates that their efficiencies of Cd sequestration decreased after anthesis.

Taken together, the unexpected stronger accumulation of Cd in grains at 100 nM Cd can be mainly attributed to the poor ability of roots and stems and to the decreased efficiency of leaves and bracts in limiting Cd transfer toward the grain. The partitioning of Cd newly taken up toward the grain is more influenced by the level of Cd exposure than that of Cd remobilization. About two times more of Cd taken up after anthesis was allocated to the grains at 100 nM Cd than that at 5 nM Cd (Figure 4-3b), while the fraction of Cd remobilized to grains was increased 1.4 folds (Figure 4-4c).

4.4.3 Effect of earlier maturing of grains

In average, remobilization contributed about half of the Cd accumulated in grains at both 5 and 100 nM Cd (Figure 4-2), which is in agreement with those have been reported in rice (Rodda

et al., 2011) and durum wheat (Yan *et al.*, 2018). The increase in the level of Cd exposure didn't alter the contribution of remobilization to the amount of Cd in mature grains, although the fraction of Cd taken up after anthesis allocated to grains was more affected than that of remobilization (see above). For uptake parts of the grain, the amount of Cd taken up after anthesis increased 15.6 folds (Figure 4-2), while its fraction allocated to grains increased 1.9 folds (Figure 4-3), from 5 to 100 nM Cd. For the remobilization parts, the amount of Cd stored before anthesis in source organs, i.e. stems and roots, was 18.3-fold higher (Figure 4-2), while the fraction of Cd remobilized from this pool to grains was 1.4-fold higher at 100 nM than at 5 nM Cd (Figure 4-4c). In total, the increase in the amount of post-anthesis Cd allocated to grains ($15.6 \times 1.9 = 29.6$) was close to that in the amount of Cd remobilized to grains ($18.3 \times 1.4 = 25.6$) between Cd exposures. In other words, the impact of Cd exposure on the grain loading of Cd derived from post-anthesis uptake can be counterbalanced by that on the remobilization of Cd toward the grain.

However, this contribution of remobilization was greatly varied at 100 nM Cd with different rate of grain maturation (Figure 4-4a). The grain development is a key event in the plant life cycle and is associated with many physiological processes such as vegetative senescence and element remobilization (Distelfeld *et al.*, 2014). For example, removal of reproductive head of wheat delayed the leaf senescence (Patterson and Brun, 1980), while accelerating the plant senescence led to an earlier drying of spikes and a stronger remobilization of nutrients from leaves (Uauy *et al.*, 2006). In the present study, the rate of N remobilized from leaves, which is tightly linked with the senescing process (Maillard *et al.*, 2015; Tegeder and Masclaux-Daubresse, 2018), increased with the decrease of KRWs (Suppl. Fig. S4-2). These results indicate that the rate of senescence, at least that of leaves, was influenced by the rate of grain maturation (i.e. KRWs). For this reason, the response of durum wheat to 100 nM Cd in the contribution of remobilization may be a combined effect of Cd exposure itself and Cd-induced differences in the rate of grain maturation. Here, the varied rate of grain maturation was used to discuss how the contribution of remobilization to grain Cd could be affected (Figure 4-4).

Cadmium was remobilized from the roots and stems, but not from the leaves (Figure 4-2). Leaves start senescing shortly after anthesis, which is simultaneous with the remobilization of elements like nitrogen (N) (Kong *et al.*, 2016; Maillard *et al.*, 2015). It seems not the case for Cd as Cd remobilization didn't occur in leaves, although the yellowing appearance indicates that their senescence had been initiated (Suppl. Fig. S4-1). Similarly, Yan *et al.* (2018) reported that Cd was not remobilized from senescing leaves but from non-senescing stems, and suggest that Cd remobilization may be not coupled with senescence. In contrast to Cd, N was remobilized from the senescing leaves at a high rate, namely 53% in average, during grain filling (Suppl. Fig. S4-2).

Meanwhile, the rate tended to be higher in the plants with earlier grain maturation (e.g. 71% at 21% KRW) which may represent an earlier leaf senescence, as compared to that in the later ones (e.g. 27% at 49% KRW). In contrast, no relationship was found between the "efficiency" of Cd remobilization and the KRWs ($p = 0.35$). These results, together with those reported by Yan *et al.* (2018), suggest that Cd remobilization may be a senescent-independent process.

Unlike the remobilization of Cd, at 100 nM Cd, post-anthesis uptake of Cd tended to be lower for plants earlier in grain maturation (Figure 4-4d). This decrease in post-anthesis uptake was not linked to the loss of Cd from plants ($p = 0.36$), but may result from the earlier degradation in root absorbing power due to the shortened period between anthesis and maturity (Schneider *et al.*, 2017). It was this negative affect on the root uptake of Cd during grain filling that increased the relative importance of remobilization with the faster grain maturation (Figure 4-4a), although Cd remobilization itself was not affected (Figure 4-4b, c).

If the effect of grain maturation rate is eliminated and comparing plants with the similar grain maturation rate (i.e. 40 < KRW <50), the post-anthesis uptake would increase ×18.8. Given the constant fraction of Cd taken up after anthesis and that of Cd remobilized to the grain (Figure 4-4c, e), post-anthesis uptake of Cd (18.8 × 1.9 = 35.7) contributed more to grain Cd than the remobilization (18.3 × 1.3 = 23.8) between exposures of 5 and 100 nM Cd.

Furthermore, the concentrations of mineral nutrients in the grain were altered between 5 and 100 nM Cd (Table 4-2). Like Cd, mineral nutrients may enter the grain through post-anthesis uptake and remobilization. The root uptake of Cd was negatively affected by the earlier grain maturation, which may also apply to the uptake of other mineral elements due to the corresponding decrease of root absorbing power (Figure 4-4d). Meanwhile, as mention above, the rate of grain maturation could influence the leaf senescence which may thereby trigger the remobilization of some elements such as N (Suppl. Fig. S4-2). Therefore, the response of grain accumulation in minerals was suspected to be explained by the rate of grain maturation (i.e. KRWs) and by the different dependencies of their remobilization to leaf senescence which is associated to grain maturing (Suppl. Table S4-2). The phloem mobility of manganese (Mn) is very low which interrupts its remobilization (Riesen and Feller, 2005). Conversely, N, and copper (Cu), and molybdenum (Mo) can be strongly remobilized from senescing tissues (Maillard *et al.*, 2015). In the present study, both these elements were significantly affected by the rate of grain maturation (Suppl. Table S4-2). Correspondingly, the grain concentration of Mn was lower in earlier maturing grains (i.e. positively correlated with KRWs), which could be attributed to the lack of remobilization for this element. In contrast, N, Cu and Mo concentrations in grain were higher in the grains earlier in maturation

(i.e. negatively correlated with KRWs), which was probably the huge remobilization from senescing tissues induced by earlier grain maturing that counteracted even surpassed the decrease in uptake. Hence, the lower Mn and the higher N, Cu and Mo concentrations in grain was observed at 100 nM Cd (Table 4-2). Compared to these elements, concentrations of Zn and Fe in the grain were not significantly linked to the rate of grain maturation. Their remobilization could be also induced (Pearce *et al.*, 2014), but the flux may be lower than that of elements like N. Indeed, the rate of zinc (Zn) remobilization is low when the supply of Zn and N is high (Kutman *et al.*, 2012), which is the case for the plants grown hydroponically (Harris and Taylor, 2013). Hence, earlier maturing of grains may induce the remobilization of these elements to the extent just counterbalancing the depressed uptake, which thus overshadowed the effect on the remobilization.

4.5 CONCLUSION

This study demonstrates the response of durum wheat plants in growth and Cd allocation by exposure to nanomolar concentrations of Cd. The results show that, first, the plant growth of durum wheat was not limited before anthesis but was limited during grain filling as the level of exposure increased from 5 to 100 nM Cd. The decrease in plant growth is attributed to the toxicity of Cd at 100 nM Cd and leads to a lower grain yield at maturity. Second, durum wheat has a poor ability to buffer changes in Cd exposures, which resulted in a more than proportional increase in the grain Cd concentration with the increase of exposure from 5 to 100 nM Cd. The weak Cd buffer ability is mainly due to the low efficiency of roots and stems in sequestrating Cd and to the decrease sequestration power of nodes and high-transpiring tissues during grain filling. Third, at both 5 and 100 nM Cd, remobilization contributes in average around half of the grain Cd. However, the importance of remobilization tends to increase with the higher rate of grain maturation which lowers the post-anthesis uptake. By contrast, the grain maturation rate, which may be linked to leaf senescence, has no impact on Cd remobilization. As suggested by Yan *et al.* (2018), Cd remobilization may be a senescent-independent process. Last, the increase in the level of Cd exposure to 100 nM tends to accelerate the grain maturation, through which the concentration of nutrients in the grain is affected negatively or positively, depending on their efficiencies of remobilization.

The results of this study highlight the importance in controlling the availability of Cd in agricultural soils. It is not only because the poor ability of durum wheat in restricting the transfer of Cd towards the grain, also considering the impact of Cd on the grain yield and the grain nutrient quality. The control needs to be carried out in priority during grain filling period. One reason is

that the increase in the level of Cd exposure favors the direct transfer of Cd newly taken up toward the grain. Moreover, at this growth stage, high-transpiring organs can readily accumulate Cd to levels that exceed the toxic threshold. Furthermore, the lower post-anthesis uptake with earlier grain maturations enlightens the possibility to restrict the root uptake through modulating the root absorbing power

4.6 ACKNOWLEDGMENTS

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4.7 SUPPLEMENTARY MATERIAL

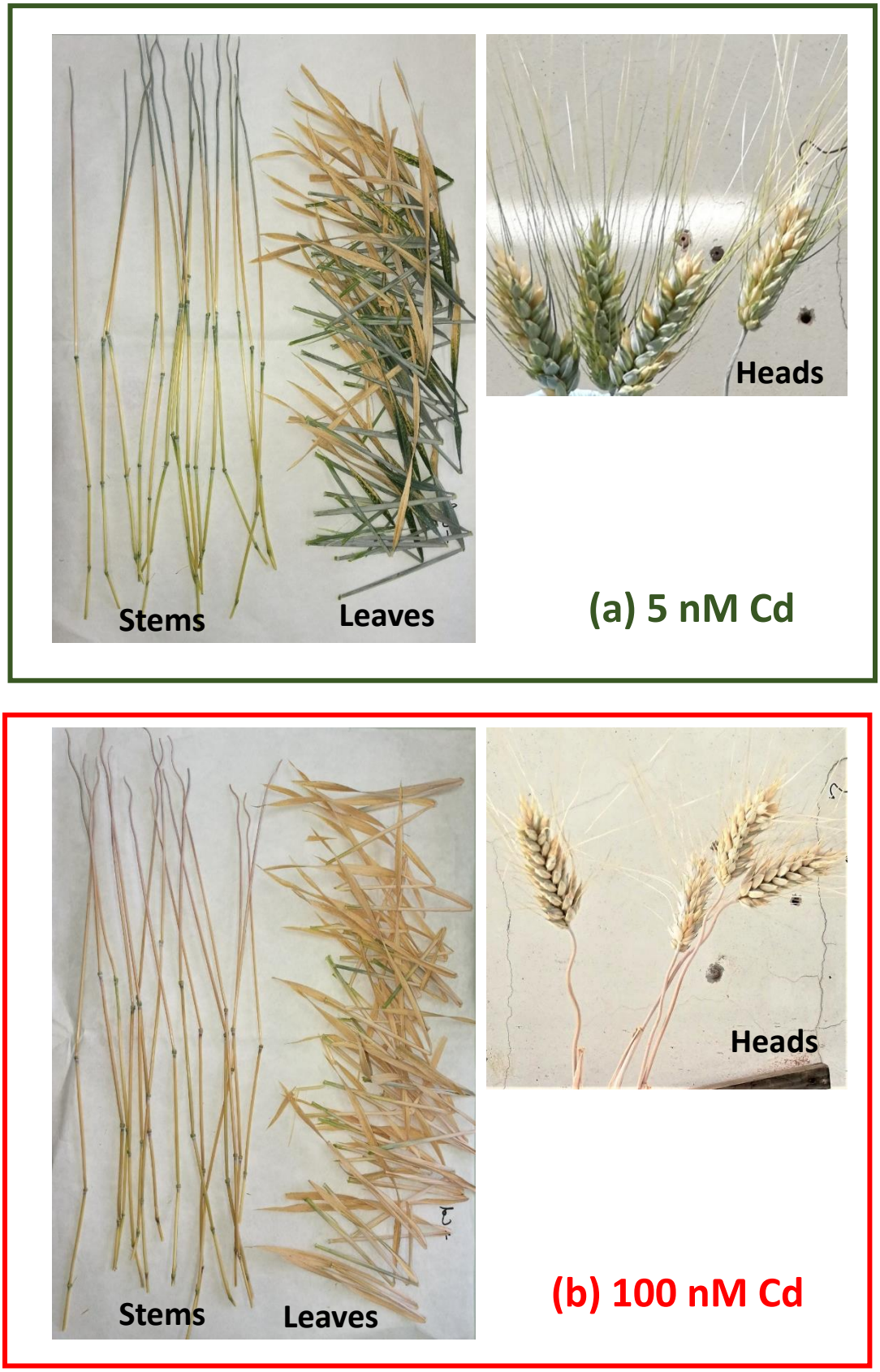
Suppl. Table S4-1 Isotopic abundances (%) of the different sources of Cd

	^{106}Cd	^{108}Cd	^{110}Cd	^{111}Cd	^{112}Cd	^{113}Cd	^{114}Cd	^{116}Cd
Standard (A_s %)	1.25	0.89	12.49	12.80	24.13	12.22	28.73	7.49
Enriched (A_{ei} %)	0.02	0.02	0.50	95.50	2.12	0.59	1.10	0.15

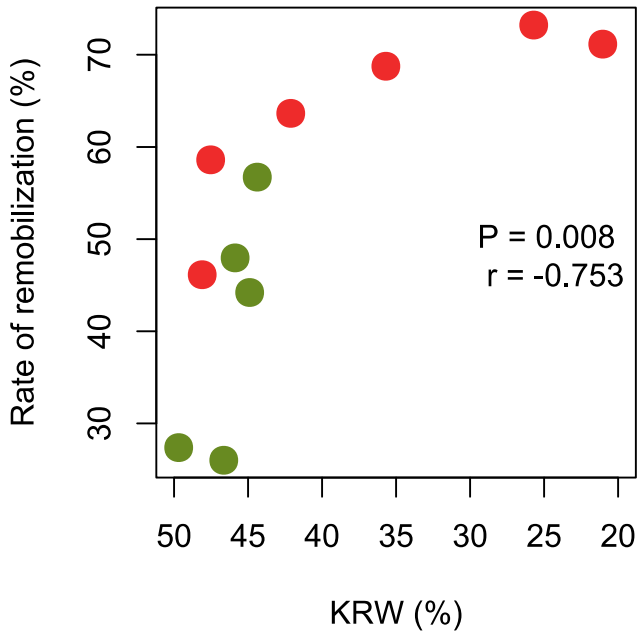
Suppl. Table S4-2 Linear correlation coefficients (r) between kernel residual water (KRW) and grain concentrations of N, Zn, Mo, Cu, Fe, Mn in durum wheat grown in hydroponics in presence of Cd at 5 and 100 nM

	N	**Zn**	**Cu**	**Fe**	**Mn**	**Mo**
KRW	-0.78**	-0.22	-0.75**	-0.55	0.81**	-0.61*

* and ** indicate significant correlations at the probability of $P < 0.05$ and $P < 0.01$ respectively.



Suppl. Fig. S4-1 Appearances of stems, leaves, and heads in hydroponically grown durum wheat (cv. *Sculptur*) exposed to (a) 5 or (b) 100 nM Cd on the day of harvest at maturity.



Suppl. Fig. S4-2 Relationships between the kernel residual water (KRW) and the rate of N remobilized from leaf pre-anthesis stores in hydroponically grown durum wheat (cv. *Sculptur*) exposed to 5 (●) or 100 nM Cd (●). The statistics in both figures are relative to the correlation of all data points in the figure.

Chapter 5
Imaging of cadmium distribution in mature durum wheat grains using laser ablation-ICP-MS

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The previous chapters discussed the partitioning flux of Cd between plant compartments of durum wheat. The results provide implications for better control the level of grain Cd on a whole-plant scale. From another perspective, this chapter goes deep into the grain to know how Cd is distributed among grain tissues, in order to provide clues in developing post-harvest strategies to minimize the level of Cd in durum wheat-based foods. Meanwhile, the localization of mineral nutrients in grains is investigated to estimate the impact of removing Cd-rich parts on the grain nutritional value and to build hypothesis in the internal loading and storage of Cd that may be associated with or different from other minerals.

The high-sensitive elemental imaging technique, laser ablation (LA)-ICP-MS, was used to map the distribution of Cd and mineral nutrients in the longitudinal section and in the cross section of the grain. A grain dissection experiment was carried out to provide the quantitative information in addition to the elemental maps.

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Highlight

- Cd was highly distributed in the periphery, the germ, and particularly in the crease of durum wheat grain.
- Cd was distributed similar to Zn and Mn.
- Cd was more extended inward as compared to Fe and Zn.

Abstract

Understanding the distribution of cadmium (Cd) and mineral nutrients in durum wheat grains is pivotal for post-harvest treatments to maximize Cd removal without too much affecting the nutrient quality. Laser ablation inductively coupled mass spectrometry, together with a dissection approach, were conducted to investigate comprehensively the distribution and association of Cd and essential elements in the grain. Elements were generally concentrated in the germ, the inner periphery (incl. aleurone layer and other maternal tissues), and the crease. Cd distribution was characterized by a strong accumulation of Cd in the crease and by a non-negligible dissemination in the starchy endosperm, as compared to Fe and Zn. Grain Cd would thus be less affected by pearling compared to grain nutrients. Cd was distributed similar to Zn and Mn. Their concentrations would be impacted in parallel by the removal of grain parts. High variability in elemental distribution was shown within the germ and crease in the maps, revealing various mechanisms of internal loading, of storage and functions between elements.

Key words

Durum wheat, cadmium, mineral nutrients, embryo, laser ablation, grain processing

Abbreviations

IP inner periphery; *OP* outer periphery; *SE* starchy endosperm

5.1 INTRODUCTION

Cadmium (Cd) is a heavy metal contaminant. Its extreme toxicity to humans has long been recognized and concerned. Dietary intake is the principal source of Cd exposure for general non-smoking population (EFSA, 2012). The European Food Safety Authority (EFSA) established a provisional tolerable weekly intake for Cd of 2.5 μg Cd kg^{-1} body weight considering its long-term low-dose effects harming human health (EFSA, 2011). Though the average dietary exposure is below this level in the European population, some subgroups such as children, young women and vegetarians are more frequent to exceed it (Clemens *et al.*, 2013). In France, for example, 14.9% of the children intake more Cd than the tolerable value (Arnich *et al.*, 2012). People eat cereals as a staple food, amongst which, wheat, rice and corn account for about 60% of the caloric intake (Tilman *et al.*, 2002). In Europe, about 27% of the dietary exposure to Cd is from cereals (EFSA, 2012). Durum wheat has a greater tendency to accumulate Cd in grains compared to common wheat and other cereals (Grant *et al.*, 2008; McLaughlin and Singh, 1999). Cd in durum wheat grains is frequently above 0.1 mg kg^{-1} and can sometimes exceed the regulation limit of 0.2 mg kg^{-1} set by the European Union (EC, 2008; Grant *et al.*, 2008; Mench and Baize, 2004). Therefore, special attention shall be paid to reduce the level of Cd in durum wheat-based foods.

If Cd is not homogeneously distributed in wheat grains (He *et al.*, 2000; Pieczonka and Rosopulo, 1985), their Cd concentrations of derived food products could be reduced by processing the grain to eliminate the parts rich in Cd. Giordano and Blandino (2018) showed that 44% of the Cd content in wheat grains can be excised by removing 25% of the grain mass by pearling. Similarly, Guttieri *et al.* (2015) found that the concentration of Cd was higher in the whole grains compared to the milled grains. This finding suggests that the outer layers, i.e. the combined aleurone and pericarp that make the bran, concentrate Cd in a greater extent. However, the authors documented that removing the bran fraction also led to a stronger loss of zinc (Zn) as compared to that of Cd, and they concluded that Cd extended more inward in the grain than Zn. Durum wheat is an important source of dietary minerals. Insufficient dietary intake causes micronutrient deficiency, especially for Zn, in a large amount of world population (Cakmak and Kutman, 2017). It is thus necessary to keep the durum wheat nutritional value when trying to remove Cd. A different pattern of spatial distributions between Cd and mineral nutrients, if any, would give the possibility to reduce the concentration of Cd in durum wheat grains without too much affecting that of nutrients because milling processes allow to discard more or less specifically grain parts. For instance, a processing method was designed to remove the outer pericarp and the crease material while retaining as much as possible the aleurone fraction in the final product (Delcour *et al.*, 2012). Therefore, the localization of Cd, as well as mineral nutrients, in the grains needs to be better

known for optimizing such post-harvest treatments to maximize Cd removal whilst minimizing nutrient losses.

Significant advances in imaging techniques make it possible to obtain high definition elemental maps in wheat grains (Lombi *et al.*, 2011a). For instance, synchrotron-based methods have been used to investigate the distribution of phosphorus (P), sulfur (S), manganese (Mn), iron (Fe), zinc (Zn), and copper (Cu) (Ajiboye *et al.*, 2015; De Brier *et al.*, 2016; Singh *et al.*, 2013). However, contrary to micronutrients, the concentration of Cd in wheat grains for food consumption is below the detection limit for this method. In general, under field conditions, durum wheat grains concentrate at least 10 times more Cu and 100 times more Zn compared to Cd (Clarke *et al.*, 2002). The detection limit of synchrotron-based methods of around 1 mg kg^{-1} (Lombi *et al.*, 2011a) is thus too high for mapping of grain Cd, the concentration of which is theoretically below the regulation limit of 0.2 mg kg^{-1} in whole grains and may even lower in some grain parts. By contrast, though the spatial resolution is lower than synchrotron-based technique, mass spectrometric imaging technique such as a laser ablation inductively coupled mass spectrometry (LA-ICP-MS), would be more effective in assessing the distribution of Cd isotopes in grains lower than the ppm level, thanks to the high sensitivity of ICP-MS. Indeed, depending on the element monitored, detection limits in the range of 10^{-4} to 10^{-1} mg kg^{-1} are reported (Becker, 2002). In recent years, it has been widely applied to map trace elements, such as molybdenum, Cd, arsenic (As), and lead (Pb) in plant samples (Lombi *et al.*, 2009; Pessôa *et al.*, 2017; Thyssen *et al.*, 2018; Van Malderen *et al.*, 2017; Wu *et al.*, 2013; Wu and Becker, 2012). However, a detailed map of Cd spatial distribution in the grain of durum wheat is still lacking.

In the present study, LA-ICP-MS was set up to produce images of Cd and mineral nutrients in transversal and longitudinal thin sections of mature durum wheat grains at the micron scale spatial definition. Additionally, a grain dissection was performed to provide quantitative information complementary to the maps. The objectives were (i) to precisely characterize the spatial distribution of Cd in the grain of durum wheat, (ii) to investigate the difference between the distribution of Cd and mineral nutrients within durum wheat grains with respect to the current knowledge about loading and storage of essential elements and Cd in the grain, (iii) to assess the possible impact on the nutritional component by removing Cd-enriched parts from the grain of durum wheat.

5.2 MATERIALS AND METHODS

5.2.1 Grain production

Mature grains of durum wheat (*Triticum durum* cv. *Sculptur*) were harvested from hydroponic plants exposed to 100 nM Cd. The composition of nutrient solution and the procedure of hydroponic culture were the same as described in Yan *et al.* (2018). The average concentration of Cd in these grains was 2.2 ± 0.3 μg g^{-1} DW. As a control for the mapping, grains with background level of Cd (0.0024 ± 0.0019 μg g^{-1} DW) were collected from plant grown under the same condition but not supplied with Cd in the nutrient solution. Neither Cd toxicity nor mineral deficiency symptoms of the plant were observed during the whole culture period. The spikes were oven dried at 60 °C for 72 hours before separating grains from the heads.

5.2.2 Cryomicrotomy

Grains with median size (50 ~ 60 mg) were selected for the elemental mapping. They were soaked 6 hours at. 4 °C Then grains were embedded in O.C.T compound (tissue-teck) at low temperature (~ -30°C) and transversal (across root or leaf primordia) as well as longitudinal (alongside the crease) sections (40 μm-thickness) were prepared by cryomicrotomy (Leica CM 1950). The thin sections were directly deposited on glass slides. Before LA-ICP MS analysis, the integrity of the grain section structure was verified under a microscope (Figure 5-1; Figure 5-2; Figure 5-3).

5.2.3 LA-ICP-MS imaging

Elemental analysis of 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. 800 mL min^{-1} of He was used to transport the ablated material directly into the dry plasma of ICP-MS. ICP-MS parameters (torch position, carrier gas flow rate and ion lenses voltage) were tuned for each set of experiments by ablating the 612 NIST glass reference material while monitoring ^{238}U and ^{232}Th signals. Detection limits of monitored isotopes are estimated in the range of 0.1 μg g^{-1}.

Ablation of grain sections was performed my means of a Nd: YAG laser source at 213 nm with a 20 Hz repetition frequency, a square laser beam of 10 μm x 10 μm, a fluence of 12 J cm^{-2} (20% of delivered energy), and a scan speed of 10 μm s^{-1}. Samples were 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 1s 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. Isotope maps were generated using a homemade program. Isotope profiles could then be extracted from the maps.

Table 5-1 Size and elemental concentrations of the durum wheat grains used for the dissection

Size	**Cd**	**Zn**	**Cu**	**Mn**	**Mo**	**Fe**	**Ca**	**K**	**Mg**	**P**
g DW	**μg g^{-1} DW**						**mg g^{-1} DW**			
69.6 ± 0.9	2.48 ± 0.12	131 ± 4	9.78 ± 0.35	50.3 ± 4.0	2.93 ± 0.17	44.6 ± 2.7	0.47 ± 0.02	5.98 ± 0.24	1.52 ± 0.04	2.60 ± 0.60

Values are means of five independent replicates
Grain size is expressed as TKW (thousand kernel weight)

5.2.4 Grain dissection and analysis

Fifty grains with similar sizes were selected for hand dissection using ceramic scalpels under a stereomicroscope. The sizes and mineral contents of the grains are summarized in Table 5-1. The dissections were conducted as two rounds of 25 grains (5 replicates, each being a pool of 5 grains). The first one was dedicated to separating the outer parts and therefore the duration of grain soaking in ultrapure water was restricted (4 hours at 4 °C) to limit the potential losses of elements from these parts by diffusion to the water. Grains were separated into three parts: the germ (composed of the embryo and the scutellum); the outer periphery of the grain (*OP*) that loosely attaches to the grain and probably corresponded to the outer pericarp and the remaining parts (*RP*), composed of the starchy endosperm, the crease, and the inner periphery of the grain (likely the aleurone layer, the seed coat, the inner pericarp, and other maternal peripheral layers).

The second round aimed at separating specifically the crease from the starchy endosperm (*SE*) which required a longer soaking time (24 hours at room temperature). Each grain was cut longitudinally through the crease area. The black strand part was collected as the crease. For each half of grain, the core of the *SE* was then collected, paying attention to not sample envelope parts.

Grain parts were freeze-dried overnight at -60 °C and weighed before wet digestion. A mixture of 0.8 mL of ultra-pure 30% H_2O_2 and 0.2 mL of 67.5% HNO_3 was added to the sample. The mixture was left at room temperature overnight for preliminary digestion. The digestion was carried

out by heating the mixture in an oven following the temperature gradient: 60 °C for 30 min, 80 °C for 45 min, and 100 °C for 90 min. The solutions were diluted to 10 mL with ultrapure water after cooling and filtered through 0.2 μm polycarbonate membrane filters. 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 (http://www.ehu.eus). The results were validated using procedural blanks and the NIST Standard Reference Material (NIST SRM 1573a tomato leaves). The recovery rates of reference values were (mean ± standard deviation): Cd 95 ± 5%; Zn 92 ± 8%; Cu 95 ± 5%; Mo 89 ± 10%; Fe 80 ± 3%; Ca 96 ± 5%; K 92 ± 4%; Mg 91 ± 4%; and P 108 ± 10%.

5.2.5 Budget of element partitioning between grain parts from dissection results

The objective was to use the results from the dissections to establish a quantitative budget for the partitioning of elements between grain parts to further estimate the losses resulting from tissue removal by milling processes. The grain parts targeted for the budget were the outer periphery (*OP)*, the inner periphery (*IP)*, the starchy endosperm (*SE)*, the germ and the crease. It was not possible to directly establish the budget from our data because i) the inner periphery was not individually sampled and was included in the remaining parts *(RP)* of the first round of dissections, ii) only the core of the *SE* was sampled and therefore its contribution to the grain dry weight was not determined. Therefore, the fraction of the grain dry weight (DW) corresponding to the *SE* and to the *IP* were derived values reported in the literature for which similar dissections of wheat grains were performed (Table 5-2) (Barron *et al.*, 2007; Hemery *et al.*, 2009; Pieczonka and Rosopulo, 1985). The grain DW fraction for SE derived from the literature together with the element concentrations in that part determined from our dissections gave the quantity of each element in the SE. The quantity of each element in the *IP* was calculated by subtracting the quantity of the element in the *SE* and in the crease from that in the *RT*. These quantities divided by the DW of *IP* estimated from the grain DW fraction for *IP* derived from the literature gave the concentrations of each element in the *IP*. Finally, the whole grain concentration was then calculated by dividing the sum of the quantity of an element in all grain parts by the grain DW.

Because the data used to establish the budgets were derived from two rounds of dissections and from literature, in order to estimate the standard deviation for each term of the budgets, calculations were performed by the Monte Carlo method. A sample of 10 000 replicates was generated by random sampling from a lognormal distribution based on the mean value and

standard deviation obtained experimentally from the dissections of from literature, depending on the variable (see above).

5.2.6 Statistical analysis

Data were processed using ©R 3.4.2 statistical software. Principal component analysis (PCA) was used to study correlations and groups for the ionomic data of grain parts (R package FactoMineR, version 1.38).

5.3 RESULTS

5.3.1 LA-ICP-MS imaging of elements in durum wheat grains

The average concentration of Cd in grains was 2.2 μg g^{-1} dry weight (DW) which is about 10 times higher than the regulatory limit (0.2 μg g^{-1} fresh weight ≈ 0.23 μg g^{-1} DW considering 12% of residual water) set by the European Union for durum wheat (EC, 2008). The maps of cadmium (Cd), zinc (Zn), copper (Cu), manganese (Mn), molybdenum (Mo), potassium (K), and phosphorus (P) distributions are shown for the longitudinal section alongside the crease (Figure 5-1) and for the cross sections at the level of the leaf (Figure 5-2) and root (Figure 5-3) primordia. It must be noticed that the maps show the relative abundances of an element between grain parts. The relative scale of intensity was established by normalizing the signal by the maximum of each map, the maps cannot be directly compared, even for a given element between longitudinal and transversal sections.

The germ, the crease, the grain periphery, and the starchy endosperm (*SE*) were identified. Two layers were visible at the grain periphery. The outer periphery (*OP*) is most likely the outer pericarp, while the inner periphery (*IP*) is likely to mainly represent the aleurone and other maternal tissues including the seed coat and inner pericarp. In the germ, it was possible to distinguish the scutellum, and in the embryo, the coleoptile and coleorhiza surrounding the leaf and root primordia.

Overall, all the elements considered in the present study were not evenly distributed in the grain of durum wheat (Figure 5-1; Figure 5-2; Figure 5-3). They were preferentially distributed in the germ, in the periphery and in the crease of the grain. Cd was more intensively localized in the germ (incl. embryo and scutellum), the *IP*, and especially, in the crease but was also localized in the *SE*. The distribution pattern of Cd and Zn were quite similar, except that Zn was relatively less localized in the *SE* compared to Cd. Notably, Zn, and to a lesser extent Cd, were more abundant in the area of the scutellum in the vicinity of the endosperm. Mn was mainly localized in the germ and in the

narrow layer of the *IP*. In the germ, Cd, Zn, and Mn were equally distributed between the scutellum and the embryo. Mo was mainly distributed in the outer layer of the root primordia and in the coleorhiza, while Cu dominated in the scutellum. P and to a lesser extent K were more abundant in the *IP* and the scutellum. By contrast, K is more evenly distributed within the germ as compared to other elements.

The periphery parts of the grain were especially rich in Cd, Mn, K, and P (Figure 5-1; Figure 5-2; Figure 5-3). For Cu, Mo, and P, the *IP* was richer than the *OP* which can hardly be identified in the maps for these elements. By contrast, the presence of Cd, Zn, and Mn in the *OP* were clearly visible although the *OP* was still less concentrated than the *IP*. Whatever the element, no clear differences were observed between the ventral and dorsal side for the richness of these peripheral parts.

The crease at the level of leaf primordia (Figure 5-2) was especially rich in Cd, Zn, and Mn. Mn was mainly found in the outer region of the crease, likely in the vascular bundle. By contrast, Zn was dominating in the inner region of the crease. Cd was highly present throughout all regions of the crease. Cu, Mo, K, and P were not particularly accumulated in the crease compared to the other parts of the grain. Particularly, the intensity of P in the outer region of the crease was at the background signal.

The *SE* constitutes the main part of the grain biomass, but none of the element was dominant in this part (Figure 5-1; Figure 5-2; Figure 5-3). The lower abundance of elements in the *SE* compared to the peripheral layers of the grain was clear on the maps and on the signal intensity profile from inside to outside of the grain (Suppl. Fig. S5-1). However, Cd and to a lesser extent Cu and Mo showed a relatively greater intensity in the *SE* compared to the other elements. The significant presence of Cd in the *SE* was also confirmed by comparing the Cd map with that in the non-contaminated control sample (Suppl. Fig. S5-2). A gradient in Cd intensity can be seen in the map, being high in the *IP*, medium in the outer endosperm, and low in the inner endosperm (Figure 5-2). For Cu, however, the high-to-low gradient of intensity appeared to be from the crease toward the *SE*, which explains the high signal in the middle of the profile line (Suppl. Fig. S5-1). By contrast, Mn followed by Zn and P were the elements that were the least intensive in the *SE*.

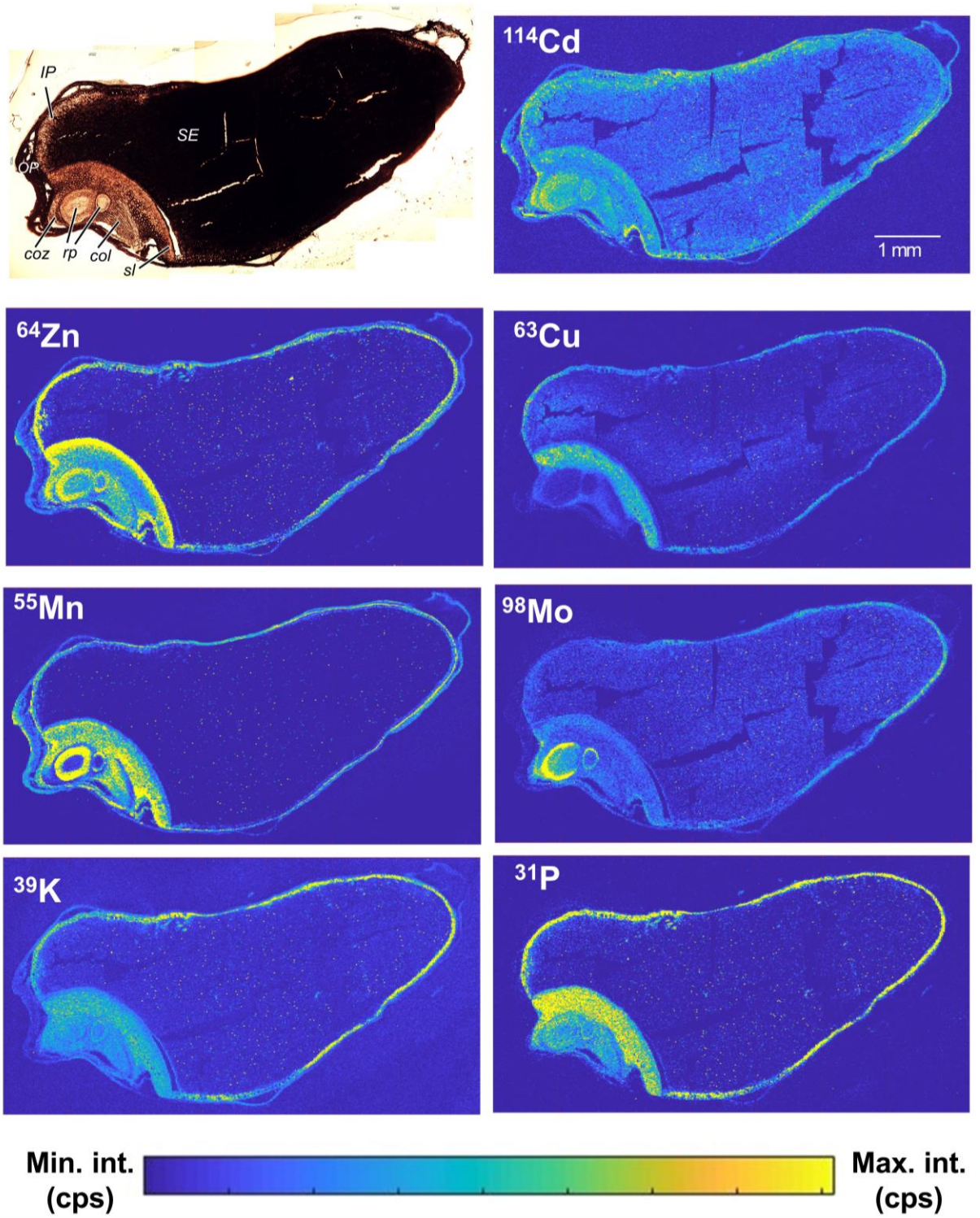

Figure 5-1 Elemental maps of a longitudinal section alongside the crease of a durum wheat grain. The color scale represents the different intensities (*int.*, in counts per second (*cps*)) detected by LA-ICP-MS, with blue and yellow respectively corresponding to the lowest and highest relative intensity for each element. A microscopy image presents the grain tissues. *col*, coleoptile; *coz*, coleorhiza; *IP*, inner periphery (likely the aleurone layer, the seed coat, the inner pericarp, and other maternal peripheral layers); *OP*, outer periphery (likely the outer pericarp); *rp*, root primordia; *sl*, scutellum; *SE*, starchy endosperm.

5.3.2 Concentrations of elements in dissected grain parts

The analysis of the dissected grain parts provided quantitative information complementary to the maps and for Fe, Ca, and Mg that were not mapped by the LA-ICP-MS (Figure 5-4). The grain concentration of micronutrients (Zn, Cu, Mn, Mo, and Fe) and macronutrients (Ca, K, and Mg) were higher than those generally reported for grain from the field (Clarke *et al.*, 2002) or from the market (source: USDA, ref.: 20076). By contrast, the concentration of P in our grain was lower than those reference values. The concentration of Cd was the lowest in the *SE* (1.2 $\mu g\ g^{-1}$ DW) but was above the whole grain concentration (2.5 $\mu g\ g^{-1}$ DW) in all the other parts. The crease was by far the most concentrated in Cd (41.3 $\mu g\ g^{-1}$ DW) followed by the *IP* (7.4 $\mu g\ g^{-1}$ DW), the *OP* (6.5 $\mu g\ g^{-1}$ DW) and the germ (5.5 $\mu g\ g^{-1}$ DW).

Consistent with the LA-ICP-MS maps, the germ, the *IP*, and the crease were generally more concentrated in elements compared to the *OP* and the *SE* (Figure 5-4). In the *SE*, the concentration of Cd, Cu, P, and Mo, was more than half of that in the whole grain. These elements were more accumulated in the *SE* compared to other elements (Table 5-2). By contrast, Fe, Mn, and Mg were the least accumulated in the *SE*. The crease concentrated in first Cd, Zn, and Mn (Figure 5-4). The concentration was 17-fold, 18-fold, and 19-fold higher in the crease than in the whole grain for Cd, Zn, and Mn, respectively. Considering the mass, the crease constituted less than 1% of the grain mass, but it represented 16% Cd, 17% Zn, and 18% Mn of the total quantity in the grain (Table 5-2). The concentration of these elements in the crease was far higher than that in any other grain parts. Apart the crease, Cd was concentrated more evenly among the other grain parts compared to the other elements. Within the peripheral layers (i.e. *IP*+*OP*), elements were mostly concentrated in the *IP* (Figure 5-4). The *IP* concentrated around 3 times more P and Cd and up to 5-8 times more Zn, Ca, K, Fe, and Mg than the whole grain. Elemental concentrations in the *OP* were in comparison low, particularly for P, Cu, Mg, Fe, and Mo the concentrations of which were less than half of the whole grain concentrations. By contrast, the *OP* concentrated more Zn, Ca, Cd, and Mn compared to the whole grain. In the germ, all the elements were highly concentrated (Figure 5-4). This was especially true for Cu, Mn, and P which were more than 5 times more concentrated in the germ than in the whole grain. The germ accounted for about 3% of the grain biomass but it contained more than a quarter of the total P and close to 20% of the total Cu, Mn, and Fe (Table 5-2). By contrast, the germ accumulated 7% of the total Cd, which was the lowest among elements investigated.

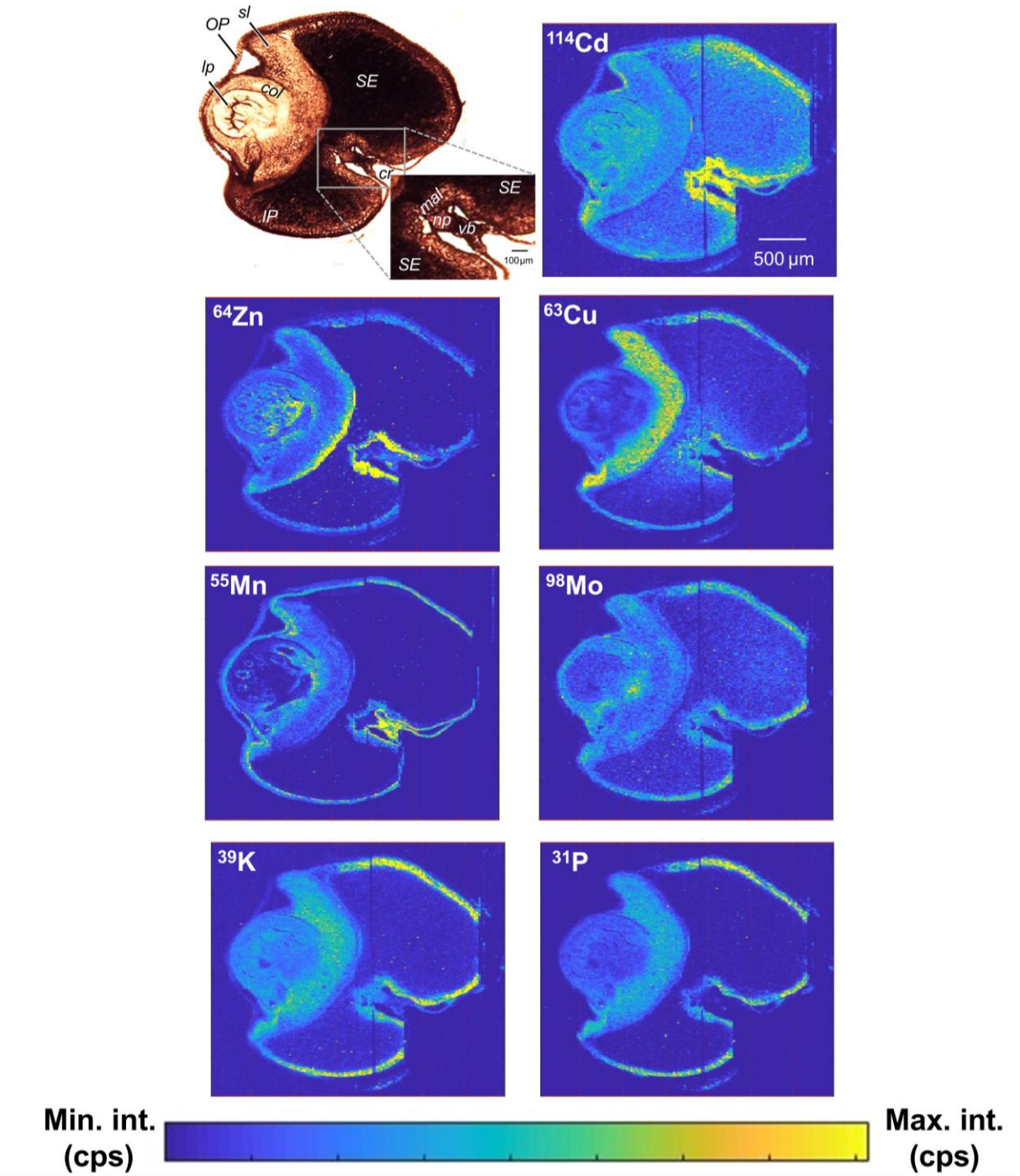

Figure 5-2 Elemental maps of a cross section of a durum wheat grain at the level of leaf primordia. The color scale represents the different intensities (*int.*, in counts per second (*cps*)) detected by LA-ICP-MS, with blue and yellow respectively corresponding to the lowest and highest relative intensity for each element. A megascopic image presents the crease region marked with possible tissues in addition to the microscopy image of the grain. *col*, coleoptile; *cr*, crease region; *IP*, inner periphery (likely the aleurone layer, the seed coat, the inner pericarp, and other maternal peripheral layers); *lp*, leaf primordia; *mal*, modified aleurone; *np*, nucellar projection; *OP*, outer periphery (likely the outer pericarp); *sl*, scutellum; *SE*, starchy endosperm; *vb*, vascular bundle.

A principle component analysis (PCA) was performed to study correlations between the concentration of elements (Figure 5-5). The first two dimension (Dim1 and Dim2) explained 85% of the total variance. All the elements exhibited positive loads in Dim1. Three groups of positive correlations were clearly identified: (Cd, Mn, Zn), (Cu, Mg, K, P), these two being uncorrelated and (Ca, Mo, Fe), which is slightly positively correlated with the two previous groups. This illustrates regarding the grain part concentrations (Cd, Mn, Zn) and (Cu, Mg, K, P) form two distinct patterns of repartition and (Ca, Mo, Fe) is a third one, intermediary compared to the previous ones. Plotting the grain parts on the first two components shows three distinct groups: the *SE* and the *OP* low in all elements, the crease, characterized by relatively high concentrations of Cd, Mn and Zn and the germ+*IP* that are rich in Cu, Mg, K and P.

5.3.3 Loss of biomass and elements by removing grain parts

Altogether the *SE* accounted for the largest part of the grain biomass (Table 5-2). In line with the biomass partitioning, the largest fraction, 39%, of the Cd in the grain can be found in this part. By removing all the other parts of the grain, the concentration of Cd in grain could be lowered to 48% along with a loss of 17% of the grain biomass (Figure 5-6). Similar proportions (~3%) of the grain biomass was allocated to the *OP* and to the germ. The *OP* was little concentrated in elements. Only the concentration of Cd, Mn, and Ca could be slightly lowered by removing this part. The germ was low in Cd compared to the other elements. Removing this part would slightly reduce the concentration of Cd by 4% but would strongly decrease that of mineral nutrients especially Cu, Mn, Fe, and P. The *IP* constituted a larger proportion of grain biomass and of grain Cd. Removing this part would discard 10% of the grain biomass but would lower the grain Cd concentration by 25%. However, in parallel it would also strongly decrease the concentration of mineral nutrients, particularly Zn, Mn, Fe, Ca, K, and Mg. Finally, the crease was particularly rich in Cd, Zn, and Mn. Removing the crease would reduce the concentration for Cd by 18% along with a loss of biomass of only 1%. However, in parallel, 20% of the total Zn and 21% of the Mn, would be also lost, while the other elements would be less affected.

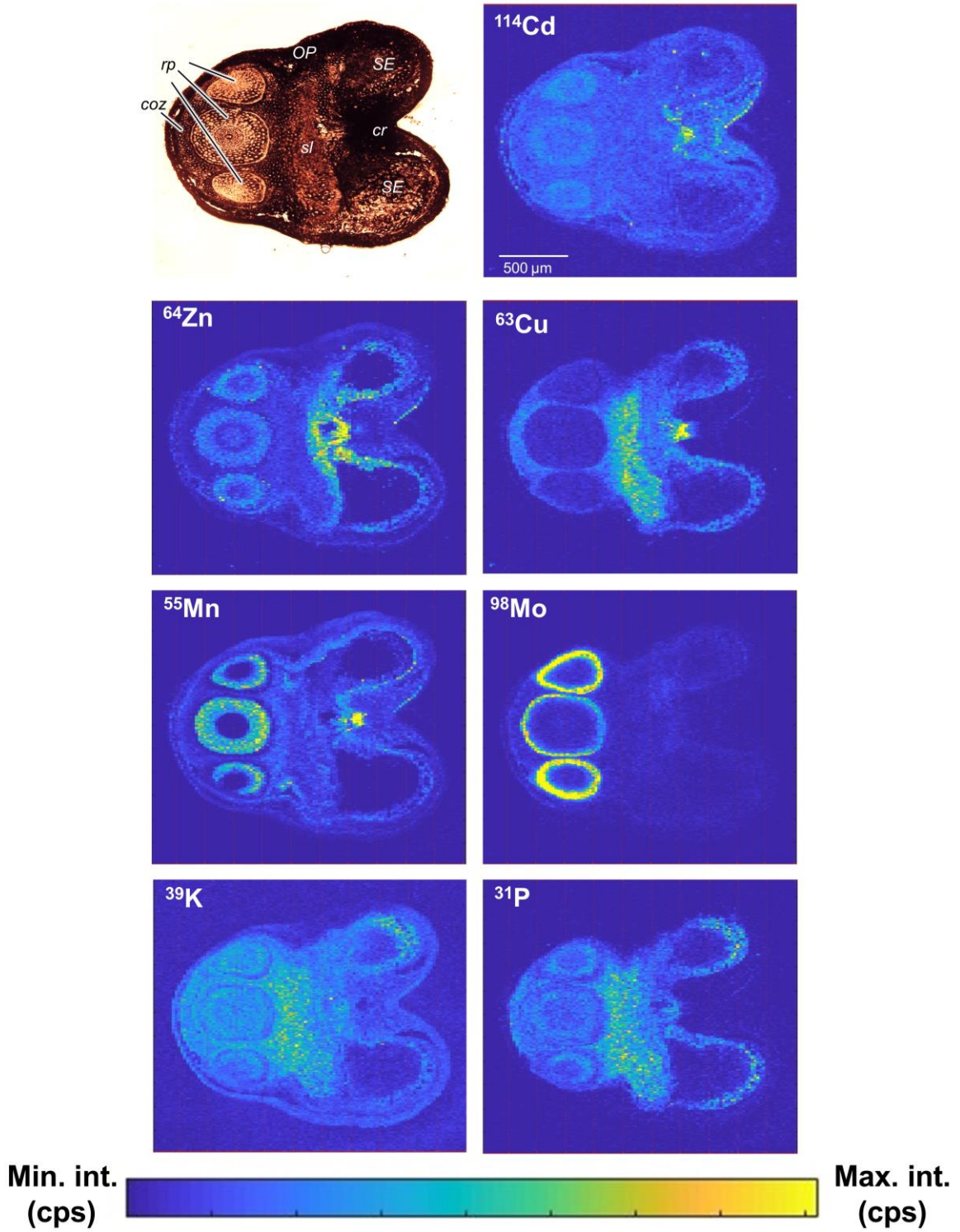

Figure 5-3 Elemental maps of a cross section of a durum wheat grain at the level of root primordia. The color scale represents the different intensities (*int.*, in counts per second (*cps*)) detected by LA-ICP-MS, with blue and yellow respectively corresponding to the lowest and highest relative intensity for each element. A microscopy image presents the grain tissues. *cr*, crease region; *coz*, coleorhiza; *OP*, outer periphery (likely the outer pericarp); *rp*, root primordia; *sl*, scutellum; *SE*, starchy endosperm.

5.4 DISCUSSION

5.4.1 Localization of cadmium and essential elements in durum wheat grain assessed by LA-ICP-MS and grain dissection

Only a few studies have reported the mapping of Cd distribution in cereal grains. It is probably due to the difficulty to detect Cd by imaging techniques in grains for ultra-trace level concentration of this element (Clemens and Ma, 2016). Van Malderen *et al.* (2017) mapped Cd in wheat grains purchased from the market, for which the concentration of Cd was 0.05 μg g^{-1} DW. However, the signal intensity of Cd in some parts fell the background level, making difficult the identification of the localization of Cd in the grain tissues. Samples with higher content of Cd could strength the signal and facilitate the imaging. For example, Cd distribution were imaged on the rice grain (4 μg Cd g^{-1} DW) collected from mining contaminated regions (Basnet *et al.*, 2014) and on sunflower seeds (5 μg Cd g^{-1} DW) cultivated in soils with added Cd (Pessôa *et al.*, 2017). In like manner here, Cd-contaminated grains (2.2 μg Cd g^{-1} DW) were used for the mapping to enhance the definition for Cd. To our knowledge, it is the first time that fine maps of Cd distribution is shown particularly in the embryo and the crease region of wheat species (Figure 5-1; Figure 5-2; Figure 5-3).

The chemical analysis of dissected grain parts is another approach to investigate elemental distributions (Detterbeck *et al.*, 2016; Kutman *et al.*, 2011b; Pieczonka and Rosopulo, 1985). It provides quantitative information at the whole grain scale, compared to the imaging that only reflects the distribution of elements in a grain section. In the present study, LA-ICP-MS analysis from three sections combined with a dissection approach provided a more comprehensive view about distributions of Cd and essential elements in the grain. Both mapping and dissection showed that elements were preferentially concentrated in the germ, the crease, and the peripheral parts of the grain, while the starchy endosperm (*SE*) was low in minerals (Figure 5-1; Figure 5-2; Figure 5-3; Figure 5-4; Figure 5-5). This result is also supported by earlier reports showing that the wheat bran, which mainly contains the peripheral parts and sometimes the germ, contains a higher concentration of Cd and essential elements than the inner kernel fraction which was likely to also include the crease (De Brier *et al.*, 2015; Emanuelli *et al.*, 2014; Guttieri *et al.*, 2015). Indeed, the crease region extending deeply inward through the whole length of a grain, and it can hardly be removed with the bran by peeling and pearling processes (Evers and Millar, 2002). The present study showed that the crease was particularly enriched in Cd, Zn, and Mn. The concentration of these elements in the crease was far higher than that in the grain (Figure 5-4), which is mirrored in the maps (Figure 5-2). Compared to our observation, similar patterns of element distribution within the crease were reported by De Brier *et al.* (2016). In this study, consistent with our results,

the authors observed that Mn was mostly present in the external part of the crease close to pigment stand which is part of the vascular bundle, that Zn was more inward in the modified aleurone of the crease, whereas P was almost absent in the outer region of the crease.

Table 5-2 Histological and mineral compositions of mature durum wheat grain (% of total amount)

	Crease	Germ	Outer periphery	Inner periphery	Starchy endosperm
				%	
DW	0.9	3.4	3.1	9.9[a]	82.7[a]
Cd	15.6	7.4	8.1	29.6	39.3
Zn	16.7	12.1	4.2	48.6	18.5
Cu	1.8	16.8	0.5	35.6	45.3
Mn	18.0	19.4	10.7	41.4	10.5
Mo	3.2	8.5	0.8	33.7	53.8
Fe	6.1	16.2	0.7	67.3	9.7
Ca	4.0	9.5	7.0	54.6	24.9
K	0.7	11.1	2.4	58.4	27.3
Mg	0.5	11.0	0.5	77.1	11.0
P	1.3	28.5	0.1	21.2	48.9

[a] Values reported by literatures
Values are means of the data (n = 10 000) generated as presented in Materials and methods
DW refers to dry weight
Outer periphery: likely composed of the outer pericarp; Inner periphery: likely composed of the aleurone layer + seed coat + the inner pericarp + other maternal peripheral layers

High concentrations of Zn, Cu, Mn, and P were found in the dissected germ (Figure 5-4; Figure 5-5). This finding is supported by the maps showing that these elements were more abundant in the scutellum (Figure 5-1; Figure 5-2). By contrast, Cd was more evenly distributed in the germ, which is reflected by its lower ratio of germ-to-grain concentrations as compared to other elements (Table 5-2). The distribution of elements within the germ as shown here is rarely studied especially for trace element like Cd and Mo. However, the distinct localization of Mo in the outer layer of the root primordia presented in Figure 5-3 is in agreement with the results reported by Wu *et al.* (2013) using LA-ICP-MS. However, the laser size (25 μm) used by these authors for the mapping was larger than the one used in the present study, and the longitudinal section was not considered. For the other essential elements, their distributions in the germ are supported by synchrotron-based analyses of wheat (De Brier *et al.*, 2016). These studies reported that Fe was highly distributed in the scutellum, which is in agreement with the rather high concentration of Fe in the dissected germ (Figure 5-4).

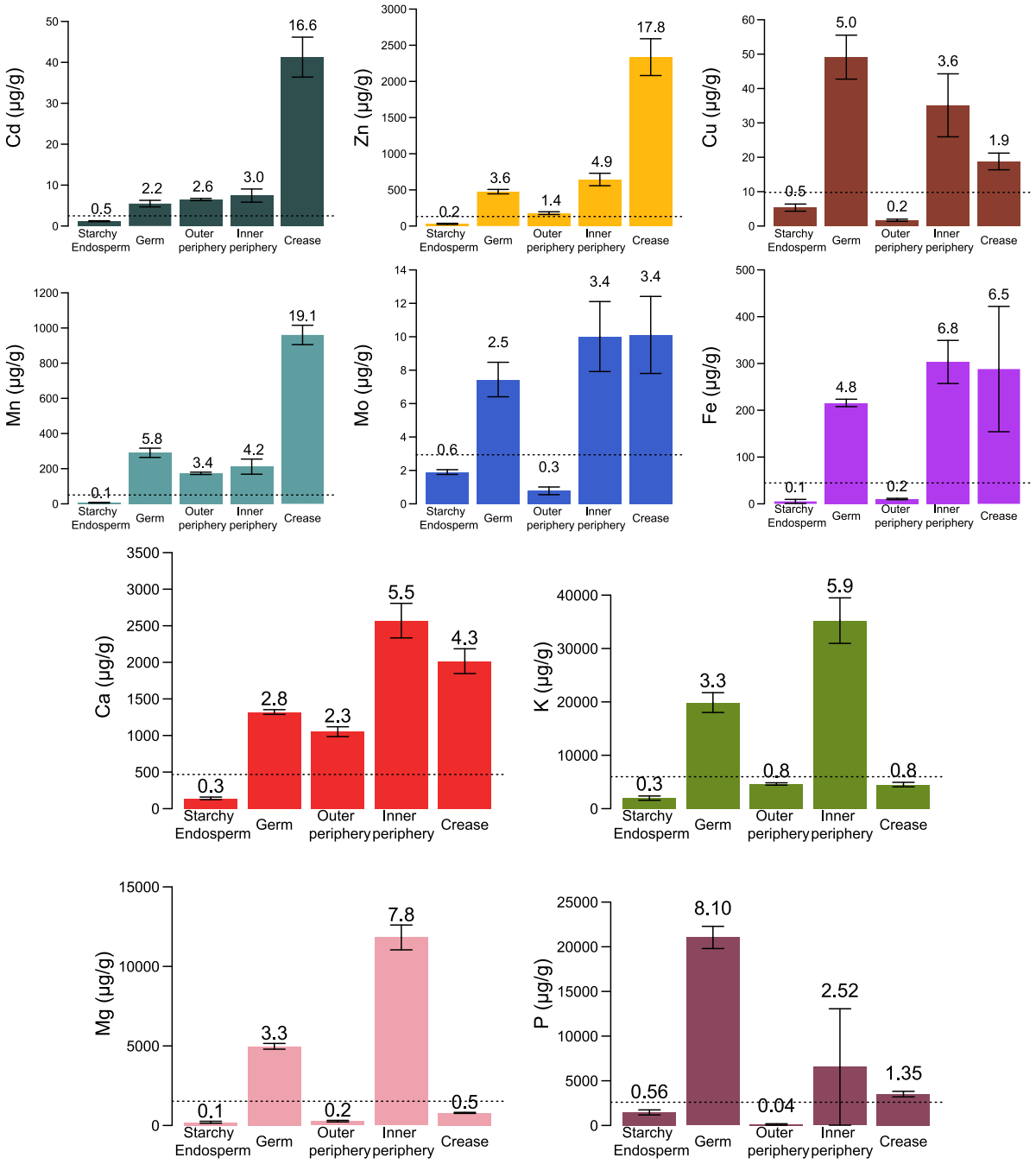

Figure 5-4 Quantitative information about the elemental distribution in durum wheat grains. Data (*n* = 10 000) are generated as presented in Materials and methods. Concentrations of Cd, Zn, Cu, Mn, Mo, Ca, K, Mg, and P in dissected parts are shown as mean ± standard deviation. The dash line in each plot represents the mean value of the whole grain concentration of the element. Numbers in each plot indicate the ratio of concentration between a grain part and the whole grain. Inner periphery likely contains the aleurone layer, the seed coat, the inner pericarp, and other maternal peripheral layers. Outer periphery likely contains the outer pericarp.

Contrary to the other elements, for Cd, Zn, and, Mn, the outer periphery of the grain (*OP*) was clearly visible in the maps of Cd, Zn, and, Mn (Figure 5-1), reflecting a significant accumulation of these elements. Similarly, the analysis of the grain dissection showed the relatively high *OP*-to-grain ratios for these elements as compared to the others (Figure 5-4). The richness of the inner periphery (*IP*) in all elements was observed in both the maps and the dissection results (Figure 5-1; Figure 5-2; Figure 5-3; Figure 5-4), although for the latter the concentration of elements in the *IP* was estimated (see Materials and methods). This agreement gives a good confidence in these estimates. It is likely that the aleurone constituted the main part of the *IP*, since the aleurone layer is commonly recognized as an important storage tissue for minerals (Palmgren *et al.*, 2008).

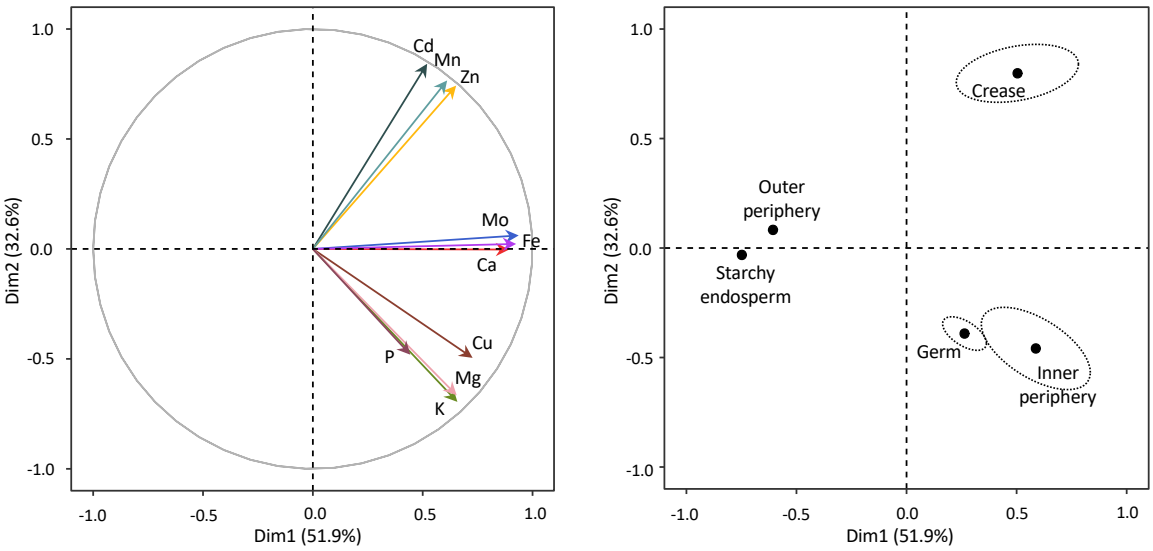

Figure 5-5 Principal component analysis (PCA) on the concentration of elements in dissected grain parts. The distribution of elemental concentration in each grain part along the first two principle component was shown as the ellipse covering 95% of the individual. Different elements are indicated by colors. Inner periphery likely contains the aleurone layer, the seed coat, the inner pericarp, and other maternal peripheral layers. Outer periphery likely contains the outer pericarp.

Cadmium, Cu, and Mo were extended more into the *SE* as compared to other elements, which is evidenced both by their significant signal in this part in the maps and by their relative low concentration in the *IP* and high concentration in the *SE* (Figure 5-1; Figure 5-2; Figure 5-3; Figure 5-4). Consistent with the Cu map of Figure 5-2, Cu was reported to be mobile from the crease to the endosperm in the map of sections of wheat and barley grains (Ajiboye *et al.*, 2015; Lombi *et al.*, 2011b). Similar observations were made by Pieczonka and Rosopulo (1985) using dissection of wheat grain. They showed that Cd and Cu were more evenly distributed between the *IP* and the

SE. The analysis of wheat milling fractions also showed that the concentration of Zn and Fe was more decreased by the removal of outer parts as compared to that of Cd (Guttieri *et al.*, 2015).

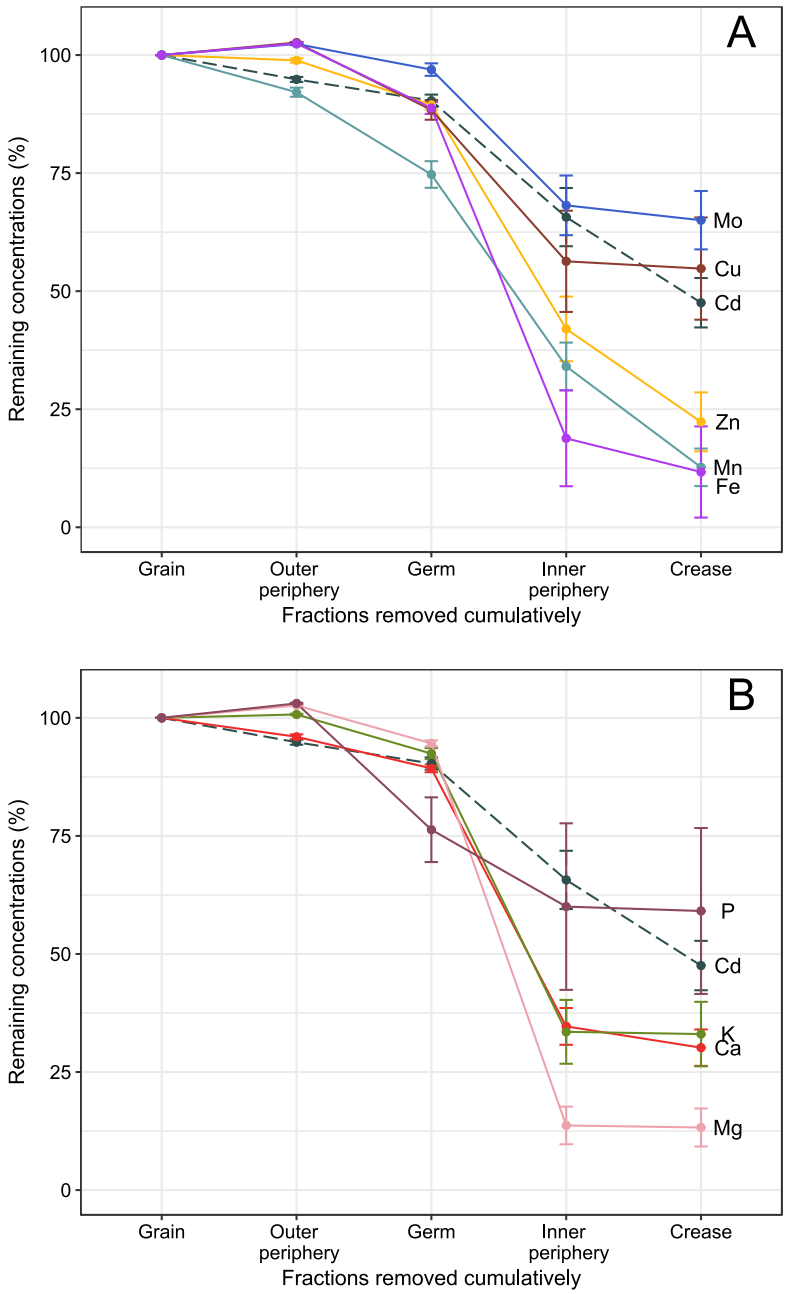

Figure 5-6 Theoretical cumulative curves of remaining concentrations as the percentage of the initial whole grain level by removing fractions of durum wheat grains for Cd, (A) micronutrients and (B) macronutrients. The remaining concentration was calculated by dividing the remaining quantity with the remaining dry weight for each element after removing given tissue(s). Values are mean ± standard deviation (n = 10 000) calculated from the data generated as presented in Materials and methods. Different elements are indicated by colors. The curves of Cd are highlighted by dashed lines. The outer periphery likely contains the outer pericarp. The inner periphery likely contains the aleurone layer, the seed coat, the inner pericarp, and other maternal peripheral layers.

5.4.2 Localization of cadmium and essential elements in durum wheat grains with respect to the mechanisms of grain filling

The differential accumulation of an element between grain tissues may mirror its internal loading, storage and functions within the grain (Lombi *et al.*, 2009). The crease represents the major route for nutrients entry into the grain (Fisher and Gifford, 1986). In the crease, nutrients can be unloaded from the phloem of the main vascular bundle to the apoplastic space of the maternal tissue including the seed coat, absorbed by the nucellar projection, transferred inward and discharged into the endosperm cavity (Patrick and Offler, 2001; Wang *et al.*, 1995; Yu *et al.*, 2015a). During grain maturation, the transfer of nutrients from the phloem to the nucellar projection can be limited by differentiation of the pigment strand (Zee and O'Brien, 1970a). The endosperm cavity is an apoplastic structure at the interface between the maternal and filial grain tissues, where transporters are required for the efflux from nucellar projection and for the influx by the modified aleurone layer (Patrick and Offler, 2001). Nutrients in the aleurone cells are transferred through the extensive plasmodesmata circumferentially to the dorsal side of the grain or radially into the *SE* (Morrison *et al.*, 1975; Patrick and Offler, 2001; Pearson *et al.*, 1998). The pericarp and the seed coat enveloping the aleurone obtains nutrients primarily from the main vessel within the crease but also from non-crease protophloem strands located in the pericarp (Fisher, 1990).

Grain Cd, Zn, and Mn were particularly concentrated in the crease, which reveals suggests that the transfer of these elements further to the filial tissues may be a limiting or regulated step for their loading into the grain. Indeed, it has been suggested that the symplastic isolation between maternal and filial grain tissues is a major bottleneck for the phloem unloading (Palmgren *et al.*, 2008). Among the three elements, our results show that Mn seems to be the one that is the most restricted or regulated with respect to the loading of the filial tissues of the grain. Pearson *et al.* (1995) suggested that the large sequestration of Mn in the crease would be due to the massive release of Mn from the phloem to the apoplastic spaces of the seed coat instead of loading into the nucellar projection transfer cells for further transport into the grain. Such a strong restriction is probably linked to the lack of transporter or to the maturation of pigment strand, which remains to be elucidated. Zn seems to be controlled or limited one step forward, most probably in the modified aleurone layer. The aleurone was shown as a source for Zn for the endosperm (Wang *et al.*, 2011). Therefore, the reason for this retention has to be investigated in the perspective of biofortification which aims at increasing the content of Zn in the endosperm (Clemens, 2014). The distribution of Cd in the crease was more homogeneous compare to that of Mn and Zn (Figure 5-2). Since Cd has no biological function for higher plants, this pattern could be the result from a

limitation of the transport due to the lack of specificity of transporters mediating the loading of Cd into the filial tissues.

We observed that the *IP* part of our grains concentrated more elements than the *OP* and, therefore, it is likely that the *IP* included the aleurone layer. Aleurone cells have myriad globoids that can strongly store minerals as reserve for the seedling growth (Regvar *et al.*, 2011). Phytic acid, which is highly concentrated in the globoids, is the major form of P storage in wheat grains and strongly forms insoluble complexes with metal elements such as Zn, Mn, Cu, Fe, and Mg (Cakmak, 2007; De Brier *et al.*, 2016; Ficco *et al.*, 2009; O'Dell *et al.*, 1972). In the maps (Figure 5-1; Figure 5-2; Figure 5-3), both P and Cd were highly present in the *IP*, indicating that phytate may also bind part of the Cd in the grain.

Once loaded into the grain, Cd appeared to be more distributed in the *SE* (Table 5-2; Suppl. Fig. S5-1). First, this pattern may be related to the capacity of endosperm cells to accumulate proteins. It has been suggested that Cd was associated with biological macromolecules in cereals (Günther and Kastenholz, 2005). In mature wheat grains, He *et al.* (2002) reported that Cd was bound to proteins with molecular weights of 54.5 kDa and 5.5 kDa. The inward extension of Cd may be related to protein gradients across the *SE*, being more concentrated in the subaleurone than the inner parts (Tosi *et al.*, 2011). Indeed, Cd has a high affinity for sulfur (S)-containing compounds and, accordingly, the distribution pattern of Cd mirrored that of S observed in wheat, barley and rice grains (De Brier *et al.*, 2016; Detterbeck *et al.*, 2016; Lombi *et al.*, 2009). In rice grains, Cd is associated with S-containing amino acids including cysteine and methionine (Wei *et al.*, 2017). Taken together, it can be speculated that, apart from phytic acid, Cd may be complexed by thiol containing proteins, which facilitates the movement and the storage of Cd toward the inner *SE*. The richness in protein of durum wheat grain may be a reason of the relatively high content of Cd in the endosperm. Further works are highly needed to investigate the speciation of Cd in durum wheat grains.

The relative high presence of Cd in the *SE* may be also due to a restriction of Cd transfer from the endosperm to the germ. The germ can receive nutrients from the *SE* or from the rachilla directly through the suspensor in the early development stage (Yu *et al.*, 2015c). The contribution of these pathways could differ between elements. For instance, Mn in the germ may mainly derived from the direct supply of rachilla, since its high accumulation in the crease and low content in the endosperm suggest a limitation of Mn transport in the crease (Figure 5-1; Figure 5-2; Figure 5-3; Figure 5-4; Table 5-2). By contrast, for Cd and Zn, the concentration gradient between the crease, the IP and the *SE* (Figure 5-4), together with the high-intensive band in the scutellum facing the

crease (Figure 5-2), indicate that these elements are mainly transferred to the germ from the main vascular in the crease passing through the endosperm. Unlike for Zn, no specific transporters are likely to exist for the transfer of non-essential Cd into the germ, which therefore, accumulated via non-specific pathways for mineral nutrients (Borg *et al.*, 2009; Tauris *et al.*, 2009). It is probable that the loading of Cd into the germ was limited by the competition with nutrients that are much more concentrated. It is also possible that the binding of Cd to endosperm proteins makes the transport to the germ difficult if transporters require the free Cd ion. These results reveal that the passage from the *SE* to the germ may be another step, apart from the one in the crease, that limited the internal transfer of Cd in the grain.

The germ is composed of the embryo with the root and shoot primordia that will develop into the plant of next generation. The scutellum in the germ supplies nutrients to the embryo during grain germination (Lu *et al.*, 2013). Cu was mostly localized in the scutellum and much less concentrated in most of the embryo parts. Cu is involved in several critical physiological processes such as photosynthesis and respiration. Furthermore, the adequate range of Cu concentration in plant tissues is relatively narrow for the plant metabolism, and the toxic level is quite close to the optimal level (White and Brown, 2010). Hence, the intense accumulation of Cu in the scutellum can be interpreted as a strategy to deliver the adequate quantity of Cu to the different growing seedling tissues while maintaining the Cu homeostasis during germination. In contrast, Mo typically dominated in the root primordia. This pattern can be related to the essential role of Mo in nitrate reduction by roots and to the fact that the range of Mo concentration in tissues below toxicity is large (Marschner, 2011). Zn and Mn were highly presented in both the embryo and the scutellum, which matches with their central role in the function of numerous enzymes involved in plant growth and metabolism (Marschner, 2011). Zn is required for the functioning of over 300 enzymes (Palmgren *et al.*, 2008). K is the element the most homogeneously distributed within the germ, which may reflect the key role it plays in the cell osmoregulation (Marschner, 2011).

5.4.3 Interest of post-harvest treatment for lowering the level of cadmium in durum wheat-derived products

Through setting up new milling technologies, grain processing could improve the sanitary quality of durum products by removing the grain tissues that are rich in Cd while minimizing the impact on the process yield and on the nutritional value of the products. Common milling processes include debranning through friction, namely peeling, or through abrasion, namely pearling (Hemery *et al.*, 2007). The *OP*, which is the outermost layer, would be the easiest to be

removed by these processes. A higher degree of pearling can remove the germ, while the *IP* can be reached through a more intense pearling. The crease extending inward cannot be removed by peeling or pearling.

Based on our results, removing the peripheral parts (bran; incl. *OP* and *IP*) and the germ, which is manageable through pearling or peeling would reduce the concentration of Cd by 34% in the products (Figure 5-6). However, the cost is important with respect to the nutritional value. Together with Cd, this treatment would lower the yield by 16% (loss of dry weight) and negatively affect the nutritional value especially for Zn (-58%) and Fe (-81%). Such treatment may also lower the technological qualities of durum semolina, since the level of proteins will decrease as well due to the loss of the aleurone layer (Tosi *et al.*, 2011). De Brier *et al.* (2015) also reported that the concentration of almost all essential elements in wheat flour was negatively affected by pearling. By contrast, removing the crease would be of great interest since it would lower the concentration of Cd by 18% without much affecting the yield (-1%) and only the level of Zn (-20%) and Mn (-21%) would be the most affected. Delcour *et al.* (2012) reported a wheat fraction procedure for limiting the presence of the crease. Grains need to be pearled first to recycle the aleurone, then milled and sieved to separate the crease fraction. However, the feasibility of this procedure at large scale of production needs to be assessed.

5.5 CONCLUSIONS

The detailed map of Cd distribution in durum wheat grains was obtained using the high-sensitive imaging technique, LA-ICP-MS. Cd was highly concentrated in the germ, the outer parts of the grain, and, particularly, in the crease. Eliminating these parts in the final product would reduce the presence of Cd in diets. Unfortunately, compared to other elements, Cd was observed to extend relatively more inward in the endosperm, making it difficult removing the majority of grain Cd. The distribution pattern of Zn and Mn is similar to that of Cd. Therefore, their concentrations would be strongly impacted by the removal of grain parts for reducing the grain Cd level. The germ and the inner periphery of the grain were rich in all the elements, removing these parts may thus result in a great loss for the grain nutritional value. Different distribution patterns between elements were also observed within the germ and within the crease region, thanks to the high-definition of LA-ICP-MS. These details provide clues with respect to the physiological mechanisms of Cd and mineral nutrients loading and storing within the grain of durum wheat, which are important for further researches targeted for lowering the loading of Cd in grains or for enhancing the grain nutritional value.

5.6 ACKNOWLEDGMENTS

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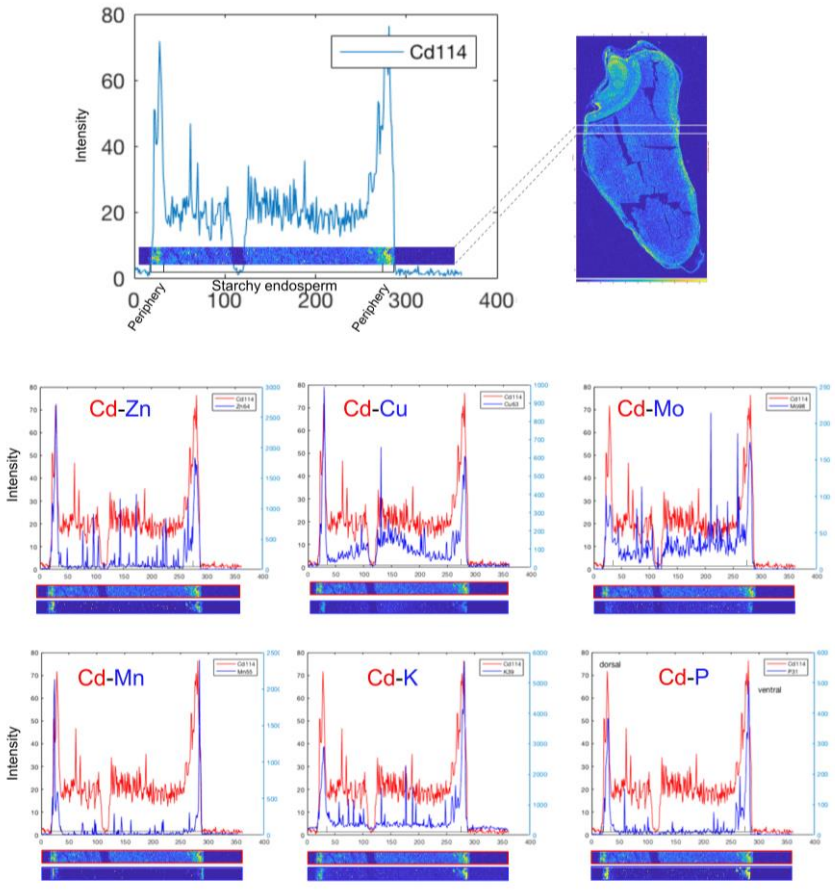
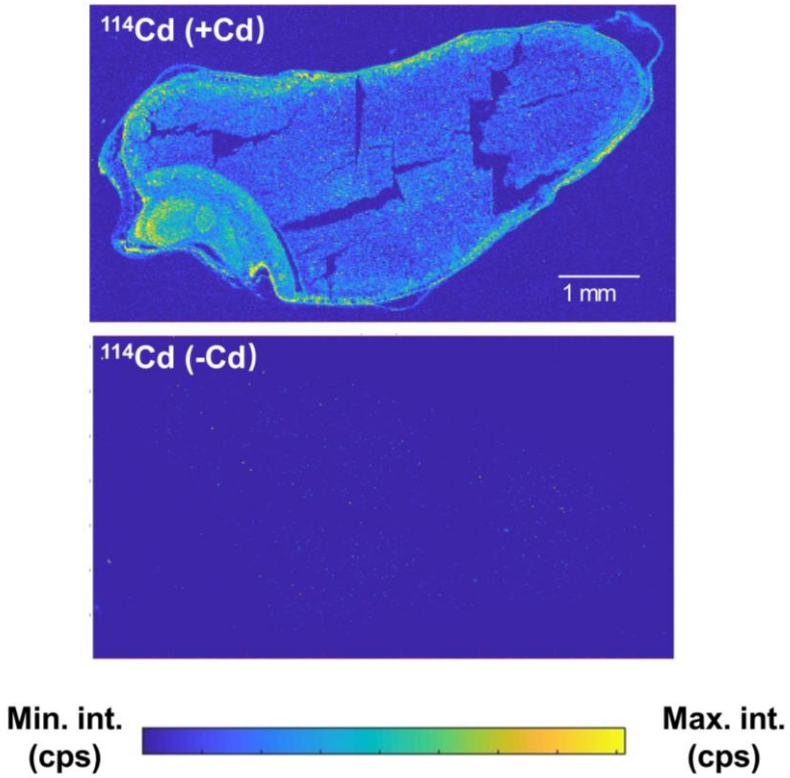
5.7 SUPPLEMENTARY MATERIAL



Suppl. Fig. S5-1 Profiles for the intensity of Cd and cross that of Zn, Cu, Mo, Mn, K, and P from the selected area (the box in the Cd map) with focus on the peripheral parts and the starchy endosperm. Images shown below each plot are extracted from the maps in Figure 5-1, with red frame line for Cd and blue frame lime for other elements. X-axis values are number of lines in LA-ICP-MS, which corresponds to a total length of 2654 µm. The scale lines below the X axis indicate the different parts.



Suppl. Fig. S5-2 Cd maps of a longitudinal section away from the crease of a durum wheat grain contaminated with Cd (+Cd) or containing background level of Cd (-Cd). The color scale represents the different intensities (*int.*, in counts per second (*cps*)) detected by LA-ICP-MS, with blue and yellow respectively corresponding to the lowest and highest relative intensity.

Chapter 6
General discussion

This thesis mainly discusses the ecophysiology of Cd allocation to durum wheat grains, with a particular focus on the partitioning and remobilization of Cd during the period of grain filling. The previous chapters described how agronomic practices such as the varietal choice (Chapter 2) and the level of nitrogen (N) supply (Chapter 3) as well as the level of exposure to Cd (Chapter 4) may affect the allocation of Cd to the grains in durum wheat. The last part of this work (Chapter 5) investigated the localization of Cd in mature grains and evaluated to what extent the level of Cd in durum wheat grains (as well as that of mineral nutrients) may be reduced when removing specific grain parts through dedicated grain-milling processes.

In response to the objectives set in Chapter 1, this chapter recalls the main outputs of this thesis (Figure 6-1) and discuss its main limitations and agronomical implications.

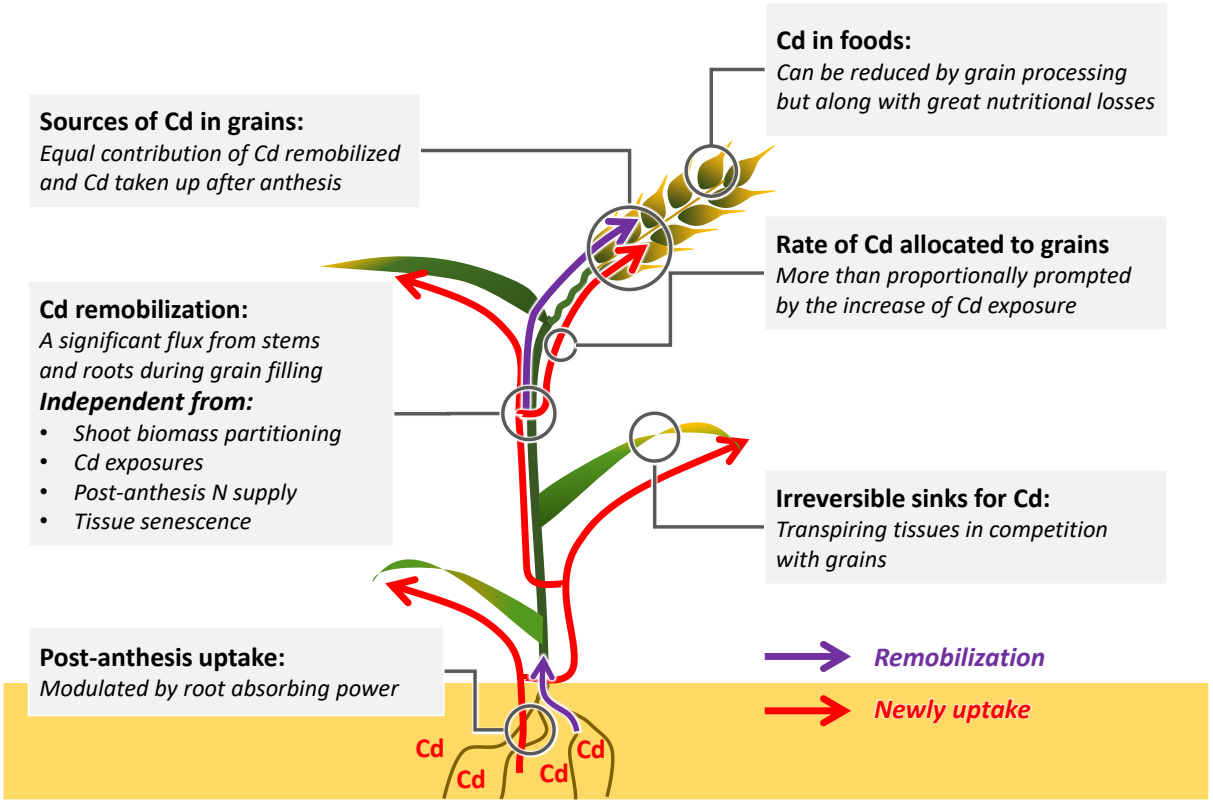


Figure 6-1 Main outputs of the thesis.

6.1 LEAVES ARE CADMIUM SINKS IN COMPETITION WITH DEVELOPING GRAINS

The study presented in Chapter 2 tested the hypothesis of a link between the allocation of Cd to the grains and the shoot allometry in durum wheat. The results showed that the cultivars which allocate a higher proportion of their shoot biomass to the straws (leaves + stems) relatively to grains tend to accumulate less Cd in their grains. This finding underlines the role played by vegetative organs in capturing part of the Cd flow delivered by the roots during grain filling. Chapters 3 and 4 confirmed this ability and clarified the role played by leaves and stems in this Cd sequestration process. In both experiments, leaves sequestrated a large majority of Cd newly delivered by roots during grain filling. Moreover, the isotopic labeling revealed the lack of net Cd remobilization from leaves, contrary to stems. These results strengthen the idea that leaves are terminal sinks for Cd regardless of its origin – i.e. either transported by the transpiration flow or remobilized from other vegetative tissues. Therefore, our work suggests that the level of Cd accumulation in durum wheat grains could strongly depend on the biomass partitioning between grains and leaves. The story is less clear for stems because, on the one hand, stems can sequestrate Cd, in particular in nodes as demonstrated in our work (Chapter 4) and for Zn in rice (Suzui *et al.*, 2017), but, on the other hand, they were shown to remobilize Cd during the stage of grain filling. Depending on the experiments, stems acted as either a net source or a net sink for Cd, so that their status as well as the impact of their relative biomass towards the level of Cd accumulation in grains is less direct and more difficult to predict than that of leaves. Applying plant growth regulators to modulate the straw growth would be a way to check the impact of stem length on grain Cd.

6.2 HALF OF GRAIN CADMIUM ORIGINATES FROM CADMIUM TAKEN UP DURING GRAIN FILLING WHILE THE OTHER HALF FROM CADMIUM REMOBILIZED

Cadmium in durum wheat grains is derived from both the uptake during grain development and the remobilization from pre-anthesis accumulations. The equal contribution of direct uptake and remobilization to the grain Cd accumulation was evidenced on two durum wheat cultivars differing in their shoot biomass partitioning (Chapter 3), on two levels of post-anthesis N supply (Chapter 3), and on two levels of Cd exposure (Chapter 4).

The phytoavailability of Cd in soils during grain filling determines the amount of Cd in grains from uptake, while the amount of remobilized Cd depends on the quantity of Cd accumulated before anthesis and therefore on the Cd phytoavailability during vegetative phase (Chapter 4). Therefore, the relative contribution of remobilization and direct uptake is expected to depend on

the relative availability of soil Cd during pre- and post-anthesis periods. Our results suggest minimizing Cd phytoavailability in soils throughout the whole growth cycle. In the field, the phytoavailability of Cd during grain filling is more likely to be lower than that before anthesis because of a lower water content of soil, a lower ionic strength of the soil solution due to lower amount of fertilizers. Hence, attention must be payed to the pre-anthesis phytoavailability of Cd which could be controlled using common drivers such as maintaining a neutral soil pH, using Cd-free fertilizers and irrigation water, and applying organic matters.

The flow of Cd taken up post-anthesis can be restricted through modulating the root absorbing power at this stage (Lux *et al.*, 2011; Schneider *et al.*, 2017). In Chapter 4, the post-anthesis uptake of Cd decreased with the earlier grain maturation, which was suspected to result from the earlier degradation of root function in absorbing Cd. This finding enlightens the possibility to limit the root absorbing power through factors that accelerate the maturation or the degradation of roots which includes, for instance, N and water deprivation. This strategy shall be combined with the use of cultivars with a high efficiency of N remobilization to maintain a proper grain protein level which is critical for the technological quality of durum wheat in the pasta industry. On the other hand, the stay green trait in durum wheat is expected to be risky, which prolongs the root activity and thereby promotes the Cd uptake. Furthermore, stay green is often achieved through late N fertilization which is a common used strategy to increase the grain protein level. However, late N supply can also contribute to maintain a high Cd phytoavailability in soils during grain filling. Given the significant contribution of post-anthesis uptake to grain Cd loading, the stay green is definitely not a good option. Chapter 3 showed that the remobilization of N was not linked with that of Cd. Therefore, instead of a stay-green strategy, it is better to work at increasing the efficiency of N remobilization.

In contrast, the driver of Cd remobilization is poorly understood. Conceptually, there is no sink demand of grain for toxic elements like Cd. Hence, the remobilization of Cd may be not driven by the sink demand but by the source supply. Even so, the loading of Cd in developing grains can follow the pathway for essential elements (Palmgren *et al.*, 2008). In other words, there may be a sink demand for Cd that derived from that for essential elements like Zn, a Cd analogue. In Chapter 3, however, Cd remobilization was not affected by post-anthesis N supply, which is different from Zn remobilization which was strongly favored by the low nutritional status of durum wheat (Kutman *et al.*, 2012). It would be interesting to test the impact of grain demand for essential elements on the allocation of Cd. One way would be to decrease the sink demand for Zn through applying Zn on spikes. Isotopic labeling can be conducted at specific times during grain filling to check if the remobilization is linked between Zn and Cd. There is a need for future research to

identify the driver of Cd remobilization and to find out what element Cd is moving with during remobilization, which would help for a better management of the flow of Cd remobilized from pre-anthesis pools, a significant source for grain Cd.

Furthermore, the results of Chapters 3 and 4 suggest that Cd remobilization is poorly linked to the senescence of leaves. In both experiments, no net Cd was remobilized from the senescing leaves, which was different from that for N, the element known to be remobilized at the start of leaf senescence (Maillard *et al.*, 2015). Further study is required to confirm and understand the link between tissue senescence and Cd remobilization by means, for example, regulating the expression of NAC genes which encode transcription factor that accelerates senescence (Uauy *et al.*, 2006) or applying senescence-regulating phytohormones such as ABA (Distelfeld *et al.*, 2014). It was reported that senescence manipulations would be a strategy to improve the crop yield and nutrient use efficiency by enhancing the rate of nutrient remobilization from vegetative tissues to grains (Distelfeld *et al.*, 2014; Uauy *et al.*, 2006). It was also proposed in this thesis as a mean of reducing post-anthesis uptake (see above). If the senescent-independent character of Cd remobilization being confirmed in durum wheat, these senescent-regulating practices would not favor the remobilization of Cd toward the grain.

In the long term, breeding low-Cd cultivars of durum wheat would be a way to reduce the content of Cd in durum wheat grains. The Canadian have bred cultivars harboring a low-Cd locus *Cdu1* that can strongly limit the transfer of Cd from roots to shoots. However, till now, most of the European cultivars did not hold this locus (Zimmerl *et al.*, 2014). The results of this thesis indicate that, apart from root uptake and root-to-shoot transpiration, aboveground processes are of importance in controlling the allocation of Cd to durum wheat grains. Stem nodes are the hub for Cd distribution in shoot. The xylem-to-phloem transfer in the node would be another step that can be targeted in breeding low-Cd cultivars.

6.3 DURUM WHEAT IS SENSITIVE TO CHANGES IN THE LEVEL OF CADMIUM EXPOSURE IN THE RANGE OF THOSE ENCOUNTERED IN AGRICULTURAL CONTEXT

The level of phytoavailable Cd in agricultural soils are often at nanomolar range (Sauvé *et al.*, 2000). It was shown in Chapter 4 that the root influx of Cd was not saturated at 100 nM Cd which represents the upper limit of Cd in agricultural soils. However, leaves kept accumulating Cd both before and after anthesis, which resulted in the leaf Cd above the toxic threshold during grain filling, and thereby a decrease in grain yield at 100 nM Cd. Our results indicate that, besides the far

higher level of exposure (Shahid *et al.*, 2016), nanomolar range of Cd that presents in the field could be toxic to durum wheat. Therefore, soil Cd phytoavailability must be reduced in durum wheat production basin.

Stem nodes behaved as buffers for the allocation of Cd to grains (Chapter 4). They can sequestrate more Cd when more Cd was present in the plant. However, this buffer ability seemed weak, at least at 100 nM Cd. As a consequence, the increase in the level of Cd exposure favored the allocation of Cd taken up post-anthesis to the grain. These results suggest that the importance of direct root uptake in grain Cd accumulation may increase at a higher phytoavailability of Cd in soils. Though, in the field, the Cd availability is often low during grain filling as mentioned above, while more attention should be payed to the post-anthesis uptake when it is not the case. Meanwhile, the maximum level of Cd that the node can buffer need to be assessed for better predict the impact of Cd exposure on the flow of Cd taken up post-anthesis. Furthermore, in Chapters 3 and 4, stems were the source organ of Cd remobilization. Whether this buffer ability comes from a promoted sequestration or from a limited remobilization needs to be understood further.

6.4 TOWARDS THE EFFICIENCY AND THE SELECTIVITY OF GRAIN MILLING PROCESS TO LOWER THE CADMIUM CONCENTRATION IN DURUM WHEAT-BASED PRODUCTS

This thesis was conducted based on the idea of protecting durum wheat-based foods from Cd contamination. Grains are the edible part of durum wheat. They need to be processed by milling and by removing different grain parts before human consumption (Sissons *et al.*, 2012). The results in Chapter 5 showed that Cd was localized mainly in the peripheral parts, the germ, and particularly, in the crease, which makes it possible to reduce the content of Cd in foods by removing these Cd-rich parts.

However, the similar distribution was observed for manganese (Mn) and zinc (Zn) which would be lost together with Cd during grain processing. For the perspective of biofortification, it would be interesting to increase the ratio between Zn+Mn and Cd of wheat grains. One can imagine that if this ratio is increased, loosing Zn+Mn when removing Cd by milling processes would not impact too much the final nutritional quality of the products. Increasing the (Zn+Mn)-to-Cd ratio can be achieved by foliar application of Cd-free Zn+Mn fertilizer. However, as mentioned above, it needs to investigate to what extent remobilization contributes to the loading of Zn and Mn in grains and how it is linked to the remobilization of Cd and to that of N (Cakmak and Kutman, 2017). Moreover, Cd was shown to be more extended inward as compared to Fe and Zn. Increasing the

storage of Fe and Zn in the endosperm would help to limit the loss of these elements during grain pearling, which are important in terms of biofortification (Palmgren *et al.*, 2008).

Cadmium is highly present in the grain periphery, and thus the concentration of Cd would be low in larger grains with a lower periphery-to-volume ratio. In Chapter 2, a negative relationship between grain size and grain Cd content was observed among cultivars. Therefore, it would be relevant not to breed cultivars that produce more but smaller grains.

More information is needed for better understanding the internal loading of Cd in durum wheat grains. LA-ICP-MS can determine the abundance of multiple isotopes of Cd. In Chapter 5, for instance, both ^{114}Cd and ^{111}Cd maps were obtained though only the former ones were shown. As the labeling approach used in Chapters 3 and 4, further studies can take the advantage of LA-ICP-MS to trace the in-grain transfer of Cd isotopes introduced at different times of grain development, as that has been used for Zn (Wang *et al.*, 2011). It would be also interesting to differentiate the localization of Cd taken up during the first two weeks of development, when the direct transfer to the germ is possible (Figure 1-10), from that taken up later on.

Furthermore, analyzing the speciation of grain Cd may help to reveal the storage of Cd and the element with which Cd is associated in the grain. It could be achieved through X-ray absorption near edge spectroscopy (XANES) and high-pressure liquid chromatography (HPLC)-ICP-MS as that introduced by Lombi *et al.* (2009) for the speciation analyses of arsenic in rice grains. Moreover, the speciation of Cd may be useful in assessing the bioavailability of Cd in food stuffs for dietary intake. The maps in Chapter 5 showed that both P and Cd are concentrated in the aleurone layer, which indicates the possible chelation of Cd with phytate, a form can be poorly absorbed by humans (Lopez *et al.*, 2002). Regulating the bioavailable level of Cd in durum products would be also a way to improve the food safety.

Not only for foods, grains of durum wheat are also produced for seeds. Cd is a toxic element which could affect the seed germination and seed dormancy (Kranner and Colville, 2011). High level (at micromolar range) of external Cd was shown to reduce the germination frequency of wheat seeds (Liu *et al.*, 2007). In Chapter 5, Cd is intensively present in the germ which carries the embryo that will develop into the plant of next generation. Further study is needed to test whether the level of internal Cd in durum wheat seeds may affect the seed quality which includes the seed dormancy, the vigor of germination as well as its dependence toward thermal and hydric conditions.

6.5 POSSIBLE LIMITATIONS OF THE THESIS

All experiments in the thesis were carried out in hydroponics to maintain the exposure to Cd as constant as possible, which was necessary to avoid confounding effects. The plants grew healthily, and no visual symptoms of Cd toxicity or nutrient deficiency occurred. Grain Cd was lower than the EU regulatory standard when plants were grown at 2 or 5 nM Cd in the nutrient solution (Chapter 2 and Chapter 4). These results indicate that the growing conditions would be assumed to be comparable between hydroponics and field. The solution culture facilitates the harvest of entire roots, as well as the manipulation of elemental compositions (e.g. Cd isotopes) in the growth medium. However, given its sufficient nutrient supply and the stress-free condition, the plants could remain green and vigorous longer than those in the field (Harris and Taylor, 2013). In Chapter 2, post-anthesis N deprivation was expected to trigger tissue senescence (Juraniec *et al.*, 2017) but it did not, which was probably due to the high supply of N during the vegetative stage. Hence, the less restricted transpiration and root functioning may have artificially increased the contribution of direct uptake to grain Cd. In this case, the competitive power of transpiring tissues, namely leaves, for Cd may have been strengthened. In addition, the high tillering rate as compared to the field have forced us to remove extra tillers which may lead to uncertainties in the results.

The main idea of the long-term isotopic labeling was to distinguish between the Cd taken up pre- and post-anthesis. This long-term approach is necessary to assess the contribution of remobilization throughout the whole grain filling period. However, Cd remobilized was assimilated as Cd taken up pre-anthesis. However, the remobilization of Cd taken up post-anthesis, if any, is unknown. It may have led to an overestimation of the contribution of direct uptake. In these experiments, either all leaves (incl. flag leaf and lower leaves) or all stems (incl. nodes and internodes) on a plant were pooled, among which there would be disparities in the Cd remobilization. For instance, it may mask the possible remobilization of Cd from flag leaves where the remobilization of Cd was reported on rice and durum wheat (Harris and Taylor, 2001; Kashiwagi *et al.*, 2009). Otherwise, in Chapters 3 and 4, the loss of Cd occurred during reproductive stage. This is one difficulty of long-term labeling that growing plants until late maturity. This loss of Cd introduced some uncertainty in the quantification of Cd taken up post-anthesis, as well as in the contribution of roots to the flux of Cd remobilized to the grains. The conclusion about the relative contribution of remobilization to grain Cd thus needs to be further tested in the field or in soil by changing Cd phytoavailability pre- or post-anthesis to see the consequences on the grain Cd. Detailed investigation is also required to assess the remobilization of Cd from vegetative tissues.

In the maps provided by the LA-ICP-MS, the sub-tissue distribution of elements in peripheral parts and in the crease cannot be clearly identified (Chapter 5). Most of the results from grain dissection matched well with the elemental maps, although some discrepancies were found, notably regarding the level of Cd accumulation in the starchy endosperm. Hand dissection may have introduced some uncertainty in the grain parts isolated. Further works are thus needed to identified tissues that is contained in the dissected grain parts through, for example, biochemical markers (Hemery *et al.*, 2009).

Chapter 7
General conclusion and perspectives

7.1 CONCLUSIONS

In summary of the results presented in the previous chapters, we concluded that:

- Cd content in grains could be related to plant allometry in particular the ratio of biomass between grains and leaves. Leaves are possible irreversible sinks for Cd.
- During grain filling, Cd is sufficiently mobile in durum wheat plant to be importantly allocated to grains from remobilization from the pre-anthesis pool in stems and perhaps root tissues but not from leaves. Cd remobilization is not linked to that of nitrogen (N).
- Increasing moderately Cd exposure (100 nM of total Cd) already results in toxicity, as reflected by a decrease of plant growth and grain yield and by an accumulation of Cd in grains more than proportional to the increase of the exposure.
- In grains, Cd is more concentrated in periphery tissues and in germ as most of the macro and micronutrients, which makes it difficult to remove Cd without affecting nutrients by milling processes. Furthermore, contrary to some elements like Fe and Zn, Cd is significantly disseminated in the endosperm, which limits the perspective of strongly reducing Cd in the food products. The best options would be to remove the crease but then Zn and Mn will be also lost.

7.2 SUGGESTIONS

Reducing the transfer of Cd from soils to grains is perhaps the most efficient way to achieve the low-Cd food, since the Cd distribution in durum wheat grains does not allow its selective and efficient removal through even dedicated grain milling processes. We thus suggested:

- To better control the phytoavailability of Cd in soils both before and during grain filling period.
- To select durum wheat cultivars with higher leaf-to-grain biomass ratio (but would lower

the water use efficiency), with larger grains (to lower the relative weight of grain periphery), and with higher N use efficiency (to adapt practices that regulate the root absorbing power).

- To breed low-Cd cultivars targeting not only with the root sequestration but with aboveground processes like xylem-to-phloem transfer.

7.3 WHAT WOULD NEED TO BE DONE FURTHER?

This thesis documented findings. Some of the outputs are important and worthy to be understood further, while some need to be checked in consideration of the limitations.

First, further studies are needed to test our main findings in soil for rapid application in the field/milling industry:

- About allometry: to test the effect of leaf-to-grain and grain periphery-to-endosperm biomass on grain Cd within a cultivar with respect to sink-source modification or across cultivars with respect to genetic variability. If the effect confirmed, the traits could be recommended for the selection of durum wheat cultivar.
- About Cd remobilization: to test by modulating Cd phytoavailability pre- or post-anthesis and between stay-green traits or not to see the consequences on the grain Cd.
- About milling processes: to test the effect of peeling, pearling, and crease removal from grains collected in the field.
- Cost-effectiveness analysis for different options suggested by our work.

Second, gaining knowledge about the allocation of Cd to durum wheat grains is still in progress. Mechanistic approach, which includes eco- and molecular physiology, will help to understand what governs the dynamic of Cd in plants and to target mechanisms/locus for genetic selection:

- Mechanisms of Cd partitioning in plant: leaves competition for Cd, xylem-to-phloem transfer in stem nodes, loading of Cd to the grain from maternal to filial tissues, and transfer of Cd between grain tissues.
- Accurate determination of source tissues for remobilization and driving mechanisms and speciation of mobile Cd.

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