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Doctoral thesis

CADMIUM EXCLUSION FROM BARLEY (*Hordeum vulgare* L.):

DEVELOPMENT OF PHYSIOLOGICAL AND MOLECULAR MARKERS

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Content

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Abstract

The entry into the food chain of excessive amounts of heavy metals, due to the consumption of foodstuffs from crops grown on contaminated soils, is of increasing concern for public health. Among heavy metals, Cd results particularly dangerous since it is easily taken up by roots and translocated to vegetative and reproductive organs of plants without obvious symptoms of phytotoxicity. In particular, Cd can accumulate in grains of cereals such as rice, wheat and barley. The Codex Alimentarius Commission of the Food and Agricultural Organization/World Health Organization set the maximum permissible concentration of Cd for human consumption at 0.1 μg g^{-1} for cereal grains, excluding rice (0.4 μg g^{-1}). Among the strategies to limit the risk of introducing Cd into the human food chain, the identification and/or constitution of plant genotypes able to exclude the metal from the shoot or from the edible parts seems to be the most promising line of enquiry for the future.

Among cereals, barley ranks fourth in terms of both yearly-produced amounts and cultivated area in the world. In recent years, a correlation between presence of barley in the diet and reduced risk of coronary heart diseases has been suggested, inducing a progressive increase in the demand for the cereal in countries where its consumption was traditionally limited. Although some evaluations of genotypic differences in Cd accumulation in barley grain have been described, very little information is available about the physiological basis of the observed variability.

Specific aims of the research were: a) to analyze six barley cultivars among the most cultivated in Tunisia for their tolerance to relatively high Cd concentrations and ability to limit the accumulation of the metal in shoot and grain; b) to identify the molecular and physiological basis of the behavior of the two most divergent cultivars, i.e. the highest and the lowest Cd accumulator, in order to develop markers useful in the selection of low-Cd grain cultivars.

Among the six Tunisian barley cultivars, a large variability in their sensitiveness to Cd exists**.** The concentrations of the metal in the roots of plants grown in hydroponic solution in the presence of Cd did not significantly differ among the six cultivars, whereas wide differences were apparent in the shoots, where Lemsi and Manel showed the highest and the lowest values, respectively. Despite similar transpiration fluxes, the six barley cultivars loaded into the xylem and translocated to the shoots different amounts of Cd. A close linear correlation between the concentrations of the metal in the xylem sap and those measured in the shoots was observed.

The measurements of concentration-dependent influx of Cd in the roots revealed marked differences between Lemsi and Manel. Lemsi showed a clearer saturable component in the low Cd concentration range; the maximum influx (V_{max}) for Cd was about threefold higher in Lemsi.

Although the Cd concentrations were not different in the roots of the two cultivars, the amounts of phytochelatins and the ability to retain Cd were lower in Lemsi than in Manel. The different Cd retention in roots between the two cultivars cannot be ascribed to a differential expression of the *HvHMA3* gene encoding a tonoplast-localized transporter mediating the vacuolar sequestration of the metal. In detail, the Cd-treatments decreased the steady state levels of *HvHMA3* mRNA in the two cultivars at the same extent.

Exhaustive extraction combined with a fractionation procedure showed that in the roots of Lemsi the percentage of free non-chelated Cd ions (Cd^{2+}), i.e. the form potentially available for xylem loading , was twice that present in Manel. Since the expression levels of the gene *HvHMA2*, encoding a protein actively extruding Cd^{2+} from the parenchyma cell of the root stele towards the xylem vessels, did not differ between Lemsi and Manel, it is reasonable to conclude that the larger amount of metal loaded in the xylem in the former cultivar is due to the higher amount of substrate (Cd^{2+}) available for the HvHMA2 protein.

When plants were grown on Cd-contaminated soil, the levels of the metal in the grain, as well as in flag leaves and husk, were higher in Lemsi than in Manel. This suggests that the reallocation of Cd from the leaves to the spike during grain filling does not involve mechanisms able to override the differences imposed by the differential Cd root uptake and root-to-shoot translocation described for the two cultivars.

In conclusion, the activity of mechanisms mediating the uptake of Cd into the root, and, particularly, the efficiency of the phytochelatin-dependent system chelating and sequestering Cd in the root, emerge as critical points in controlling low the concentration of Cd in barley.

Introduction

Trace elements

Chemical elements that occur in the Earth's crust in amounts less than 1000 mg kg^{-1} are defined as "trace elements". Similarly to this geochemically-derived term, the biological sciences define "trace elements" as being elements at similar concentrations within the organisms (Kabata-Pendias, 2011). Elements that are "trace" in materials may not be "trace" in terrestrial terms (i.e., iron).

Many elements occur in trace amounts in living matter where they are essential for the growth, development and health of organisms. In plants these elements, defined as the essential micronutrients, are Fe, Mn, Zn, B, Cu, Mo, Cl, Se and I.

In soils, many trace elements, including micronutrients, can reach concentrations which may be toxic to microrganisms and plants. They are: mercury (Hg), lead (Pb), cadmium (Cd), copper (Cu), nickel (Ni) and cobalt (Co). As concern for human health increases, the list of toxic "priority metals" has been extended to: arsenic (As), beryllium (Be), antimony (Sb), chromium (Cr), selenium (Se), silver (Ag) and thallium (Tl). In organisms, the quantitative difference between amounts playing essential physiological roles and the excess triggering toxic effects is very small.

Several trace elements are defined as heavy metals (HMs). Altough the term "heavy metal" has never been defined by any authoritative body such as IUPAC it is widely utilised for metals with a atomic density higher than 3.5 for some authors or 7 g cm^{-3} fo others (revised by Duffus, 2002). The term "heavy metal" is linked in many people's minds to metals (or their compounds) that are toxic, nevertheless, no correlation between the density of a metal and its physiological or toxicological effects is known (Appenroth, 2010).

More in-depth consideration reveals a huge amount of problems with the simple definition of "heavy metals". This definition is meant to suggest that the density of a heavy metal is high, but this physical property is quite meaningless in a biological context, including plants and other living organisms. Plants, as well as other organisms, do not deal with metals in their elemental forms; they are not accessible to plants. Metals are only available to them in solution, and it is necessary for metals to react with other elements and form compounds before they can be solubilised. Once such a chemical compound is formed, the density of the metal does not play any role.

The following are commonly referred to as heavy metals/metalloids: a) transition elements, all of which are metals, even though some of them form slightly amphoteric oxides; b) rare earth elements, which are subdivided into the lanthanide series and the actinide series; c)

some elements from the p-group (called sometime as “lead group” that are either metals (Al, Ga, In, Tl, Sn, Pb, Sb, Bi and Po) or metalloids/borderline elements (Ge, As and Te).

Trace elements, including heavy metals and metalloids, which are present in the soil, have different origins. These elements are generally ubiquitous in nature, but they may accumulate in the soil due to both geogenic and anthropogenic processes. Geogenic processes concerned are: a) the weathering of metal-rich parental material; b) marine aerosols; c) volcanic activities; d) wood/plant burning. Anthropogenic sources accelerating the natural fluxes of trace elements are: a) fuel combustion; b) waste disposable and incineration; c) agricultural, mining and industrial activities (Lombi et al., 2001).

Whether an element is present naturally in the soil or has been introduced by pollution, a measure more useful than total element content for most purposes is an estimation of its availability, since it is this property that can be related to mobility and uptake by plants and extractability by chemical treatments. Chemical soil tests are developed to extract a quantity of the element from the soil solids that correlates statistically to the size of the available pool in the soil, defined by the quantity of the element taken up by plants. The extractability of an element depend on its properties, such as its tendency to: a) chemisorb on minerals; b) complex with organic matter; c) precipitate as insoluble sulfides, carbonates, phosphates or oxides; d) co-precipitate in other minerals.

The better measure of availability in the soil for an element can be considered its concentration in soil solution. In natural soil solutions concentrations of trace elements are in the range 1-1000 µg L^{-1}. In the soil, trace elements undergo a series of reactions that increase or decrease their solubility, availability and mobility.

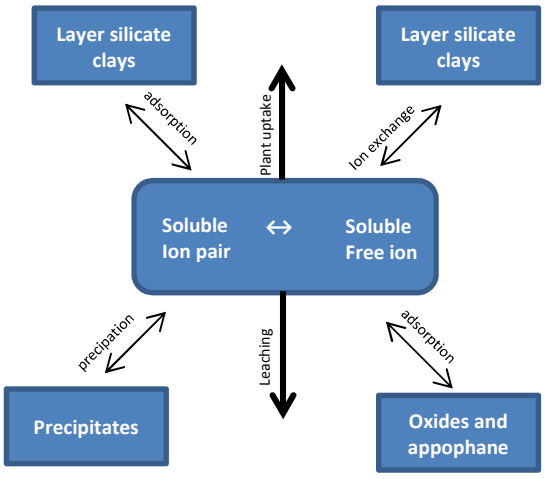


Figure 1. Dynamic interactive processes governing solubility, availability and mobility of elements in soil (modified by McBride, 1994)

Cadmium

Cadmium (Cd), atomic number 48, is in Group IIb of the Periodic Table of Elements. It is commonly associated in natural geological settings with Zn and Hg, the other elements of the Group IIb, with which it has strong chemical similarity. Cd(II) is the most common valence of Cd in natural environments and the only valence of Cd in aquatic system (Baes and Mesmer, 1976). Elemental Cd is a white, lustrous and tarnishable metal. It is relatively volatile with melting and boiling points of 321 and 767 °C, respectively and a heat of vaporisation of 26.8 kcal mol^{-1}. This latter property makes it susceptible to atmospheric transport which is the a major component of the global cycle of Cd (Laws, 1993).

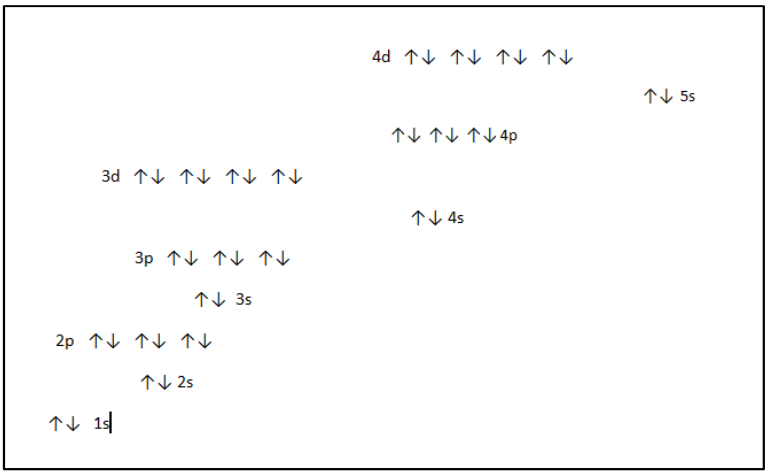


Figure 2. Electronic structure of cadmium

The absence of multiple valence and the lack of any Cd compound in which the outer d shell is in any state other than full prevent Cd from being included in the group of transition elements.

The Pauling radius of Cd (97 pm) is similar to that of Ca (109 pm). This similarity, along with its preference for six-fold coordination, facilitates the substitution of Cd into specific *Ca sites* in phosphate minerals.

The tendency of Cd to form stable solution complexes with organic and inorganic ligands can be estimated from the *Hard-Soft Lewis Acid-Lewis Base* principle. According to this principle Cd, that is a soft Lewis acid with a Misono softness parameter (Sposito, 1984) value of 3.04, in solution forms strong complex ions with S^{2-}, HS^{-}, the halide ions and organic sulphides and thiols that are soft Lewis bases. The latter complexes are responsible for much of the biological activity of Cd as discussed in this thesis. Cd forms comparatively weak complexes with hydroxyl ions and forms soluble solution complexes with borderline Lewis basis such as amines, imidazoles, bromide and

chloride and soluble complexes with hard bases such as sulphate, nitrate, carboxyls and organic hydroxyls. Altough considered to be hard Lewis bases, HPO_4^{2-} and CO_3^{2-} also form strong solution complexes with Cd.

Natural sources of Cd in the soil

Cadmium is a trace element in the lithosphere, with an estimated average content of 0.2 mg kg^{-1} (Lindsay, 1979). The highest Cd concentrations are found in sedimentary rocks. Cadmium ores are rare; it typically occurs in association with the Zn ore sphalerite, and is recovered as a by-product of Zn mining. Specific natural Cd solids of interest are greenockite and hawleyite, both diamorphs of CdS, cadmoselite (CdSe), monteponite (CdO), otavite (CdCO3), and Cd inclusions in natural apatite ores. The presence of Cd in phosphate ores is of particular interest due to their potential as Cd-sources for agricultural soils.

Natural geochemical processes have been known to concentrate Cd in surface soils. Also when introduced in soil as result of cycling through vegetation or external applications, either through agricultural or industrial activities, Cd tends to concentrate in the topsoil, the layer richest in organic matter to which Cd ions are adsorbed. In the long term, similarly to Zn, it moves downwards, concentrating in the lowest horizons of the soil profile (Alloway & Steinnes, 1999). Horizontally, Cd movement in the soil matrix is suggested to be dependent on the transpiration-driven mass flow of the soil solution, taking into account the low diffusion coefficient for Cd^{2+} in aqueous solutions (Sterckeman et al., 2004). This is also consistent with reports that Cd accumulation by plants grown in soil is directly related to the transpiration rate (Ingwersen & Streck, 2005).

Cadmium level may be high in poorly drained soils or in soils of arid and semiarid climates. This has created a problem in some irrigated farming regions where the climate is too dry for leaching to deplete the naturally high levels of Cd in the soil (McBride, 1994).

In most soils, more than 99% of the Cd content is associated with the solid phase and less than 1% is found in the soil solution. Per hectare of agricultural land, the fluxes of Cd in plants and in leachate are of the order of a few grams per hectare annually and the pool of Cd in the soil water of the root zone is also of the order of a few grams per hectare. The retention times for Cd in the upper soil layers are of the order of hundreds of years.

Cadmium may be present in soils as chemical precipitates in various forms of Cd minerals or as associations with other soil components. Sulphide (pKs=27.9), carbonate (pKs=12.1), hydroxide (pKs=13.7) and phosphate (pKs=32.6) are solid chemical species present in soils.

Mineral precipitation does not control the soil solution concentration of Cd (Christensen and Haung, 1999). The relationship between adsorption, the processes that may bind Cd to the solid phases of soil, and desorption, the processes that release the metal from the solid phase into the soil solution, determines the distribution of Cd between the solid phases of the soil and the soil solution.

Metal oxides possessing surface metal OH groups (e.g. oxides of Fe and Mn), layer silicates, calcite and hydroxyapatite, organic matter and biological colloids play roles in Cd adsorption on the solid phases of soils. The metal-organic complex-fraction of Cd is most abundant in surface soils and seems to play a vital role in influencing the labile pool of soil Cd. The distribution between soil solid phase Cd and soil solution Cd is controlled by many factors such as pH, redox potential, cationic composition, competing heavy metals, presence of dissolved organic and inorganic ligands and the natural properties of soil components.

The actual effect of pH on soil adsorption of Cd depend on soil characteristsc and pH range, but undoubtedly pH is dominant to all other solution factors, increasing adsorption dramatically with increasing pH. Cd availability is inversely related to soil pH (Mengel et al., 2001; Tudoreanu & Phillips, 2004; Kirkham 2006), as increasing acidity causes the dissolution of hydroxides and their co-precipitated metals, causing, in turn, reduced Cd adsorption on colloids because of a decreased pH-dependent negative charge (Alloway & Steinnes, 1999).

The redox potential of the soil medium also has an important impact on Cd availability: in reductive conditions, in fact, Cd ions tend to precipitate in the form of insoluble salts, such as CdS, and thus are not available for plant uptake. On the contrary, in oxidative conditions, Cd is mainly present in the free ionic form Cd^{2+} or as a soluble salt in the soil solution and therefore likely to be taken up by plants.

Cations present in the soil solution at the mM level can affect Cd adsorption at a level comparable to the effect of pH, but since Ca solution concentration usually does not vary among soils as dramatically as pH, the overall effect of Ca is somewhat lower. Magnesium, potassium and sodium also affect Cd distribution, but in comparison with Ca, with a less competitive strength (Temminghof et al., 1995). Other metals present in the soil solution may compete with Cd for adsorption sites. This has been demonstrated for Zn, Ni, Pb, Co and Cu. Since in soil solution the

concentration of Zn is usually more than a hundred times higher than that of Cd, apart from the fact that Zn is chemically similar to Cd, this makes Zn the most important trace metal competitor for Cd adsorption sites in many soils (Christensen, 1987).

The presence of inorganic as well as organic ligands in the soil solution may decrease soil adsorption by the formation of dissolved Cd complexes. Among inorganic ligands chloride has received particular attention. The ability of chloride to enhance the concentration of Cd in soil solution has been demonstrated in field experiments (McLaughlin et al., 1996). The effects of sulphate on Cd adsorption are less clear and will depend on the sorbing surface present in the soil (Naidu, 1994). Cd can also be present in soil bound to a broad range of organic compounds resulting in various organic complexes (Tudoreanu & Phillips, 2004). Natural low-molecular-weight-organic-acid (LMWOAs) originating from root exudates, canopy drip, oxidative decay of plant and animal matter and microbial activity are present in the soil solution at concentrations of 10^{-3} - 10^{-4} M. Higher concentrations are expected in the rhizosphere of plants. Desorption is related to the stability of the constant of the Cd-LMWOAs complex (Krishnamurti et al., 1994).

From an environmental perspective it is very important to know whether the adsorption of Cd onto soil is fully reversible or if a certain fraction of the adsorbed Cd is bound so strongly that, within naturally occurring conditions, it will never return in solution. Not all the Cd present in a soil turns out to be extractable by the extractants normally utilised, indicating the existence of unavailable fractions of Cd in soils.

Anthropogenic additions of Cd to soils

Cadmium concentrations in soils range from low values for natural or uncontained materials to high values for localised sites receiving historically large quantities of the metal through agricultural or industrial activities.

According to Kabata-Pendias and Pendias (1992) the average concentration of Cd in soils not exposed to obvious source of pollution varies between 0.06 to 1.1 mg kg^{-1} with a minimum of 0.01 mg kg^{-1} and a maximum of 2.7 mg kg^{-1}.

According to the FOREGS geochemical database for European soils (http://eusoils.jrc.ec.europa.eu/foregshmc/) the level of total Cd concentration in European agricultural soils ranges between 0.06-0.60 mg kg^{-1}. The higher Cd concentrations are found in the North, however the values are always below the most limiting threshold of 1.0 mg kg^{-1}

In addition to the geochemical composition of the parental material, agricultural soils will have received inputs from anthropogenic sources including (Alloway and Steinnes, 1999): a) atmospheric emission (transported in air and deposited on soil and vegetation) due to metalliferous mining and smelting, metal-using industries, phosphatidic fertilizer manufacture, general urban/industrial emissions; incineration of municipal solid waste, coal combustion and road dust; b) direct placement by the application of phosphatidic fertilizers, phosphogypsum and other by-products of gypsum, sewage sludge, composted municipal solid waste and residual ashes of combustion of coal or wood; c) accidental/fugitive contamination.

The normal concentration of Cd in air is in the range 0.1-4 ng m^{-3} in rural areas and 2-150 ng m^{-3} in urban/industrial areas (OECD, 1994). The main forms of Cd in aerosol-size suspended particles are the oxide, sulphide, sulfate and chloride. The sources of Cd emission to the atmosphere are: industrial processes, production of iron and steel, fossil fuel combustion, waste incineration, wear of tread on motor vehicles' tyres and natural sources including forest fires and volcanic activities. In some of these processes Cd is associated with the small-size aerosol fraction: thus a considerable part of the emitted Cd is subject to long range atmospheric transport and may thereby contaminate territories very far from the emission sources.

Recently, model simulation developed by the project UE 6th FP "ESPREME" (coordinated by the University of Stuttgart) showed that in the EU and in the North African regions the input of Cd to the soils from the atmosphere ranging from 0.005-more than 0.2 kg km^{-2} y^{-1} http://espreme.ier.uni.stuttgat.de/More in detail, the deposition in the North of Tunisia is in the range 0.01-0.04 kg km^{-2} y^{-1} (Figure 3).

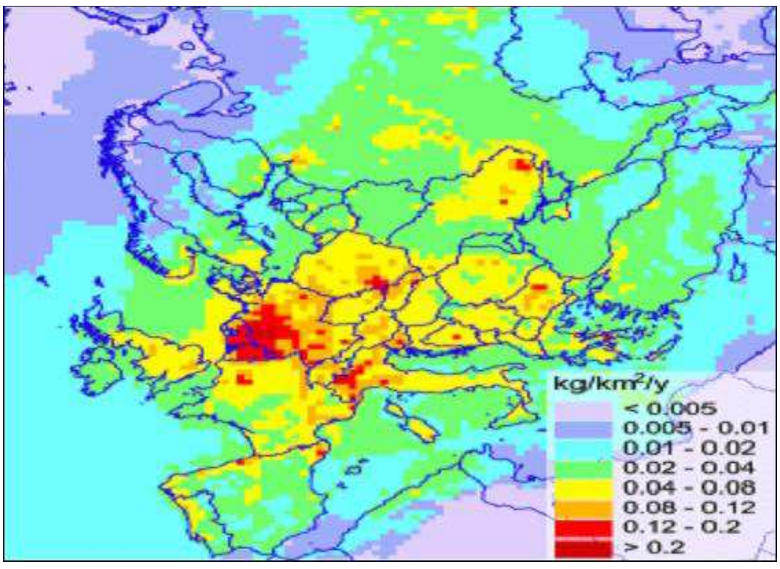


Figure 3. Deposition of Cd (http://espreme.ier.uni.stuttgat.de/)

Mines are sources of both ionic forms, the particulate and the soluble, of metal. The soluble forms tend to drain into watercourses and/or groundwater, potentially causing indirect soil pollution if these waters are utilised for irrigation or when a flood occurs. The particles of Cd contained in waste from mines are relatively large and are not transported over long distances over 4 km from the source, and the largest amounts are deposited within 500 m of tailings heaps (Alloway and Steinnes, 1999).

Phosphatic fertilisers can contain up to 300 mg kg^{-1} of Cd and thus they are the most ubiquitous sources of Cd contamination in agricultural soils, contributing to an input of the metal estimated to be in the range 1-9 g ha^{-1} y^{-1} (Alloway and Steinnes, 1999). The amount of the input depends on the sources of rock phosphate used for fertilizer manufacture and the amount applied. Phosphate rocks tend to contain relatively high amounts of Cd due to the natural isomorph substitution of Cd^{2+} for Ca^{2+} in the apatite crystal. Phosphorite deposits around the world vary markedly in Cd concentrations (Alloway and Steinnes, 1999). The lowest values are reported for deposits in the Russian Kola peninsula (0.3 mg kg^{-1}) and the highest for the deposits of the Pacific island of Nuru (243 mg kg^{-1}). Deposits in North Africa (Gafsa in Tunisa, Boucra and Youssoufia in Morocco) show high contents , (> 100 mg kg^{-1}). The current average Cd content in phosphate fertilizers used in European countries is ca. 35 mg Cd kg^{-1} P_2O_5 or 79 mg Cd kg^{-1} P_2O_5 (EU Risk Assessment Report, 2007). Since phosphate ores are a non-renewable resource, in the near future it is not unlikely that the increased use of low-grade phosphate rocks for fertilizer production could cause progressive accumulation of Cd in soils. In response to the recognition of the problem due to the input of Cd into the agricultural soil with phosphatic fertilizers, many countries suggest limiting values (from 20 to 75 mg kg^{-1} P_2O_5 in Europe) for cadmium concentration in these agrochemicals. The EU Commission is evaluating the introduction of a general threshold limit at 20 mg kg^{-1} P_2O_5.

Phosphogypsum is a by-product from the manufacture of phosphoric acid from phosphorites. This phosphoric acid is used for the manufacture of P fertilizers such as monoammonium phosphate (MAP) and diammonium phosphate (DAP). Large quantities of phosphogypsum are produced annually and the problems of its disposal find a solution in agriculture as it can be used as a P, Ca and S source and in the reclamation of both acid and sodic soils applied at rates of up to 30 t ha^{-1}. It has been reported that, depending on the quality of the phosphorites utilised, phosphogypsum can contain 3-4 mg Cd kg^{-1}. It means that in the case of an average application of phosphogypsum of about 20 t ha^{-1} of, inputs of 3-4 g ha^{-1} Cd are expected.

Cadmium input to agricultural soils from sewage sludge, the solid residue formed during the treatment of domestic/municipal waste water, is estimated to range from 30-40 g ha^{-1} y^{-1} . As a result of the highly varied range of possible sources and contaminants in waste water, sewage sludges vary considerably in their Cd content. In order to reduce the risks of Cd accumulation in agricultural soils many countries have fixed statutory permissible limits for Cd in sewage sludge and other organic waste products used for land application.

Industrial plants (electroplating; Cd-containing pigments; chemical plants; plastic manufacture; Ni-Cd battery manufacture; novel semiconductor manufacture; non-ferrous metal smelters) using Cd or Cd-containing materials are likely to be contaminated with the metal. Spillages of the soluble form of Cd onto soil and then into underground water could cause serious contamination.

Cadmium exposure and risks for human health

Cd represents one of the most potentially toxic substances for human health (ATSDR, 2008; Nordberg, 2009). Since once it has entered, Cd is strongly retained in the human body (t ½= 10–30 years), low-level chronic exposure to this metal results in its cumulative increase in the body with increasing years.

Research carried out on workers occupationally exposed to Cd suggest that the metal should be classed in Group 1 of carcinogenic substances (Nordberg, 2009). Moreover, epidemiological studies published in the last ten years have found associations between the almost ubiquitous low-level Cd exposure and serious health risks such as renal dysfunction, osteoporosis and cardiovascular diseases (Kobayashi et al., 2002; Bhattacharyya, 2009; Järup and Akesson, 2009; Nawrot et al., 2010; Satarug et al., 2010).

Other than due to occupational exposure, Cd can enter into the human body by direct inhalation and by the consumption of contaminated foods and/or beverages. It has been evaluated that, with the exception of smokers who inhale the metal present in tobacco leaves (Lugon-Moulin et al., 2006), food accounts for about 90% of Cd exposure (FAO/WHO, 2001; UNEP, 2008).

The first documented cases of disease due to Cd exposure through food arise from studies carried out on the so called *Itai-Itai* syndrome in Japan. It was established that this bone disease was caused by the consumption of rice contaminated with Cd as a result of uncontrolled Cd discharge into the Jenzuin River basin of the Toyama Prefecture (Kobayashi et al., 2002 and 2009).

Recently experts deeply involved in Cd research as biologists, biochemists, physiologists, physicians and epidemiologists, have revisited and extended various facets of Cd toxicity for humans (Moulis and Thévenod, 2010 and within articles). They focused on data which have been obtained with cutting-edge, wide ranging methods and they analysed them with contemporary biological concepts. As a result, recurring actual topics of Cd toxicity and new fields of investigation have emerged which forecast previously unsuspected health hazards of Cd and, moreover, it is increasingly clear that far more people could be affected by Cd exposure than previously thought and that Cd toxicity appears to increase mortality in a linear fashion without a measurable threshold.

On the basis of the last statement the European Food and Safety Authority (EFSA) and the US Agency for Toxic Substances and Disease Registry (ATSDR) have revised the provisional tolerable weekly intakes (PTWI) of Cd. In particular, the EFSA members of the Panel on Contaminants in the Food Chain invited the appropriate authorities to reduce from 7 to 2.5 μg kg^{-1} body weight the PTWI limit (EFSA, 2009). This value is quite close to the average PTWI for the European adult population, whereas it is below those of many populations worldwide as well as those of some European subgroups such as children and vegetarians (EFSA, 2011; Clemens, 2013). From a general point of view, since no clear margin of safety between the point of departure for adverse effects of Cd on health and the exposure levels of the population exists, environmental exposure to Cd should be reduced. In order to prevent risks for human health both the EU and the Codex Alimentarius Commission of the Food and Agriculture Organization/ World Health Organization set the official maximum allowable limits of Cd concentration for certain contaminants in foodstuffs (COMMISSION REGULATION-EC No 629/2008, 2008; CODEX STAN 193–1995; 2009).

Cadmium intake via food is a function of the Cd concentrations in the food and the amount consumed. Often it is not the food with the highest Cd levels, but foods that are consumed in larger quantities that have the greatest impact on cadmium dietary exposure. In Europe this was true as the broad food categories of grains and grain products (26.9%), vegetables and vegetable products (16.0%) and starchy roots and tubers (13.2%) were identified as major contributors (EFSA, 2012). These results are in accordance with previous studies in other regions of the world (UNEP, 2008; FAO/WHO, 2010). Thus, most of the chronic Cd exposure is a direct result of the intake of plant-derived food containing the element as a consequence of its uptake and accumulation in the plants grown on contaminated soils.

The mean values for Cd concetrations in cereal products, vegetables and potatoes in Europe are 0.023, 0.067 and 0.021 mg kg^{-1}, respectively, but depending on the level of pollution, much higher values can be reached (Mclaughlin et al., 1999; ATDSR, 2008); for example for cereal products and potatoes in Europe, values of 0.22 and 0.14 mg kg^{-1}, respectively, have been reported (EFSA 2012).

Techniques and strategies for reducing Cd content in plant-derived food

Legislation and technological improvements have already significantly reduced Cd emissions in the atmosphere and the risks of further water and soil pollution due to anthropogenic activities. Other important preventive measures include the decline in the transfer of Cd from the environment to the food chain. They involve reduction of the availability of soil Cd to plants and/or the introduction into the crops of traits able to exclude Cd from the edible plant organs.

Concerning soil management as a possible intervention to reduce the likelihood of Cd accumulation by crops, a number of strategies are available to minimize the effects of contamination. With this aim, liming is often suggested as a primary management tool. Beneficial effects of liming in reducing plant Cd concentrations have been clearly demonstrated in glasshouse experiments, where Cd is added as metal salt in hydroponic conditions, or in the form of sewage sludge in soil trials (Street et al., 1978; Eriksson, 1989). More recently, the same positive results have been confirmed in field experiments, where a decrease in rice plants' Cd concentration was observed after lime application (Cattani et al., 2008). The effect of liming is due to the increase in soil pH which stabilizes Cd adsorbed to soil particles and also to the competition effects deriving from the increase in Ca ions in the soil solution, limiting Cd uptake by the root system. Moreover, it is well documented that lime produces a rise in cation adsorption capacity of soil (Bolan et al., 2003), as well as a precipitation of Cd as $CdCO_3$ (Holm et al. 2003), thus reducing its bioavailability.

The application of organic matter has been reported to have contrasting effects on Cd solubility and consequent uptake by plants. On one hand, the organic matter added increases the cation adsorption capacity of the soil, providing additional surfaces onto which Cd ions can be adsorbed and thus immobilized; on the other hand, it is also possible that low molecular fractions, such as hydrophilic phases, have a strong affinity towards forming soluble Cd complexes. Cd-dissolved Organic Carbon (DOC) complexes, in fact, are more labile in soil and can soon release weakly bound Cd (Grant et al., 1999).

Additions of Zn to soil have been shown to significantly reduce crop Cd concentrations (Cataldo et al., 1983; Abdel-Sabour et al., 1988; McKenna et al., 1993; Moraghan, 1993; Chaney &Ryan., 1994; Oliver et al., 1994; Choudhary et al., 1995). This effect has been demonstrated in both common and durum wheat and it is particularly evident under conditions of Zn deficiency (Oliver et al., 1994; Choudhary et al., 1995).

Another factor deeply affecting Cd bioavailability is the water regime of the soil. It has been shown that lowland conditions reduce the content of Cd in rice in comparison with upland conditons (Cattani et al., 2008). This is may be due to the redox potential, stable around -400 mV under flooded conditions and fluctuating between +300 and -400 mV under dry conditions; indeed, the low value of the redox potential of the soil in submersion causes the sulfate ions to reduce to sulfide ions that forms complexes with Cd ions, immobilizing them as CdS_2 insoluble salts (Cattani et al., 2008; Gimeno-Garcìa et al., 1996).

Soil dressing techniques have also been taken into consideration but they are often hard to implement because of their high cost and the difficulty in obtaining unpolluted soil. Similarly, electronic thermodynamic remediation and on-site soil washing/clean up techniques could be interesting in terms of efficiency but there are some factors to take into consideration, such as (Mulligan et al., 2001; Murakami et al., 2007; Makino et al., 2008): a) selection of chemicals that have high effectiveness but also low environmental impact in that they could result in destruction of the physicochemical properties of soils and in secondary pollution of soil and groundwater; b) development of an on-site washing and wastewater-treatment system; c) ensuring favorable post-washing soil fertility and plant growth; e) maintenance of the washing effect. Taken together, these observations underline how interventions on soil are not always feasible, nor cost-effective, thus do not solve the problem of Cd accumulation in plants grown especially on low Cd contaminated soils.

Phytoextraction has been proposed as a promising technique for decontaminating soil characterized by low levels of Cd pollution. It basically consists in a cost-effective, environmentally friendly green technology that utilizes the capacity of hyperaccumulator plants to extract heavy metals from soil (Pilon-Smits, 2005; Krämer, 2005; McGrath et al., 2006). Nevertheless, field trials or commercial operations that demonstrate successful phytoremediation of metals have been rather few so far (Robinson et al., 2006; Maxted et al., 2007). Only *Alyssum*, a hyperaccumulator species used for Ni phytoremediation has been developed into a commercial technology (Chaney et al., 2007). Therefore, most of the hyperaccumulators tested so far cannot be unequivocally

considered as commercially viable for phytoremediation (Robinson et al., 1998). However, among the Cd hyperaccumulators, *Solanum nigrum* L., *Populus* spp., *Salix 'calodendron'* and *Arabis paniculata* (Wei et al., 2005; French et al., 2006; Maxted et al., 2007), have been found to be valuable candidates for field conditions due to their potentially high biomass, which, along with accumulation capacity and growth rate are the main determinants of success of the phytoextraction process (Salt et al., 1998). Other plants commonly known to be able to accumulate high metal concentrations in the shoots, as *Thlaspi caerulescens* and *Brassica juncea*, might not be suitable for large-scale phytoextraction, the former for being easily infected by diseases whose development is favored by humid and warm weather conditions (McGrath et al., 2000), the latter for its slow growth and the difficulty of mechanical harvesting, which is also an issue for other hyperaccumulator plants (Ebbs et al., 1997; Ishikawa et al., 2006).

Natural variation in plant Cd accumulation

The identification and/or the constitution through traditional or biotechnological approaches of low Cd-accumulating elite genotypes within crops can be considered a medium-term challenge for limiting the risks of introducing dangerous amounts of Cd into the human food chain. The feasibility of this hypothesis is sustained by the demonstrated existence of a substantial natural variation in the uptake, distribution and accumulation of Cd in crop species (Guo et al., 1995; Grant et al., 1998; Cakmak et al., 2000), and in cultivars within species (Clarke et al., 2002; Dunbar et al., 2003; Grant et al., 2008; Uraguchi et al., 2009).

Nevertheless, data about interspecific variation in Cd accumulation are quite fragmentary and sometimes confused. Tobacco plants are considerated efficient accumulators of Cd into the leaves and for this reason in smokers the blood concentration of the metal is particularly high (Lugon-Moulin et al., 2004). Some plant families (e.g. Compositae) have a greater number of strongly accumulating species than others (Abe et al., 2008). Concerning Cd in plants, the existence of strong intraspecific variation is well known, also considering plant tissues and organs that contribute most to food-derived human environmental Cd exposure (Wang et al., 2007; Grant et al., 2008).

Examining 237 wheat varieties, Kubo et al. (2008) found a great variation among the genotypes in the range 0.01-0.07 mg kg^{-1}, whereas in Canadian trials (Grant et al. 2008; Gao et al., 2011), variability in the ranges 0.010-0.045 mg kg^{-1} and 0.06-0.145 mg kg^{-1} were detected for bread wheat and durum wheat grains, respectively. Higher levels and large variation were found in

near-isogenic lines of durum wheat differing in the root-to-shoot Cd translocation capacity (Harris et al., 2004). The major locus justifying the observed variation and named *Cdu1* has been located on chromosome 5B.

The variation in grain Cd concentrations was evaluated among 600 barley genotypes grown in the same field conditions to select Cd accumulation genotypes (Chen at al., 2007). The results showed the existence of a very large genotypic variation (0-1.21 mg kg^{-1}). To date no barley genetic element has been associated with the observed natural variation.

Large variations in Cd concentration have been reported for potato (McLaughlin et al., 1997; Öztürk et al., 2011) but to date no QTL explaining the observed variability has been identified in this species.

Since rice consumption is the major exposure to Cd in food, in recent years a large number of studies have been carried out in order to describe the natural variation existing among several genotypes of this species with the aim of identifying the genetic basis of the character. Several QTLs explaining variation in Cd accumulation in the grains of rice have been detected (Ishikawa et al., 2005; Kashiwagi et al., 2009; Ueno et al., 2009; Ishikawa et al., 2010; Tezuka et al., 2010) thus allowing the subsequent identification of the gene/s determining the character potentially useful for marker-assisted selection of low Cd accumulation genotypes.

Other than QTLs approaches, the identification of gene/s responsible of the low-Cd accumulating traits in plants can also be achieved by a wide genome association mapping approach, but also by a traditional gene candidate approach. The former case requires phenotyping of very large natural or mutant populations; latter takes advantage of the characterisation of divergent genotypes, allowing the scientists to identify the biochemical and/or physiological mechanisms underlying the observed difference. From these points of view a deep knowledge of the mechanisms involved in Cd transport and tolerance in plants is an important perspective for reducing accumulation of Cd in the edible organs of the plants.

Cadmium uptake and long distance transport in plants.

Plants' roots draw water from the surrounding soil, which results in a convective transfer of solutes toward the roots. Therefore, depending on how this flux of solutes matches with its uptake by the root from soil solution, solutes will either accumulate or be depleted in the rhizosphere. For those elements that occur at low concentrations, such as Cd or other trace elements, in the soil solution, mass flow replenishes only a portion of the actual flux taken up by plant roots. As a result

of this, these elements are expected to be depleted from the rhizosphere; in other words the concentration of the element in the soil solution of the rhizosphere is lower than the concentration of the element in the solution of the bulk soil. A direct consequence of this situation is a diffusion of the element towards the roots along the created gradient. A further consequence, according to the mass action law, is a shift in the various reaction equilibria involving it, in order to replenish the soil solution in the rhizosphere. For Cd that is present in the soil as an exchangeable cation, the shift consists in an enhanced desorption from soil reactive surfaces (Hinsinger, 2001).

Rhizosphere acidification take place due to: a) root respiration and the resulting build up of pCO_2; b) differential rates of uptake of cations and anions by plant roots; c) active release of protons from the roots; d) exudation of organic acids from the roots. All these processes result in severe pH changes in the rhizosphere which are directly involved in the dissolution of minerals such as carbonate, phosphate and metal oxides known for their role in the immobilisation of Cd in the soil. In addition to the effects on dissolution/precipitation equilibria of minerals, pH is also a key factor in sorption of Cd on the soil constituents. In conclusion, by altering soil pH in their rhizosphere, plants can affect the chemistry and bioavailability of Cd through a wide range of chemical processes.

Cadmium solubility in the rhizosphere is increased by root exudates (Zhu et al., 1999) such as organic acids (eg., citrate, malate) and non-protein amino acids (eg., the phytosiderophores or phytometallophores in cereals). Root exudates are believed to play an important role in the acquisition of heavy metals from the soil. Several studies have shown the ability of exudates to mobilize and/or bind heavy metals (Mench and Martin, 1991). Specifically, exudates of maize (*Zea mays* L.) roots were found to mobilize Cd (Han et al., 2006). Exudates of wheat cultivars (Cieśliǹski et al., 1998) and maize enhanced the solubility of Cd, with the extraction of Cd by the root exudates being similar to the order of Cd bioavailability of the three species when grown on soil (Mench and Martin, 1991).

Phytosiderophores (PS) facilitate not only Fe but also Zn soil solubilisation and uptake by graminaceous plants (Hopkins et al., 1998). Since Zn and Cd are both members of the group IIb of the periodic table, PS have also been suspected of mediating Cd uptake in grain crops that accumulate this metal. However, if a solubilisation effect of PS on soil Cd has been demonstrated, no clear evidence on the permeability through plasmamembranes of the Cd-PS complexes exists.

6.2. Uptake of cadmium by roots

Apart from aquatic plants, where the metal is also taken up by the shoot system (Ornes & Saiwan, 1993), Cd enters plant organisms through the roots. The driving force for Cd^{2+} absorption across the plasma membrane of root cells is generated by the electrochemical potential difference between the activity of Cd^{2+} in the cytosol and that in the root apoplasm (Kabata-Pendias, 2011). Over a broader concentration range for Cd the uptake kinetics of Cd can be clearly represented by the sum of a single Michaelis–Menten component plus a linear component; therefore, it can be described as biphasic uptake kinetics. Since Cd does not have a biological function within the organism, no specific transporters have developed through evolution and, probably, its uptake is mediated by transporters specific for bivalent micronutrients such as Ca^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} or Mn^{2+}. This is indirectly suggested by the inhibition that the presence of these elements in the rhizosphere solution exercises on Cd uptake and accumulation in plant roots (Cataldo et al., 1983; Costa & Morel, 1993, 1994; Lombi et al., 2001; Hart et al., 2002; Zhao et al., 2002; Berkelaar & Hale 2003; Han et al., 2006; Zhao et al., 2006). Thus, in plants and mammals, many of the transporters for divalent transition metals (for a review see Colangelo and Guerinot, 2006) have a Cd^{2+} uptake activity.

The ZIP (ZRT-*I*RT-like Protein) family of membrane transporters has been shown to play a role in the transport of Zn, Fe, Mn, Cu, and also for heavy metals such as Cd in *Arabidopsis thaliana* (Eide et al., 1996; Grotz et al., 1998; Connolly et al., 2002; Wintz et al., 2003; Lin et al., 2009), *Thlaspi caerulescens* (Pence et al., 2000), *Pisum sativum* (Cohen et al., 2004), *Hordeum vulgare* (Pedas et al., 2008, 2009) and *Oryza sativa* (Ishimaru et al., 2006; Nakanishi et al., 2006). Not all the members of the ZIP family (15 in *A. thaliana*) are involved in plasma membrane micronutrient uptake (Vert et al., 2009; Milner et al., 2013). ZIP family member may also play a significant role in how heavy metals, both essential and toxic, are taken up and translocated throughout the plant (Guerinot, 2000; Pence et al., 2000; Rogers et al., 2000).

Transporters of Natural resistance associated macrophage *p*roteins (Nramp) family (for a review see Nevo and Neson, 2006; Colangelo and Guerinot, 2006) have also been proved to take part in Cd transport in yeasts, (Supek et al., 1996; Liu et al., 1997; Chen et al., 1999), in *A. thaliana* (Thomine et al., 2000) and in *Oryza sativa* (Sasaki et al., 2012; Takahashi et al., 2011; Nakanishi et al., 2012) where the OsNramp5 member has recently been identified as the major Cd uptake transporter localized in the plasmalemma at the distal side of the exodermis and endodermis of the root cells (Sasaki et al., 2012). In the same study it was shown that an Os*nramp5* knockout

mutant and RNAi lines grown in soil show a strongly reduced Cd concentration of the straw and the grain, corresponding with a much lower short-term rate of rice root uptake for Cd.

In addition to the free ion form, Cd might also enter root cells as a Cd-chelate with plant-derived organic compounds, such as phytosiderophores or non-proteic aminoacids. However, to date no conclusive results concerning this aspect or more generally about the possible role played of Yellow-Stripe 1-Like proteins (YSL) in the uptake from the rhizosphere of metal-chelated compounds have been obtained (Curie et al., 2001; Colangelo & Guerinot 2006; Curie et al., 2009).

Due to the similarity to Ca, Cd is also taken into the cell through the commonly named "cation channels", such as depolarization-activated calcium channels (DACC), hyperpolarization activated calcium channels (HACC) and voltage-insensitive cation channels (VICC), all of which are relatively non-selective between cations (White & Broadley, 2003; White, 2005; DalCorso et al., 2008; Pedas et al., 2008; Verbruggen et al., 2009). It is important to note that this type of transport is particularly significant in the case of relatively low Cd concentrations, which is the most widespread condition in contaminated agricultural soils.

6.3. Cadmium xylem loading and root-to-shoot translocation

P_{1B}-type ATPases form a distinct evolutionary sub-family of P-type ATPases, transporting transition metals such as Cu, Zn, Cd, Pb and Co across membranes in a wide range of organisms, including plants (Williams and Mills, 2005; Arguello et al., 2007; Zorrig, 2011). Arabidopsis has eight P_{1B}-ATPases (AtHMA1–AtHMA8), which differ in their structure, function and regulation. They perform a variety of important physiological tasks relating to transition metal transport and homeostasis. There are eight, nine and ten members of P_{1B}-ATPase in *A. thaliana*, rice and barley, respectively (Williams and Mills, 2005). They are divided into two groups: zinc (Zn)/cadmium (Cd)/cobalt/lead (Pb) and copper (Cu)/silver transporters (Williams and Mills, 2005). AtHMA1 to AtHMA4 in *A.thaliana* and OsHMA1 to OsHMA3 in rice belong to the former group, while AtHMA5 to AtHMA8 and OsHMA4 to OsHMA9 belong to the latter group, although AtHMA1 has also been shown to transport Zn, Cu, and calcium (Axelsen and Palmgren, 2001; Williams and Mills, 2005; Seigneurin-Berny et al., 2006; Moreno et al., 2008; Kim et al., 2009). All members of HMAs in *A. thaliana* have been functionally characterized. In particular, AtHMA2 and AtHMA4 localized at the pericycle are partially redundant and responsible for the release of Zn into the xylem (xylem loading) as well as Cd (Hussain et al., 2004; Verret et al., 2004; Wong and Cobbett, 2009; Wong et al., 2009), while AtHMA3 localized at the tonoplast plays a role in the detoxification of

Zn/Cd/cobalt/Pb by mediating them into the vacuole (Morel et al., 2009; Chao et al., 2012). By contrast, only three out of nine P-type ATPase members have been functionally characterized in rice. In detail, OsHMA2 seems to be involved in the root-shoot translocation of Zn and Cd (Nocito et al., 2011; Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013). OsHMA3 is localized to the tono-plast of the root cells and is responsible for the sequestration of Cd into the vacuoles (Ueno et al., 2010; Miyadate et al., 2011). On the other hand, OsHMA9 was mainly expressed in vascular tissues, including the xylem and phloem (Lee et al., 2007) and its knockout lines accumulated more Zn, Cu, Pb, and Cd, suggesting its role in the efflux of these metals from the cells (Lee et al., 2007). Some members of P-type ATPase have also been identified in other plant species, including barley, wheat, *Thlaspi caerulescens* and *Arabidopsis halleri*. HvHMA1 from barley might be involved in mobilizing Zn and Cu during the stage of grain filling (Mikkelsen et al., 2012). HvHMA2 from barley and TaHMA2 from wheat showed similar functions to OsHMA2 in rice (Mills et al., 2012; Tan et al., 2013). AhHMA3 in *A. halleri*, a Zn hyperaccumulator, is probably involved in high Zn accumulation (Becher et al., 2004; Chiang et al., 2006).

The mass flux generated by the transpiration process is the driving force determining the movement of Cd along the xylem vessels up to the shoots (Senden et al., 1995). Nevertheless, in which form Cd is translocated to shoots and distributed is not absolutely clear. In *B. juncea,* by the use of K-edge XAS technique it has been possible conclude that in the root, Cd is coordinated with S with an internal atomic distance of 2.53 Å. However, the spectra obtained on xylem sap fits either oxygen or nitrogen coordination at a distance slightly shorter than 2.3 Å, which probably represents Cd coordinated with six ligands. Thus, whereas at root level Cd appears mostly bound to S-containing compounds, probably phytochelatins (see below) , the transport of Cd via the xylem occurs in a phytochelatin-independent manner (Salt et al., 1995), through coordination to molecules which are still to be identified.

The chemical shifts of the stable isotope ^{113}Cd evaluated by ^{113}Cd-NMR spectroscopy technique change as a function of the ligand coordinating the metal (Kostelnik & Bothner-By 1974). Thus, after analysing by this technique the xylem sap of the Zn- and Cd-hyperaccumulator *Arabidospsis halleri* after exposure to 1 µM Cd, Ueno et al. (2008) concluded that Cd in the xylem sap of this species is predominantly present in the free ionic form and only small amounts were complexed with citrate, malate and histidine. Nevertheless it is not possible to exclude that in plants more sensitive to Cd toxicity than *A. halleri* Cd have to be moved complexed with organic ligands.

Cadmium phloem transport

Phloem mediated Cd transport to seeds following xylem-mediated root-to-shoot translocation is critical for accumulation of the metal in the seeds and it is of particular concern in the case of cereals. It is known that phloem mediates nearly 100% of the Cd deposition into rice grains (Tanaka et al., 2007). Despite its importance, little is known about the molecular mechanism of phloem Cd transport in plants. Studies have provided evidence of the existence of genotypic variation in Cd transport through a panicle neck (Kato et al., 2010) and in particular an investigation using a non-invasive live imaging technique to trace the transport of ^{107}Cd in intact rice plants (Fujimaki et al., 2010) suggested the importance of shoot nodes for transfer of Cd from xylem to phloem. Recently, in rice, Uraguchi et al. (2011) have identified a gene named *OsLCT1* encoding a Cd-efflux transporter on the plasma membrane of cells in leaf blades and in node I (the uppermost node). In node I *OsLCT1* during the reproductive stages is mainly expressed in the diffuse vascular bundles which are connected to panicles. RNAi-mediated knock-down of OsLCT1 reduced phloem-mediated transport and in the grain, suggesting that the gene functions at the nodes in Cd transport into the grain (Uraguchi et al., 2011). The *Triticum aestivum* homolog low-affinity cation transporter 1 (*TaLCT1*) found in the wheat cDNA library enhances the intake of various cations, including Cd in yeast. However, no direct evidence of the involment of *TaLCT1* in Cd transport into the wheat grains exists.

Cadmium toxicity and tolerance in plants

Accumulation of Cd in plant tissues may cause a variety of toxicity symptoms ranging from chlorosis, wilting and growth reduction to cell death. Cadmium cellular toxicity may result from interactions with the carboxyl or thiol groups of proteins (Sanità di Toppi & Gabbrielli, 1999), genesis of free radicals inducing oxidative stress (Schützendübel & Polle, 2002) or interference with the regulation and functionality of calcium- dependent processes (Rivetta et al., 1997; Perfus-Barbeoch et al., 2002).

Roots experience Cd damage first. Cadmium inhibits later root formation, inducing disorders in division and abnormal enlargement of rhizodermids and cortical cell layers in the apical region. In *Allium cepa*, 24-h Cd treatment induces genotoxicity causing chromosome and mitotic aberrations (Seth et al., 2008) and damaging nucleoli (Liu et al., 1995). In rice the presence of the metal altered the synthesis of RNA in the roots and inhibited ribonuclease activity (Shah and

Dubey, 1995). Cd inactivates DNA mismatch repair in yeast and human cells (Jin et al., 2008) and the same mechanisms may act in plants.

In many species photosynthesis is inhibited after both long-term and short-term exposure. The metal damages the photosynthetic apparatus, in particular the light harvesting complex II photosystem and to a larger extent photosystem II, probably affecting the water splitting system at the level of the mangano-proteins (Krupa, 1988; Siedlecka and Baszynsky, 1993; Siedlecka and Kupa, 1999; DalCorso, 2008). A large number of studies have demostrated that Cd treatment reduces the total chlorophyll content as well as the chlorophyll a/b ratio whereas carotenoids are less affected (Krupa, 1987). The fine structure of chloroplasts degenerated in Cd-treated plants, as well as altering the content of phosphoatidylglycerol hexadecenoic fatty acid, components important for the oligomerization of the chlorophyll protein complex (Krupa et al., 1993).

Cd significantly reduces the normal H^+/K^+ exchange and the activity of plasma membrane ATPase (Obata et al., 1996; Astolfi et al., 2005; Nocito et al., 2008), and strongly affects (often by inhibiting) the activity of several enzymes, such as glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, malic enzyme, isocitrate dehydrogenase (Van Assche and Clijsters, 1990; Mattioni et al., 1997), Rubisco and carbonic anhydrase (Siedlecka et al., 1997).

Cd also reduced the absorption of nitrate and its transport from roots to shoots, by inhibiting the nitrate reductase activity in the shoots (Hernandez et al., 1996).

Cd also inhibits stomatal opening, probably in an indirect way by interfering with movements of $K^{+,}$ Ca^{2+} and abscisic acid in the guard cells (Barcelo´ et al., 1986; Barcelo´ and Poschenrieder, 1990). Moreover, Cd generally decreases water stress tolerance of plants, causing cell turgor loss overall and degradation of the xylem cells, thus reducing water transport (Barcelo et al., 1988). Due to interference with the processes of regulation of plant water parameters, cellular growth as well as whole plant growth is drastically inhibited by Cd: in bean plants exposed to Cd, for instance, leaf cell expansion growth and relative water content of primary leaves decreased by about 10%, probably because of an increase in the cross linking of pectins in the middle lamellae (Poschenrieder et al., 1989). Proline accumulation, as a consequence of water stress induced by Cd, has been observed (Schat et al., 1997)

Although Cd is not a Fenton-reactive metal and does not seem to be directly involved in the production of the reactive oxygen species (ROS), Cd causes oxidative stress in plant cells (Sandman and Boger, 1980; Sanità di Toppi and Gabrielli, 1999; Schützendübel & Polle, 2002). However, Cd ions can inhibit (and some times stimulate) the activity of several antioxidative enzymes such as

superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), peroxidases (POD) and glutathione reductase (GR). Varying responses to Cd-induced oxidative stress are probably related both to levels of Cd supplied and to the concentration of thiolic groups already present or induced by Cd treatment. Indeed, thiols possess strong antioxidative proper- ties, and they are consequently able to counteract oxidative stress (Pichorner et al., 1993).

As in animals, the mitochondrial electron transfer chain of plant cells is thought to be one of the major targets of Cd toxicity, and is the site of the most rapid Cd-induced ROS production (Heyo et al., 2008). Increased ROS production induces lipid peroxidation. It was recently shown that vitamin E (α-tocopherol, the main antioxidant in membranes) is crucial in the tolerance of *A. thaliana* to oxidative stress induced by Cd (Collin et al., 2008).

Plant responses to nonessential metals are thought to be triggered by the damage occurring as a consequence of excessive exposure. Signaling of Cd stress may occur through its impact on homeostasis of essential elements. Cd exposure seems to rapidly induce apparent Zn deficiency, maybe through binding to a Zn sensor protein (Weber et al., 2004, 2006; Roth et al., 2006).

Cd-induced increase in ROS production may act as a cellular signal triggering the stress response. Stress-responsive MAP kinases seem to be involved in transcriptional responses to Cd as they are activated possibly by ROS under Cd^{2+} excess. The observation that the Cd-induced MAPK activation was slower than upon Cu^{2+} treatment (Ye et al., 2004), that directly induces ROS accumulation by the Fenton reaction, supports the conclusion that oxidative injury by Cd is a secondary effect.

Similar to other abiotic stresses, different plant hormones and growth regulators may participate in the plant response to metal stress. Exposure to Cd induces increases in ethylene, jasmonate, ABA, and salicylic acid (DalCorso et al., 2008). However, since the growth of *A. thaliana* SA-deficient *nahG* and ethylene *etr1-1* and *ein2-1* mutants in the presence of Cd^{2+} does not significantly modify their Cd^{2+} tolerance, Weer et al. (2006) concluded that neither SA nor ethylene mediate the protective response to $Cd^{2+.}$

The most recurrent general mechanism for Cd detoxification in plants is the synthesis of specific low-molecular-weight chelators to avoid binding to physiologically important proteins and thus, to facilitate the transport of the metal into the vacuoles. Among the earliest responses of plants to Cd exposure, the accumulation of non protein-cysteine-rich peptides (Phytochelatins,

PCs) arising from the tripeptide glutathione (Glu-Cys-Gly; GSH) is the most extensively characterized (Cobbett & Goldsbrough, 2002).

The tripeptide GSH, is synthesized by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS). GSH, other than to Cd, can bind to several metals and metalloids and is also a key metabolite in cellular redox balance, which is usually one of the targets of metals toxicity. Increasing GSH synthesis is considered a means of increasing metal(loid) binding capacity as well as a way to increase cellular defense against oxidative stress. Since glutathione is the precursor of PC, overexpression of γ-ECS or GS usually also leads to higher rates of PC accumulation under metal exposure.

Glutathione conjugates (Cd-GS_2) are transported into the vacuoles of *Saccharomyces cerevisiae*, by the activity of the vacuolar transporter YCF1. The overexpression of *ScYCF1* in *A. thaliana* (Song et al., 2003) increased Cd accumulation and tolerance in transgenic plants. However, to date a clear homolog of YCF1 in plants has not been identified. However, there is clear evidence for the involvement of an ABC transporter of Cd–GS2 into vacuoles of plant cells (Mendoza-Cozatl, 2005).

PCs are a family of peptides with the general structure $(\gamma\text{-Glu-Cys})_n$-Gly where n = 2–11. Phytochelatins are synthesized nontranslationally from GSH in a transpeptidation reaction catalysed by the enzyme PC synthase (PCS; γ- Glu-Cys dipeptidyl transpeptidase; Rea et al., 2004) Synthesis of PCs is induced within minutes following exposure to different metals or metalloids; among these, Cd is the strongest inducer (Grill et al., 1987; Maitani et al., 1996). PCS is constitutively expressed, but requires post-translational activation by metals, of which Cd is the most effective activator. The formation of Cd–GS_2 thiolates, which act as high-affinity substrates for the enzyme, seems to be sufficient for its activation (Vataminiuk et al., 2000) PCs are found in all plants, some fungi and animals. It has been convincingly shown that massive PC production is accompanied by a coordinated transcriptional induction of activities involved in sulfate uptake (Nocito et al., 2002) and assimilation (Lee & Leustek, 1999), and in GSH biosynthesis (Schäfer et al., 1998; Xiang & Oliver, 1998; Saito, 2004). In these conditions, the need to maintain a balance between GSH biosynthesis and PCs production is suggested by the finding that transgenic plants of *Brassica juncea* overexpressing GSH synthetase or γ-glutamylcysteine synthetase were found to be more tolerant to Cd stress (Zhu et al., 1999a,b; Wawrzynski et al., 2006), whereas transgenic Arabidopsis lines overexpressing PCS were hypersensitive to Cd since these were probably

depleted in cell GSH pools and thus more susceptible to Cd-related oxidative stress (Lee et al., 2003; Li et al., 2004).

The chelation of Cd by PCs is followed by the transport of the PC–Cd complexes. In *S. pombe* it has been demonstrated that this activity is catalysed by the ABC transporter HMT1. Following a systematic deletion of vacuolar ABC transporters, two full-length ABC transporters mediating vacuolar PC uptake (MRP1/ABCC1 and MRP2/ABCC2) have been identified in *Arabidopsis thaliana.* (Mendoza-Cozatl et al., 2010)

PCS overexpression in *A. thaliana* decreased tolerance to Cd since, probably, it leads to the accumulation of γ-Glu-Cys and to a contemporaneous depletion of the GSH pool inducing a higher sensitiveness of the plant to oxidative stress induced by Cd itself (Lee et al., 2003). The interpretation is supported by observing how increasing both glutathione and PC synthesis in *A. thaliana* improved the tolerance to and accumulation of cadmium (Guo et al., 2008).

Altough PCs have long been considered molecules that mediate the transport of metals from the cytosol into vacuoles, unexpectedly they were detected in the phloem sap of *Brassica napus* (Mendoza-Cozatl et al., 2008). Furthermore, energy-dispersive X-ray microanalysis (EDXMA) in *A. thaliana* found significant levels of Cd and sulfur-Cd complexes in the cytoplasm of companion cells (Van Belleghem et al., 2007), suggesting that thiols mediate long-distance transport of Cd through the phloem. Since *PCS1* is highly expressed in *A. thaliana* companion cells (Mustroph et al., 2009) that are connected through highly permeable plasmodesmata to sieve elements, it cannot be excluded that Cd-PC, as well as Cd-GSH conjugates (Turgeon and Wolf, 2009), enter the phloem for further transport into sink tissues (e.g. seeds and roots) (Li et al., 2004; Chen at al., 2006; Li et al, 2006; Turgeon and Wolf, 2009); Since significant levels of GSH but not PCs are found in *Arabidopsis* seeds, it has been suggested that thiol-Cd detection in seeds (Vogel-Mikus, 2010) [42] may result from glutathione-Cd conjugates and that PC-Cd complexes loaded into the phloem are more likely to be sequestered in root (sink) vacuoles by the phytochelatin transporters ABCC1 and ABCC2. This model suggests that PCs may contribute to the movement of toxic metals out of the shoots where they could impair photosynthesis (Mendoza-Coztal et al., 2008; Van Belleghem et al., 2007) indeed such re-circulating mechanisms would limit the accumulation of metals in shoots.

Both plants and yeasts exposed to Cd accumulate low molecular weight (LMW) PC-Cd complexes, consisting of PCs and Cd, and high molecular weight (HMW) PC-Cd-S^{-2} complexes, containing additional acid labile sulfide (Murasugi et al., 1981; Speiser et al., 1992). Genetic and

biochemical analysis suggest that the production of the sulfide part of the HMW PC-Cd-S^{2-} complex involves the purine biosynthetic pathway (Speiser et al., 1992). The HMW complex, a CdS crystallite coated by PC peptides (Dameron et al., 1989), has higher Cd-binding capacity than LMW PC-Cd and Cd ions are less susceptible to acid displacement (Reese & Winge, 1988). The role of these two types of complexes in Cd detoxification has been elucidated in studies conducted by Ortiz and coworkers (1992, 1995). These findings allow us to hypothesize a Cd detoxification mechanism in yeast that is still widely accepted (and probably reflects what happens also in plants. According to it, the cellular uptake of Cd induces PC synthesis; the PCs produced chelate the free metal ions by forming the LMW complexes. These are then transported across the vacuolar membrane by HMT1 (*S. pombe*) or by ABCC2 (*A. thaliana*), where additional sulfur (S) in the form of sulfide is incorporated in the lumen of the vacuole to generate the HMW PC-Cd-S^{-2} complexes. In this model the LMW PC-Cd complex would function as a scavenger and carrier of cytoplasmic Cd, whereas the HMW PC-Cd-S^{-2} complexes would definitely function as storage of Cd, reducing its toxicity and thus increasing Cd tolerance of the organism (Ortiz et al., 1992, 1995). This role is consistent with the increased stability and metal-binding capacity of the HMW complex (Reese & Winge, 1988).

Carboxylic acids and amino acids such as citric, malic and histidine are potential ligands for heavy metals including Cd and so could play a role in tolerance and detoxification (Rauser, 1999; Clemens, 2001). However, strong evidence for a function in tolerance, such as a clear correlation between amount of acid produced and exposure to a metal, has not been produced to support a widespread role.

Aims of the research

Among plant-derived food, cereals are the major source for human Cd intake (Clemens et al., 2012). The Codex Alimentarius Commission of the Food and Agriculture Organization/World Health Organization fixed the official maximum allowable limits of Cd concentration as 0.1 mg kg^{-1} for the cereal grains, excluding wheat and polished rice for which they are fixed as 0.2 mg kg^{-1} and 0.4 mg kg^{-1}, respectively (CODEX STAN 193–1995 2009).

The opinion that the best cost-effective and efficient approaches to prevent Cd entrance into the human foodchain is to develop low Cd-accumulating cereal cultivars is largely shared (Chen et al., 2008; Grant et al., 2008; Clemens et al., 2013). A fundamental prerequisite to carry out this option consists in exploring the variability existing across cereal cultivars in excluding Cd from the aerial tissues and/or the grains and identifying potential processes and molecular components that underlie this faculty (Clemens et al., 2013). In the cases of rice Arao and Ae, 2003; He et al., 2006; Shi et al., 2009) and wheat (Harris et al., 2004; Kubo et al., 2008; Gao et al., 2011) substantial variation in grain Cd concentration and strong genotype influences were found. Recent progress in understanding the molecular mechanisms of Cd-accumulation in rice make realistic the development in a relatively short time of low Cd-accumulating rice cultivars (Uraguchi and Fujiwara, 2012; for a review see Clemens et al., 2013). Compared with rice and wheat, not nearly as much information exists for other major cereals, including barley.

Most plant species retain much of the Cd taken up within the roots, limiting Cd from spreading through the whole plant and thus preventing excessive Cd accumulation into the seeds (Jarvis, Jones & Hopper, 1976; Wagner, 1993; Lozano-Rodríguez et al., 1997; Puig and Peñarrubia, 2009; Verbruggen et al., 2009; Nocito et al., 2011). The processes and mechanisms involved in determining the root capacity to retain Cd ions are: i) their apoplastic adsorption (Nocito et al., 2011); ii) their transport across the epidermal and cortical root cell plasma membrane and tonoplast (Clemens 2006; Ueno et al., 2011); iii) their selective binding to phytochelatins (PCs) and subsequent sequestration of the Cd-PCs complexes into the vacuole (Rauser et al., 1995); iv) their loading in free or bound forms into the xylem (Colangelo and Guerinot, 2006). The efficiency of these processes may contribute to the natural variation in Cd distribution between roots and shoots observed in crop species, as only Cd ions escaping these trapping pathways may be potentially available to be translocated via the xylem in a root-to-shoot direction.

Among cereals, barley ranks fourth both in terms of the amounts yearly produced and the area cultivated. It is mainly utilized as livestock feed, as a malt source and as flour in several human foods. In recent years a correlation between the presence of barley in the diet and a

reduced risk of coronary hearth diseases (FDA, 2006) has been suggested. This finding has induced a significant and progressive increase in the demand for barley grain and flour in countries where their consumption was traditionally limited Altough some evaluations of genotypic differences in grain Cd accumulation in barley exist (Wu et al., 2003, 2007; Chen et al., 2007) very little information about the physiological basis of the observed variability is available.

The present work was carried out to: a) evaluate the variability existing in the principal Tunisian barley cultivars for Cd root retention capacity; b) identify the lowest and the highest root-to-shoot translocating genotypes, and verify whether their contrasting behaviour results in differential Cd grain content.

Aims of the research were:

a) to analyze six barley cultivars among the most cultivated in Tunisia for their tolerance to relatively high Cd concentrations and ability to limit the accumulation of the metal in shoot and grain;

b) to identify the molecular and physiological basis of the behavior of the two most divergent cultivars, i.e. the highest and the lowest Cd accumulator, in order to develop markers useful in the selection of low-Cd grain cultivars.

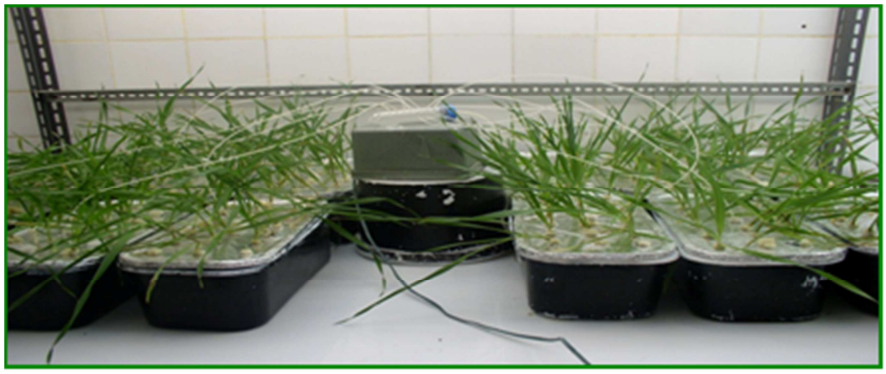
Materials and Methods

Plant material, growth conditions and sampling

Six varieties of barley (*Hordeum vulgare* L.) with six (Manel, Rihane, Martin, Souihli, Lemsi) or two rows (Roho) among the most cultivated in Tunisia, were obtained from the National Research Agronomic Institute of Tunisia,. Barley caryopses were sterilized by 20 min treatment with 0.5% calcium hypochlorite and, after a thorough washing in distilled water, were placed on a filter paper saturated with distilled water and then incubated in the dark at 20 °C. Seven days later, seedlings were transplanted into 5 L plastic tanks (8 seedlings per tank) containing the following complete aerated nutrient solution (pH 6.50): 1.5 mM $MgSO_4$, 1.6 mM KH_2PO_4, 0.4 mM K_2HPO_4, 3.0 mM KNO_3, 2.0 mM NH_4NO_3, 3.5 mM $Ca(NO)_3$, 62 µM Fe-EDTA or Fe-tartrate, 9 µM $MnCl_2$, 0.3 µM $CuSO_4$, 0.8 µM $ZnSO_4$, 46 µM H_3BO_3, 0.1 µM $(NH)_6\ Mo_7O_{24}$.

Seedlings were kept for 10 days in a growth chamber maintained at 26°C and 80% relative humidity during the 16-h light period and at 22°C and 70% relative humidity during the 8-h dark period. Photosynthetic photon flux density was about 400 µmol m^{-2} s^{-1}. At the end of this period (10 days) plants were treated with Cd supplementing the nutrient solution with different amounts of $CdCl_2$ to reach the final concentrations of: a) 25µM ; b) 10 µM; c) 0.01-10 µM. The treatment period was 30, 5 and 10 d for a, b c treatment, respectively, The hydroponic solutions in the case of treatment a were renewed twice weekly, whereas in the case of treatment b and c they were renewed daily to minimize nutrient depletion.

At the end of treatments, plants were harvested and roots were washed for 15 min in ice-cold 5 mM $CaCl_2$ solution to displace extracellular Cd (Rauser, 1987), rinsed in distilled water and gently blotted with paper towels. Shoots were separated from roots and the tissues were frozen in liquid N_2 and stored at -80 °C, or analyzed immediately.



Hydroponic culture in growth chamber under controlled conditions as described above of six investigated varieties (manel, Rihane, Martin, Souihli, Roho and Lemsi).

Plant growth on soil

Lemsi and Manel plants were grown to ripening in pots (25 cm in diameter and 30 cm in height; 4 plots per cultivar) filled with 3.5 kg of dried loamy soil collected in the neighbor of Milano in the North of Italy. Main soil properties (Astori, 1998) were showed in Table 9. Seeds (15 per plots; 4 pots per cultivar) were sown after watering the soil at 60% of its field capacity. Fifteen days after emergence the number of plant per plot was reduced to 5. Two fertilization (NPK) were carried out before sow and when plants were at the boot stage. Pots were maintained in an open and not thermo-regulated greenhouse at the Milan University experimental farm's in Tavazzano (111°55' E, 23°31' N), North Italy. Along the growth water was added to the soil twice weekly for maintaining it at about 60% of field capacity.

All plants were harvested at the maturity (grain water content about 15%). After air drying, grains were mechanically separated from the husks. Just before harvest blade leaves were collected, air dried and powdered by milling.

Determination of Cd in roots, shoots and grain

Samples of about 50 mg DW and 150 mg DW in the case of root and shoots, respectively, from hydroponics culture, about 150, 200 mg and 300 mg for husks, flag leaves and grain, respectively, from plants grown on soil were digested by a microwave digestion system (Anton Paar MULTIVAWE 3000) in Teflon tubes filled with 10 mL of 65% (v:v) HNO_3 by applying a two steps power ramp (step 1: 500 W in 10 min maintained for 5 min; step 2: 1200 W in 10 min, maintained for 15 min). After 20 min of cooling time, the mineralized samples were transferred in polypropylene test tubes. Samples were diluted 1 : 20 with MILLI-Q water and the concentration of Cd was measured by ion coupling plasma mass spectrometry (Bruker Aurora 80 ICP-MS). An aliquot of a 2 mg L^{-1} of an internal standard solution (45SC, 89Y, 159Tb) was added both to samples and calibration curve to give a final concentration of 20 mg L^{-1}. Eventual polyatomic interferences were removed by using CRI (Collision-Recation-Interface) with a H_2 flow of 45 mL min^{-1}.

Analysis of r*oot-to-shoot Cd translocation*

At the end of the treatment period, shoots were cut at 3 cm above the roots with a microtome blade. Xylem sap exuded from the lower cut surface was collected by trapping into a 1.5 mL plastic vial filled with a small piece of cotton for 2.0 h (Uraguchi et al., 2009). The amount

of collected sap was determined by weighing. The Cd concentration in the collected sap was measured by ICP-MS as described before.

Determinations of thiobarbituric acid-reactice substances

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reacting substances (TBARS) as described by Hodges et al. (1999). This modified protocol represents a rapid method for the assessment of lipid peroxidation in all plant species that contain interfering compounds like anthocyanin. Briefly: 100 mg of fresh samples (both root and shoot) were homogenized in 1 : 25 (g FW:mL) 80 : 20 (v:v) ethanol : water, followed by centrifugation at 3.000 *g* for 10 min. One mL aliquots of appropriately diluted sample was added to a test tube with 1 mL of either a) 1 20.0% (w:v) trichloroacetic acid and 0.01% butylated hydroxytoluene, or (2) the solution 1 added with 0.65% (w:v) Thiobarbituric acid (TBA). Samples were then mixed vigorously, heated at 95 °C in a block heater (Multiblok, Lab-Line Instruments, Ill., USA) for 25 min, cooled and then centrifuged at 3.000 *g* for 10 min. Absorbance was read (Ultraspec 3000, Pharmacia Biotech, Cambridge, UK) at 440 nm, 532 nm, and 600 nm. The results were reported as TBARS equivalents calculated as describe by Hodges et al. (1999).

Endopeptidases specific activity

Endopeptidase (EP; protease) activity determination was adapted from Brouquisse et al. (1998). One hundred microliters of clarified extract abtained as described by Brouquisse et al. (1998) and 100 µL of azocasein (5 mg mL^{-1} in 200 mM Mes-KOH, pH6.0) were incubated for 3h at 37°C. The reaction was stopped by addition of 100 µL 15% (v:v) trichloracetic acid. After 10 min on ice, the samples were centrifuged at 15.000 *g* for10 min. Then 250 µL of supernatant were added to 750 µL of 1 M NaOH, and the absorbance was estimated at 440 nm. The azocasein degradation activity was calculate with $\varepsilon_{1\%}$ azocasein in 1 M NaOH of 37 L cm^{-1} g^{-1}. Soluble fraction protein concentrations in the extracts were determined by the Bradford procedure using γ-globulin as the standard (Bradford, 1976).

Determination of thiols and GSH

Total nonprotein thiols (NPTs) were determined according to Nagalakshmi & Prasad (2001). Results were expressed as nanomoles of GSH equivalents. Briefly: 400mg of root powders were extracted in 600 mL of 1M NaOH and 1mg mL^{-1} $NaBH_4$, and the homogenate was centrifuged for

15 min at 13.000 *g* at 4 °C. Four hundred µL of supernatant were collected, 66 µL of 37% HCl was added and the solution was centrifuged again for 10 min at 13.000 *g* at 4 °C. For the quantification, volumes of 200 µL of the supernatant were collected and mixed with 800 µL of 1 M K-Pi buffer (pH7.5) containing or not 0.6 mM Ellman's reagent {[5,5'-dithiobis(2-nitrobenzoic acid); DTNB]}. The samples' absorbance at 412 nm were then spectrophotometrically measured The level of total GSH was determined according to Griffith (1980). All results were expressed as nanomoles of GSH equivalents.

The level of total GSH was enzymatically determined according to Griffith (1980). All results were expressed as nanomoles of GSH equivalents.

Determination of total sulfur and sulfate

Samples of about 300 mg dry weight were mineralized at 80°C in 5 mL of acid mixture (5:1) HNO_3:$HClO_3$. Later the residue was dissolved in 1 mL distilled H_2O and filtered on 0.45 µm nylon membrane. The total sulfur contents were then determined according to the turbidimetric method described by Tabatabai and Bremner (1970).

Frozen tissues were pulverized in a cold mortar with a pestle and then homogenized with ice-cold 0.1 N HNO_3 at the ratio of 2 mL to 1g tissue FW. After heating at 100°C for 40 min, the extracts were centrifuged for 10 min at 13.000 *g* and then filtered on 0.45 µm nylon membrane. The sulfate contents were then determined according to the turbidimetric method described by Tabatabai and Bremner (1970).

Determination of organic acids

Organic acids were extracted according to Rabboti and co-workers (1995). For each treatment, frozen tissues (about 2 g FW) were pulverized in a cold mortar with a pestle and then homogenized with ice-cold N_2-purged perchloric acid 10% (v:v) at the ratio of 1 ml of buffer to 1 g FW tissue and centrifuged for 15 min at 10.000 *g*. Supernatant pH was brought to 7.5 with 0.5 M K_2CO_3 to neutralize the acidity and to precipitate the perchlorate the extract was clarified by centrifugation at 15.000 *g* for 15 min and the last supernatant was filtered throughout a 0.22 µm Millipore filter. The xylem saps also were filtered with sterile filters 0.22 µm MILEX-GV (Millipore).

The determination of organic acids was performed with on a HPLC instrument (515 HPLC Pump, 2487 Dual Absorbance Detector and MILLENIUM 32 Workstation) using a Prevail organic acid column. Column effluents were monitored at 210 nm. Analyses were done in the isocratic

mode at 1 mL min-1 flow rate. The injected sample volume was 20 µL in the case of shoot, root extracts and xylem sap. A multilevel calibration method with daily prepared standard solutions was used for quantitative determination of the acids. Each sample was analyzed in triplicate. Regular recalibrations were carried out to obtain new response factors. The mobile phase was a buffer solution containing 25 mM KH_2PO_4 adjusted to pH 2.5 with H_3PO_4 and was filtered across a Nalgene nylon membrane filter (0.45-µm diameter) supplied by Nalge Company (Rochester, NY).

Enzyme assays

Root and shoot samples (about 1 g FW) were homogenized at 4°C in 2 mL of a buffer containing 50 mM 3-(N-morpholino) propanesulfonic acid-Bis-tris propane (MOPS-BTP) (pH 7.50), 3 mM ethylene glycol-tetraacetic acid (EGTA), 5mm DTT, 1 mm phenylmethylsulphonyl fluoride (PMSF) and 10 mg mL^{-1} leupeptin. The homogenate was filtered on Miracloth and then centrifuged at 13. supernatant was further centrifuged at 100000 g for 30 min and the new supernatant chromatographed through a Sephadex G-25 Fine column (1.0 cm diameter, 4 cm length; Amersham Bioscience, GE Healthcare Europe GmbH, München, Germany) equilibrated and eluted with the same buffer. The soluble extracted proteins were used for measuring the enzyme activities.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activity was determined according to De Nisi and Zocchi (2000). Malate dehydrogenase (MDH, EC 1.1.1.37) was determined in a buffer containing 50 mm Tris-HCl (pH 9.50), 0.1 mm NADH, and 0.4 mm oxaloacetate. For both the assays NADH oxidation was determined at 340 nm in a Cary-50 spectrophotometer (Varian) at 26°C.

Soluble protein concentrations in the extracts were determined by the Bradford procedure using γ-globulin as the standard (Bradford, 1976).

Fractioning of Cd in barley roots

Cadmium fractioning was carried out essentially as described by Rauser & Meuwly (1995). Briefly, frozen root tissues (about 6 g FW) were pulverized in a cold mortar with a pestle and then homogenized with ice-cold N_2-purged 100 mM Tris-HCl (pH 8.6), 1 mM PMSF and 1% (v:v) Tween 20 at the ratio of 1 ml of buffer to 1 g tissue FW. The homogenate was centrifuged at 4°C and 48.000 *g* for 6 min, the supernatant (extract 1) was collected and frozen immediately in liquid N_2, and the pellet was re-suspended in a volume of N_2-purged 10 mM Tris-HCl (pH 8.6) and 1% (v:v) Tween 20, previously used to rinse the mortar kept on ice. The suspension was centrifuged again,

the supernatant (extract 2) was collected and added to the extract 1 for freezing. Re-suspension and centrifugation of the homogenized tissue debris was repeated four more times to collect extracts 3-6. At the end of this sequence the pellet was suspended in a volume of ice-cold 100 mM HCl, centrifuged at 4°C and 48.000 *g* for 6 min and the supernatant (extract 7) was retained. This sequence was repeated two more times to obtain extracts 8 and 9. The exhausted pellet was transferred to a glass tube, mineralized at 120°C in 10 mL 14.4 M HNO_3, clarified with 3 ml 33% (v:v) H_2O^2, and finally dried at 80°C. The mineralized material was dissolved in 5 mL 0.1 M HNO_3 and filtered on a 0.45 µm nylon membrane. Extracts 1 to 6 were resolved into two fractions, referred to as anionic and cationic, by anion-exchange chromatography. Buffer extract was loaded, at 20 mL h^{-1}, onto a 0.5 x 3 cm column of DEAE-Sephadex A-25 equilibrated with 10 mM Tris-HCl (pH 8.6). After loading the column was washed with 25 mL of equilibrating buffer to remove unadsorbed solutes. All the fluid passing through the anion-exchanger was collected for Cd analysis (cationic fraction). Anionic material was eluted with 4 ml of 10 mM Hepes (pH 8.0) and 1 M KCl.)

The amount of Cd in mineralized pellets, extracts and column effluents was measured by ICP-MS.

Concentration-dependent kinetics of ^{113}Cd influx.

The enriched isotopes of ^{113}Cd (^{106}Cd, <0.04%; ^{108}Cd,<0.04%; ^{110}Cd, 0.12%; ^{111}Cd, 0.14%; ^{112}Cd,1.48%; ^{113}Cd, 95.83%; ^{114}Cd, 2.2%; ^{116}Cd, 0.2%) and ^{114}Cd (^{106}Cd, <0.01%; ^{108}Cd, <0.01%; ^{110}Cd, 0.08%; ^{111}Cd, 0.19%; ^{112}Cd, 0.4%; ^{113}Cd, 0.6%; ^{114}Cd, 98.55%; ^{116}Cd, 0.19%) used in the present study were purchased from Trace Sciences International Crop (Delaware, USA) in metallic form and dissolved in diluted HNO_3.

The procedure for evaluating symplastic Cd absorption in the roots, using enriched isotopes ^{113}Cd was carried out essentially as described by Mori et al. (2009). The roots of intact seedlings grown for 20 d in the control nutrient solutions were rinsed in ultrapure water for 2 min and then exposed to a 250 mL ^{113}Cd solution containing 0.4 mM$CaCl_2$ and 2 mM 2-morpholinoethanesulfonic acid monohydrate Tris (hydroxymethyl) aminomethane (MES–Tris) (pH 6.0) at 25°C for 15 or 30at seven different concentrations of Cd (0.01-10 µM). Each concentration was replicated four times. At the end of the incubation period te roots were quickly rinsed with the treatment solution without Cd, and then transferred to vessels containing ice-cold desorption solutions at 4°C for 20 min. The desorption solution was similar to incubation solution after

substituting ^{113}Cd with ^{114}Cd 25 µM. The excised roots were then rinsed in ultrapure water for 2 min. Harvested samples were dried in an oven at 75°C until dry. Roots were mineralized as described above and in the mineralized solution ^{113}Cd concentrations were determined by ICP-MS spectrometry

The ^{113}Cd influx isotherms were mathematically resolved using SigmaPlot (Chicago, IL, USA). The best fit for the equation ([Me], the concentration of Cd^{2+})

$$V_{\mathrm{Me}} = \frac{V_{\max}[Me]}{K_{\mathrm{m}} + [Me]} + a[Me]$$

that adds a linear component to the Michaelis–Menten model (Lasat et al., 1996; Cohen et al. 1998). The values of the kinetic parameters (V_{max}, K_m and a) were obtained matematichally form the resolution of the above equation.

Determination of photosynthetic parameters and leaf fluorescence

Measurements were made on attached, fully expanded leaves in the growth chamber with a portable gas exchange system (CIRAS-1, PP Systems, Herts, U.K.), using a PLC broad leaf cuvette in closed circuit mode. Measurements were made at the end of treatment.

Chlorophyll a fluorescence

Chlorophyll a fluorescence transients were determined on dark-adapted leaves kept for 30 min at growth chamber conditions (22°C and 70% RH), using a portable Handy PEA (Hansatech, UK). The measurements were taken on the leaf surface (4 mm diameter) exposed to an excitation light intensity (ultrabright red LEDs with a peak at 650 nm) of 3000 µmol $m^{-2}s^{-1}$ (600 W m^{-2}) emitted by three diodes. Leaf fluorescence detection was measured by fast-response PIN photodiode with RG9 long pass filter (Hansatech, technical manual). The parameters measured were F_o, F_m, F_v/F_m and P_I. The JIP test on the intermediate points of the fluorescence induction curves was carried out, and phenomenological and specific indexes were calculated (Strasser et al. 2000). Measurements were made at the end of treatment.

Semiquantitative RT-PCR analysis of HvHMA2 and HvHMA3

Total RNA was extracted from barley roots using Trizol Reagent (Invitrogen) and first-strand cDNA synthesis was carried out using SuperScript III first-strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (Promega), according to the manufacturer's instructions. The RNA amount was quantified by nanodrop (ND 2000C, Thermo scientific), and RNA integrity was electrophoretically verified by ethidium bromide staining and by OD260/OD280

nm, OD260/OD230 absorption ratio > 2.1. Hordeum vulgare RNa (1µg) was reverse transcribed with 50 U of Superscript II RT (Promega) using 100 µM random hexamer primers (Invitrogen Custom primers with certificate of analysis) according to the manufacturer's instructions.First-strand cDNA, deriving from about 150 ng of total RNA and obtained as described above, was used for the semiquantitative RT-PCR analysis of the transcripts of heavy metal P1B-ATPase 2 (HvHMA2) and 3 (HvHMA3). PCR reactions were carried out using Pfu DNA polymerase and the following couples of oligonucleotide primers:

•HvHMA2ATG (5'-GGCAAGAGATGGCCCTACACTG-3');

HvHMA2TAG (5'-GGCAAGAATGCTTACCTGCAGACG-3');

•HvHMA3ATG (5'-CGAGCCTTCTGTCCGACGAAGC-3');

HvHMA3TAG (5'-AGGACCTTCACAATACGGGACTGC-3').

The PCR primers used for HvHMA2 and HvHMA3 amplification are optimized to an equal annealing temperature of 66°C.Conditions of all PCRs were optimized in a gradient cycler (Master-cycler Gradient, Eppendorf, Germany) with regard to Taq DNA polymerase (Promega), forward and reverse primers, $MgCl_2$ concentrations (Promega) and various annealing temperatures (62-70°C). RT-PCR amplification products were separated on a 2% high resolution NuSieve agarose (FMC Bio Products, Rockland, ME) gel electrophoresis and analysed with the Image master system.

Statistical analysis

Statistical analysis was carried out using SigmaPlot for Windows version 12.0 (Systat Software, Inc., Chicago, IL, USA). Quantitative values are presented as mean ± standard error of the mean (SE). Data were subjected to one-way ANOVA analysis of variance between groups, and significant values were tested by post-hoc test using the Bonferroni correction for multiple comparisons. Statistical significance was at $P \leq 0.05$.

Section I

Results

Plant growth

After one month of treatment with 25 µM Cd no obvious symptoms of either necrosis or chlorosis were detectable in the shoots of any of the six barley cultivars. The visual observations were confirmed by chlorophyll analysis showing that the concentrations of both Chl_a and Chl_b in the shoots were unaffected by the presence of Cd in the nutrient solution (data not shown). However, the growth of the six cultivars was significantly modified by the treatment (Fig. 1). In detail, concerning the shoots: a) cv. Lemsi appeared to be the most sensitive variety with a value of TI (Tolerance Index - the average weight of shoot in treated group × 100 / the average weight of shoots in control group) which was very low at about 37%; b) cvs Roho, Martin and Souihli showed an intermediate sensitivity with TIs of about 63, 67 and 73% respectively; c) cvs Manel and Rihane were the most tolerant with TIs of about 82 and 85%, respectively. The growth of the roots was significantly reduced (about -37.5%) by the Cd treatment just in cv. Lemsi (Figure 1). Very similar behavior was observed by referring to plant fresh weight, since Cd exposure did not affect tissue water contents (data not shown).

Cd in roots and shoots

At the end of the treatment, among the six barley cultivars, very slight differences (just not more than 12% comparing Lemsi and Martin) were recorded considering the amount of total Cd accumulated on a per plant basis (Tab. 1). Most of the Cd taken up remained in the roots and only minor amounts were translocated to the shoots (Tab. 1). However, the percentage of Cd retained in the roots with respect to the total absorbed by the plant was variable among the six cultivars (Tab. 1): the lowest values were observed in cv. Lemsi (77.5%) and the maximum ones in cv. Manel (90.5%). As a consequence, although the total amount of Cd in the whole plants of these two cultivars was similar, the fraction of the metal translocated to the shoot in Lemsi was 2.5-fold higher than in Manel.

In the roots of Lemsi the concentration of Cd was higher than in all the other cultivars, in which it was statistically the same (Fig. 2). By contrast, wide differences were evident when considering the Cd concentration in the shoots. In detail, cvs Lemsi and Manel showed the highest and the lowest values, respectively: the value for Rihane was just slightly superior to that of Manel, and those of cvs Martin, Souihili and Roho were intermediate and very similar to each other.

Leaf-gas exchange and fluorescence parameters

Although limited to cvs Manel and Lemsi, the the most tolerant and the most sensitive cultivars to Cd stress, the leaf-gas exchange and leaf dark-adapted fluorescence parameters were evaluated at the end of the treatment. The results are reported in Figure 3 and in Table 2.

In detail, shoot of Manel and Lemsi plants exposed to 25 µM Cd exhibited (Fig. 3A) reduced net photosynthesis (P_n). The negative effect was slighter in Manel (-16%) than in Lemsi (-32%). At the end of the treatment, transpiration (E) was moderately enhanced (not more than +10%) in both the cultivars (Fig. 3B), whereas diffusive conductance (g_s) of leaves to carbon dioxide and water vapor transfer increased just in cv. Lemsi (Fig. 3C).

Data in Table 2 show that at the end of the Cd-treatment in both cvs Manel and Lemsi the values of fluorescence parameters of dark adapted leaves did not differ from those measured in the controls. Furthermore, the observed *Fv*/*Fm* values were in the range of the accepted norms (0.75–85) for healthy, non-stressed leaves (Bolhar-Nordenkampf and Oquist, 1993).

Metabolic responses to Cd stress

Although it is not a redox active metal, Cd indirectly produces reactive oxygen species and thus induces oxidative stress in plants. Since the levels of both TBARs and proteolytic activity in plant tissues are considered diagnostic indicators of the occurrence and of the severity of oxidative stress conditions (Hodges et al., 1999; Davies, 2001), these parameters were evaluated in both shoots and roots of the six barley cultivars at the end of the Cd exposure period. The results are reported in Table 3.

The Cd-treatment induced a marked enhancement in the levels of TBARs in the root of all the cultivars. The increases were not very different among the cultivars, ranging from a minimum of about 2.1 (Manel), to a maximum of 3.4-fold (Roho). The effect of Cd-treatment on TBARs level was even more evident in the shoots. Indeed, the increases in TBARs concentrations ranged from a minimum of 3.3-fold for Manel to a maximum of about 6.4-fold for Lemsi.

In the tissues of the Cd-treated plants the endopeptidase specific activity was enhanced with respect to that measured in the control plants (Tab. 3). Differently from the behavior described for TBARs, a marked cultivar-dependent increase of this activity was evident in the roots and, once again, the extreme values were observed for Manel and Lemsi: 1.3- and 3.5-fold increases, respectively. In the shoots of all the cultivars a generalized 2.0 fold increase in the endopeptidase specific activity resulted at the end of the Cd treatment (Table 3).

Since GSH and nonprotein thiols (NPTs) are involved in plant responses to Cd exposure their levels were evaluated in both roots and shoots of control and 25 µM Cd treated plants. Under Cd exposure the levels of the NTPs pool was dramatically enhanced in all the cultivars (Fig. 4). In detail, in the root (Fig. 4A) the effect was maximum for Manel (about 4.8-fold greater than control) and minimum for Lemsi (just about 1.8-fold greater than control). In the shoots (Fig. 4B) the increase was generally weaker than in the roots; however, the lowest value was once again observed in Lemsi (about 1.5-fold). In all the cultivars Cd induced a striking reduction in the levels of GSH both in the roots and in the shoots (Tab. 3). In the roots the effect was particularly evident for Lemsi (-50%) and was minimum for Manel (-27%), whereas in the shoots it ranged between the extreme value of -30% (Lemsi) to -18% (Martin).

Although limited to Manel and Lemsi, the effect of the Cd-treatment on both root and shoot content in the major organic acids were investigated. Malate and citrate were the most representative organic acids present; succinate, fumarate and 2-oxoglutarate were detectable too, but their concentrations were about one order of magnitude lower than those of malate and citrate. The accumulation of Cd caused a marked decrease in the levels of malate and citrate in the roots of both the cultivars (Tab. 4). Nevertheless, the effect was more evident in Lemsi (-73% and -50% for malate and citrate, respectively) than in Manel (-14%, -24% for malate and citrate, respectively). An opposite trend was recorded in the shoots of the treated plants where the concentrations of malate and citrate increased with respect to the controls. Once again the effect was more evident in Lemsi (+220% and +76%, for malate and citrate, respectively) than in Manel (+114% and +22% from malate and citrate, respectively).

Upon Cd treatment the most Cd-accumulating cultivar, Lemsi, showed slightly but significantly higher enzymatic activity in root extract for MDH (about +41%) and PEPC (+20%) when compared to the controls (Tab. 5). By contrast, in the less Cd-accumulating cultivar, Manel, no changes in the activity of both the enzymes in the roots of the treated plants were observed. In the leaves of both the cultivars the activities of the two enzymes were in no way affected by the Cd-treatment (Tab. 5).

Root-to-shoot translocation of Cd and organic acids

For each cultivar root-to-shoot Cd translocation was evaluated as the amount on a per plant basis of Cd ions loaded in 2 h into the xylem sap at the end of the treatment period. The greatest amount was recorded in Lemsi, the cultivar showing the lowest Cd root retention capacity; it was

about 5.0-fold higher that that observed in Manel, the cultivar with the maximal root retention capacity (Fig. 5A). With the exclusion of Manel (in which it was 2.2 mg L^{-1},) in all the other cultivars the Cd concentration in the xylem sap always exceeded that of the growth solution (2.8 mg L^{-1}) and in Lemsi it gave the highest concentration, i.e. 7.5 mg L^{-1} (Fig. 5B).

Considering all the six cultivars a close linear correlation (r^2 = 0.81) between the concentrations of Cd in the xylem sap and those measured in their shoots resulted (Fig. 6).

The amounts of organic acid loaded into the xylem sap in 2 h were determined in cvs Lemsi and Manel. The results summarized in Figure 6 showed that in the control, on a per plant basis, the amounts of malate, as well as that of citrate, loaded into the xylem and translocated were similar. Exposure to Cd induced significant enhancement of the amounts of malate and citrate present in the xylem of both the cultivars and the effect was more evident in Lemsi (+ 50% and +198% for malate and citrate, respectively) than in Manel (+49% and + 51% for malate and citrate, respectively).

Section I

Discussion

The aim of this first part of the research was to investigate the variability existing within six Tunisian barley cultivars in tolerating Cd and limiting its accumulation in the shoot. The physiological basis of the observed differences was also investigated.

Although a few studies have reported results on the possibility that low concentrations of Cd can stimulate plant growth (Ivanov et al., 2001; Wu and Zhang, 2002; Wu et al., 2003), usually the presence of the metal over safety thresholds in agricultural soils limits crop productivity worldwide as Cd tends to accumulate within plant organs and negatively interfere with essential physiological processes (Gill et al., 2012). The effects of Cd on parameters such as biomass production or root and shoot length are used as indicators of the toxicity induced by heavy metals in plants (Dias et al., 2012) and may be used to evaluate the variability existing among plant species and/or among genotypes within the same species in Cd tolerance or sensitivity. Such an approach was adopted by Chen et al. (2008) to identify barley genotypes tolerant to Cd by screening 105 varieties/lines from different backgrounds. In the present study, adopting as tolerance index the ratio between the dry weight of shoots in the treated group × 100 / the dry weight of shoots in the control group resulting after 30 days of growth in the presence of 25 µM Cd in the nutrient solution, six Tunisian barley cultivars were classified into three groups. In detail, the cultivar Manel was the most tolerant and the cultivar Lemsi the most sensitive (Fig. 1).

Among the worsening effects induced by Cd on plant metabolism the reduction in chlorophyll content of leaves and the consequent inhibition of photosynthesis have already been described (Han et al., 2006). Thus, the photosynthetic performances of plants growing in the presence of Cd may furnish useful criteria for evaluating the severity of the stress imposed by the metal (Kruppa et al., 1999). Indeed, the accumulation of Cd in the green tissues over tolerable thresholds induces chlorosis and damage to the PSII reaction center (Li et al., 2004). Cadmium induces chlorosis symptoms by inhibiting the biosynthesis of the chlorophylls, determining Fe and Mg deficiency (Vassilev et al., 2002), substituting Mg in the chlorophyll molecule and/or accelerating chlorophyll degradation due to the oxidative stress condition it can trigger in plant cells (Küpper et al., 1998). Regarding Cd toxicity to PSII activities, some studies suggest that the metal binds the complex both at the acceptor and the donor sites (Sigfridsson et al., 2004; Faller et al., 2005), which results in inhibition of photosynthetic oxygen evolution. Moreover, Cd at PSII level also blocks the electron transfer from redox-active tyrosine residues D1-161 (Wang et al., 2009).

At the end of the treatment, both in cv. Manel and Lemsi plants, neither evident chlorosis symptoms nor dramatic reduction in PSII activity were detectable with respect to the controls. The

treated plants seemed to develop an adaptive strategy consisting of a slowing of the growth rate (Fig. 1) due to a reduced net CO_2 fixation activity (P_n; Fig. 2A) and in somehow protecting the functionality of PSII. The last suggestion is supported by the very similar values recorded with regard to all the fluorescence parameters of dark adapted leaves in treated and control plants and in particular to the F_v/F_m ratio (Tab. 2). Indeed, this ratio would promptly decrease if Cd were to be interfering with PSII (Pagliano et al., 2004; Sigfridsson et al., 2004).

There are no definitive reports on the relationships between Cd stress and plant water relations since Cd can act in several ways on the parameters that affect leaf water potential (Poschenrieder and Barcelò, 2004). According to Barcelò et al. (1986) and Poschenrieder (1990), Cd can alter water relations by disturbing water balance through its effects on: a) stomatal conductance, probably interfering with movements of K^+, Ca^{2+}– and abscisic acid in the guard cells; b) water transport; c) elasticity of cell walls. Nevertheless, most of the investigations on the effects of Cd on plant water relations have adopted relatively short treatment periods, thus limiting the possibilities forobserving the longer term effects and noting the adaptive responses of plants. The results obtained here indicate that during the 30 d of treatment the plants of all the six barley cultivars, despite the relatively high Cd concentration in the nutrient solution, activated adaptive responses able to successfully counteract the potential detrimental effects of the metal on the water balance of plants. Indeed, at the end of the Cd-exposure, just for cv. Lemsi a very slight (-16%) reduction was detected comparing the value of relative water content (RWC) between treated and control plants. Finally, confining the observations to cvs Manel and Lemsi, after 30 d at 25 µM Cd , slightly higher (+ 11%) leaf transpiration activities were recorded in the treated plants of both the cultivars with respect to the relative controls (Fig. 3B) and in cv. Manel the stomatal conductance was reduced (Fig. 3C).

Plants adopt avoidance and/or tolerance strategies to counteract the excesses of Cd in the substrate in which they are growing (Sanità di Toppi and Gabrielli, 1999; Rauser, 1999). Avoidance involves mechanisms able to limit the uptake and the accumulation of the metal in the plant as a whole and particularly in the most sensitive tissues, such as meristems and leaves. Tolerance mainly consists in: a) maintaining at a low level the symplastic activity of Cd by binding it to specific chelators, mainly phytochelatins (Rauser, 1995; Zenk, 1996; Cobbett & Goldsbrough, 2002), and then accumulating the chelated forms of the metal into the vacuole (Clemens, 2006); b) lowering the cytoplasmic activity of the metal by its active transport into the vacuole as a free cation mediated by specific tonoplast-localized proteins belonging to the CAX (Hirschi et al., 2000)

and/or P_{1B}-type ATPase (Williams and Mills, 2005) families; c) up-regulating the antioxidant defence systems (Sanità di Toppi and Gabrielli, 1999) devoted to counteracting the dangerous ROS generated indirectly by the Cd presence in the cells (Vassilev et al., 2004).

The barley cultivars here analysed adopted both avoidance and tolerance strategies in order to face the imposed Cd-stress. Indeed, similarly to many plant species (Jarvis, Jones & Hopper 1976; Wagner 1993; Lozano-Rodríguez et al., 1997; Puig & Peñarrubia, 2009; Verbruggen, Hermans & Schat, 2009) they retain in the roots much of the Cd taken up(Fig. 2 and Tab. 1). The two varieties showing the highest (Manel) and the lowest (Lemsi) tolerance to the Cd treatment consistently showed the highest and the lowest Cd root retention capacity, respectively (Tab. 1). Since the two varieties did not differ in the total amount of Cd they accumulated on a whole plant basis in 30 d (Tab. 1), the higher root retention capacity shown by cv. Manel with respect to all the other cultivars (excluding Rihane), and in particular in comparison with cv. Lemsi, coherently explains its superior tolerance. Indeed, in the shoot of Manel the concentration of Cd was the lowest recorded among the six barley cultivars (Fig. 2), inducing in these tissues a weaker stress in comparison with the other five cultivars, and once again particularly with respect to Lemsi. This last conclusion is supported by the relatively low values of both TBARs concentrations and proteases activity recorded in cv. Manel (Tab. 3). Indeed, it is important to stress that both TBARs and proteolytic activity are diagnostic indicators of the occurrence and of the severity of oxidative stress conditions (Hodges et al., 1999; Davies, 2001). In particular, TBARs accumulation due to Cd-induced lipid peroxidation at leaf level has been often detected in rice (Chien et al., 2001; Shah et al. 2001), tomato (Ben Ammar et al. 2008; Chaffei et al. 2004), lettuce (Dias et al. 2012) and barley (Wu et al. 2003).

The highest tolerance to Cd showed here for the barley roots in comparison to the shoots, has been already reported in several studies on other species. For example, although Cd was mainly accumulated in the root growth was affected in shoot more than in root, in *Pinus sylvestris* (Kim et al.2003), tobacco (Martins et al. 2011) and rice (Uneo et al. 2011).

Several mechanisms may be involved in determining the root capacity to retain Cd ions. Once inside root cells, Cd ions are trapped by a 'firewall system' through selective binding sites with high affinity for the metal, or through transfer across a second membrane into an intracellular compartment (Clemens 2006). The varying efficiency of these processes may contribute to the variation in Cd distribution between roots and shoots observed among the six Tunisian barley cultivars considered in this study. In maize most of the total Cd retained by roots is bound in high-

molecular-weight (HMW) and low molecular-weight (LMW) complexes containing PCs and related thiol compounds, revealing these peptides as crucial for Cd root retention in cereals (Rauser and Meuwly 1995; Rauser, 2003). Recently (Akhter et al., 2012), it has been suggested that the strong difference observed between barley and lettuce in retaining Cd in the root (77 and 23% of the total amounts absorbed, respectively) is due to the higher accumulation of PCs in this organ induced by Cd in the former species with respect to the latter. Similarly, the high root retention capacity shown (Tab. 1) by cv. Manel (90.5%) and the low one shown by cv. Lemsi (77%), can be related to the marked NPTs accumulation (about 5.5-fold higher than in the control) recorded in the former cultivar in comparison with the relatively smaller accumulation (about 2.0-fold higher than in the control) detected in the latter.

As reported in maize (Nocito et al., 2002; 2006), in the six barley cultivars the level of GSH pools slightly but significantly decreased in the root of treated plants (Tab. 3), and assuming that under Cd-stress neither cysteine or γ-glutamyl-cysteine concentrations are expected to increase, the differences between the NTPs concentration in the treated plants and those of the relative controls (Fig. 4) is considered highly indicative of the root concentration of PCs-like compounds (Schat et al., 1997). The level of these PC-like compounds was higher in cv. Manel Cd treated plants compared with those observed in cv. Lemsi (figures inside Fig. 4).

Only Cd ions escaping the trapping pathways taking place in the root may be potentially available to be translocated via the xylem in a root-to-shoot direction. The concentrations of Cd measured in the xylem sap of the six Tunisian barley cultivars at the end of the treatment were markedly different and interestingly, the highest value was recorded in Lemsi and the lowest in Manel (Fig. 5B). Coherently, due to the similarity in their leaf water transpiration fluxes both in the control and in Cd-treated plants (Fig. 3B), the amount of the metal translocated toward the shoot was very much higher in Lemsi than in Manel (Fig. 5A). This conclusion explains the strong correlation resulting between the concentration of Cd in the xylem sap and the concentration reached by the metal in the shoots of the six barley cultivar at the end of the treatment (Fig. 6).

In which form Cd is translocated to shoots and distributed is not yet absolutely clear. Recent evidence (Mendoza-Cozatl et al., 2011) seems to exclude the role suggested by Gong et al. (2003) for GSH and/or low molecular weight PCs in Cd xylem translocation. Using ^{113}Cd-NMR and computer simulation approaches, Ueno et al. (2008) suggested that in rice Cd is translocated from the root to the shoot in the xylem solution mainly as the free cation and only a small fraction is present as malate and citrate salts. In cv. Manel and Lemsi plants exposed for 30 d at 25 µM Cd,

significant increases in the amount of malate and citrate ions translocated from the root to the shoot were observed (Fig. 8). For both the organic acids, the effect was more evident in cv. Lemsi, the cultivar with the highest root-to-shoot Cd translocation, than in Manel, the cultivar with the lowest root-to-shoot Cd translocation (Fig. 7). However, the concentrations of both malic and citric acids (never lower than 4 mM; Fig. 8) in the xylem sap are largely in excess compared to those of Cd (about 19.7 and 65.4 µM for Lemsi and Manel, respectively; Fig. 5B). Without excluding the possibility that the metal in the xylem is bound to those carboxylates, the increased translocation of both malate and citrate induced by Cd exposure could be due to the need of plants to support an influx of C from the root system to the shoots *via* xylem flow, as occurs in barley under Fe starvation (López-Millán et al., 2012). In other words, it can be speculated that the root acts as an anaplerotic source for carbon skeletons sustaining in the shoot the synthesis of PCs that in turn are necessary to prevent Cd interference with PSII activity. The hypothesis is strongly supported by the significant decrease of both malate and citrate levels observed in the roots of the treated plants (Fig. 7) and the concomitant increase in the level of these carboxylates in the xylem and in the shoots of the same plants (Fig. 7). Interestingly, the behaviour described is more evident in cv. Lemsi than in Manel and, moreover, according to the picture just drawn, in the root of Lemsi higher activities of enzymes related to the organic acid metabolism (i.e. PEPC and MDH) were recorded (Tab. 5). It is thus possible to suggest that the frequently reported relationships between the amount of malate and citrate and plant Cd-exposure, is due not only to their unspecific roles as Cd-ligands, but that the accumulation of these carboxylates is related to the activation of the anaplerotic metabolism leading to the synthesis of PCs (Nocito et al., 2008).

Section II

Results

Among the various efforts which are being aimed at developing strategies to limit the risk of introducing Cd into the human food chain, the identification and constitution of plant genotypes able to exclude the metal from the shoot or from the edible parts, seem to be the most promising lines of enquiry for the future (Clemens et al., 2013).

Plants readily absorb Cd from the soil, but the extent of its uptake and, first of all, the distribution pattern of the metal within plant tissues and organs varies very largely among different genotypes of the same species (Damber et al., 2003; Liu et al., 2007; Harris and Taylor, 2013). Wu et al. (2007), comparing Cd uptake and distribution in the plant between four cultivars of barley, found that root-to-shoot distribution, rather than root uptake, was the main factor affecting the observed differences in shoot Cd concentration. However, Harris and Taylor (2013) suggested that both total uptake and partitioning were important in explaining differences in the tissues' Cd concentration among the different wheat cultivars which they compared.

In the first part of this thesis, a screening test carried out by exposing six Tunisian barley cultivars to a rather high Cd concentration (25 µM) made possible the identification of a relatively low-Cd accumulator (Manel) and a relatively high-Cd accumulator (Lemsi) cultivar. The two cultivars differ in the amounts of metal that they translocate to the shoot as a consequence of their different root retention capacity.

The second part of the research is focused on: a) the confirmation of the differences existing between the two cultivars when exposed to a broad range of relatively low Cd concentrations (0.01-10 µM), simulating the actual Cd availability experienced by roots in moderately contaminated soils (Sauvè et al., 2000); b) a deeper analysis of the molecular and physiological mechanisms involved in establishing the observed differences.

Plant growth and Cd accumulation: short term exposure

Cv. Lemsi and Manel plants were grown for 10 days in a complete nutrient solution. After that, $CdCl_2$ was added to the nutrient solution at 10 µM, the highest concentration studied. Twenty-four, 48, 72 and 96 hours from the Cd addition, six plants for each treatment were sampled and their weight and Cd concentration in the roots, shoots and xylem saps were evaluated.

Neither Manel nor Lemsi plants grown 96 h in the presence of 10 µM Cd in the nutrient solution showed any visible symptoms of toxicity and neither the shoot nor the root growth was affected by the treatment (data not shown).

Figure 8 shows the time course of Cd accumulation in the root (A) and shoot (B) of Lemsi and Manel plants. In detail, in the roots of both the cultivars the concentration of Cd increased almost linearly during the 0-96 h period (Fig. 8A). However, already at 24 h from the start of the treatment, the metal concentration in the roots of Lemsi was higher (about +50%) than in those of Manel. The time-course of Cd concentration in the shoots was markedly different between the two cultivars (Fig. 8B). Indeed, in the case of Lemsi, already after 24 h the Cd concentration in the shoot was about 7-fold higher than in Manel. In the following 24-48 h period the concentration of the metal Cd increased linearly in the shoots of both the cultivars and thus the difference between them increased further. Then, in the last 48 h, in Lemsi the rate of Cd concentration increase slowed down, whereas in Manel it remained slow, but constant. It is interesting to stress that although at the end of the 96 h period the concentration of Cd in the roots was just +25% higher in Lemsi than in Manel, the concentration of the metal in the shoot of the former cultivar was about 6- fold higher than that measured in the latter.

The dynamic of root-to-shoot Cd translocation was examined by evaluating the amount of Cd translocated on a per plant basis in 2 h in the xylem sap of the two barley cultivars. As shown in Figure 9, the amount of Cd translocated by the xylem flux was always dramatically higher (4- to 5-fold) in Lemsi than in Manel. In both the cultivars the flux of Cd reached the maximum value after 48h from the beginning of the treatment and then tended to diminish.

Effect of low Cd concentrations on plant growth and photosynthetic parameters

In a further experiment, cv. Lemsi and Manel plants were exposed for 10 d to different Cd concentrations in the growing medium in the range 0.01-1 µM. In Manel none of the Cd concentrations tested affected the growth of both root and shoot with respect to the control (Fig. 10A and B). However, in Lemsi in the presence of 1 µM Cd in the nutrient solution, the DW of shoot and root were reduced compared to the control: by about 23% and 33%, respectively,.

As shown in Figure 11, the treatment conditions adopted did not affect in any way the net photosynthesis (*Pn*), the stomatal conductance (*gs*), the leaf water transpiration (E) and the functionality of PSII (*Fv/Fm*) both in Manel and Lemsi.

Cadmium accumulation in plants exposed to low Cd concentrations

The concentration of Cd in the roots of both Manel and Lemsi increased as Cd availability in the nutrient solutions did (Figure 12B). At the lowest Cd concentration tested (0.01 µM) the level of the metal in the roots of Lemsi was higher than in those of Manel. This difference vanished in the roots of the plants grown in the presence of the other two higher (0.1 and 1 µM) Cd concentrations tested. However, in the shoots the concentration of the metal was always higher in Lemsi than in Manel (Fig. 12 A): 2.6-, 2.0- and 1.9-fold higher at 0.01, 0.1 and 1 µM Cd, respectively. It is interesting to stress that in both the cultivars, with a change in the Cd concentration in the nutrient solution from 0.01 to 0.1 µM, the concentration of Cd in the shoot increased by the same factor as it did in the root, i.e. 5- and 10-fold in Manel and Lemsi, respectively. However, on increasing the treatment concentration from 0.1 to 1 µM the concentrations of the metal in the roots of both the cultivars [did what? Something missing here] whereas on changing from 0.1 to 1 µM the Cd concentration in the roots of both the cultivars increased by about 3.5-fold whereas in the shoots it did not rise by more than 2-fold.

The total amount of Cd absorbed by the plants increased by increasing the metal concentration in the nutrient solutions (Tab. 6). At the end of the treatments with the lowest and the highest Cd concentrations, cv. Lemsi accumulated more Cd on a whole plant basis: about 3.0- and 1.3-fold at 0.01 and 1 µM Cd, respectively (Tab. 6). At the intermediate concentration, no significant difference was found between the two cultivars in the total amount of Cd accumulated on a whole plant basis(Tab. 6).

Most of the Cd taken up by plants remained in the roots and only minor amounts were translocated to the shoots (Table 6). The percentage of the metal retained in the roots of Lemsi changed from less than 60% to 77% as a function of the Cd treatment level, whereas in those of Manel, the percentages retained were constantly more than 80% (Tab. 6). At the lowest Cd concentration tested, the amount of the metal translocated from the root to the shoot was markedly higher (about 8-fold) in Lemsi than in Manel. Although minor (about 2-fold), the difference also remained evident at the two other higher Cd exposures (Tab. 6).

Concentration-dependent uptake kinetics of ^{113}Cd in Manel and Lemsi roots

Eight different concentrations of Cd (0.01–10 µM) were used to study the influx kinetics of Cd into the roots of Manel and Lemsi. Because ^{113}Cd uptake was measured over a short period (20 min), the results mainly represent unidirectional Cd influxes.

In Lemsi the concentration-dependent uptake kinetic for ^{113}Cd showed a saturable (hyperbolic) component and a non-saturable linear component, whereas in Manel only the saturable component was clearly evident (Fig. 13). Both the curves were mathematically resolved using SigmaPlot (Chicago, IL, USA). In order to evaluate K_m, V_{max} of the saturable component and the slope (a) of the linear component, the uptake kinetics of both the cultivars were best fitted to the following equation that adds a linear component to the Michaelis–Menten model:

$$V_{\mathrm{Me}} = \frac{V_{\max}[Me]}{K_{\mathrm{m}} + [Me]} + a[Me]$$

where [Me] is the concentration of Cd. A similar approach was used by Cohen et al. (1998) to resolve the concentration-dependent kinetics of Cd influx in pea seedlings. In Table 7, the values of the parameters obtained for the two barley cultivars are summarized. Saturable Cd influxes were characterized by similar K_m values of 0.47 (± 0.12) and 0.1.38 (± 0.22) µM for the cvs Lemsi and Manel, respectively. The maximal influx (*V*max) for Cd^{2+} was significantly different between the two cultivars. The value of V_{max} in Lemsi was about 2.8 fold greater than in Manel as well as the slope for the linear component (about 7.5-fold higher).

Thiol levels and HvHMA3 expression in Manel and Lemsi exposed to low Cd concentration.

Since the activity of homeostatic mechanisms based on thiol biosynthesis (i.e. PCs) and direct vacuolar sequestration may potentially allow a greater proportion of Cd to be retained in roots, a study was made to find out how the presence of increasing Cd concentrations in the nutrient solutions affects, in the roots of both the barley cultivars: a) the levels of NPTs and GSH; and b) the expression in the roots of a gene codifying for a tonoplast-localised protein (HvHMA3) involved in Cd vacuolar sequestration.

In the roots of both Manel and Lemsi the level of NPTs was progressively increased by increasing the Cd concentration in the nutrient solution (Fig. 14B). However, in Manel at the highest Cd concentration (1µM) the level of NPTs was nearly doubled (+86%) in comparison with the control whereas in Lemsi it was only +46% with respect to the control.

A similar behavior was observed in the shoots of both the cultivars (Fig. 14A).

When plants were grown in control conditions, both in the root and the shoot the level of GSH was significantly higher in Lemsi than in Manel (Fig. 14C and D). Such a difference was also maintained when plants were exposed to 0.01 and 0.1 µM. Moving from 0.1 to 1 µM Cd, in both the cultivars and both in shoot and root, the level of GSH diminished (Fig. 14C and D). The effect

was more evident in Lemsi, in so much that the GSH level became in the root similar to or even lower than that observed in Manel.

In agreement with several studies suggesting that Cd exposure induces in the plant cells the biosynthesis of PCs with a concomitant contraction of the GSH pool and no effect on cysteine and γ-glutamylcysteine concentration (Nocito et al., 2002; 2006) the difference between the level of NTPs and that of GSH may be reasonably considered indicative of the level in the tissue of PC-like compounds (Schäfer et al., 1997). In both Manel and Lemsi the progressive increase of the Cd concentrations in the nutrient solution induced a progressive accumulation of PCs-like NPTs in the roots (Figure 14F). However, at each Cd concentration the level of these compounds was much higher in the root of Manel than in those of Lemsi (Fig. 14F). A very similar picture was evident considering the level of PCs-like NTP in the shoots (Figure 14E).

The treatments with Cd affected the root total sulfur content in Manel and Lemsi (Fig. 15B). Basically, the total sulfur level in this organ increased with the increase of Cd in the nutrient solution in both the cultivars. However, in the shoots no clear trend was detectable (Fig. 15A). The enhancement of root total sulfur content due to Cd treatments was accompanied by a progressive reduction of the sulfate levels (Fig. 15D). Considering the shoots only, in Manel under Cd treatment a slight reduction in the sulfate levels was detectable (Fig. 15C).

An important component of the capacity of plants to retain Cd in their roots is the vacuolar sequestration of the free cationic form of the metal mediated by tonoplast-localized proteins, members of the P_{1B}-type ATPase transporter family (Korenkov et al., 2007a,b). In rice it has been clearly demonstrated that the tonoplast-localized protein OsHMA3 mediating the transport of Cd^{2+} ion into the vacuole plays an important role in the capacity of the plant to retain the metal in the roots (Ueno et al., 2010; Miyadate et al., 2011). The sequence of the *HvHMA3* gene, the barley ortholog of *OsHMA3*, has been recently submitted to NCBI (Mills et al., 2012). RT-PCR analysis performed on cDNA obtained from roots of Manel and Lemsi plants grown for 10 d in the presence of Cd 1 µM in the nutrient solution showed that the levels of the transcript of the *HvHMA3* gene was lower in the Cd-exposed than in control plants (Fig. 16). Nevertheless, no appreciable differences in the amount of the down-regulation of HvHAM3 expression in the treated plant were detectable between Manel and Lemsi.

Root Cd fractioning

To identify the relative amounts of the different chemical forms of Cd accumulated in the roots of Manel and Lemsi plants grown in the presence of Cd in the nutrient solution a fractioning procedure was adopted. Since at 1 µM Cd the maximum level of PC-like compounds was registered in the roots, both in Manel and Lemsi, the fractioning experiments was carried out only on the plants so treated.

Fractioning was carried out using the sequential extraction procedure with buffer and acid previously described by Rauser and Meuwly (1995). Table 8 summarizes the results obtained. Following extraction, three main Cd fractions were obtained from roots: buffer soluble; acid soluble; ash (non-soluble Cd). The buffer soluble fraction was further resolved into two fractions, named anionic and cationic, by anion-exchange chromatography. Such a separation allows one to distinguish Cd ions which are potentially free (cationic) from those tightly retained in complexes with thiol-peptides or other soluble molecules negatively charged in the extraction buffer (anionic). The buffer extracts accounted for 76.9% and 68.3% of Cd ions retained by the roots in the cases of cvs Manel and Lemsi, respectively. Despite the fact that the total amounts of buffer-soluble Cd present in Manel and Lemsi roots were nearly similar, the distribution of the metal between the two components of the fraction turned out to be very different. Indeed, (Tab. 8), in Manel the cationic fraction (eluted from an anion-exchange resin) accounted just 14.1% of the total Cd, whereas in Lemsi these components account for 29.6% of the total Cd present in the roots. As expected, the anion fraction removed from the anion-exchange column accounted for 60% and 38% of the total Cd present in the Manel and Lemsi roots, respectively. Concerning Cd remaining in the pellets of the buffer extracts, it was largely extracted in ice-cold 100 mM HCl both in Manel (about 20%) and in Lemsi (about 29.%) and then a small amount of the metal was found in the ashes (about 6.0 and 4.7% for Manel and Lemsi, respectively).

Cadmium root-to-shoot translocation

The dynamic of root-to-shoot Cd translocation was examined by evaluating the amount of Cd ions loaded into the xylem sap for 2 h in the plants exposed to increasing Cd concentrations. As reported in Figure 17 the translocation isotherms of Cd gave a saturating curve and in both the cultivars the amount of Cd ions transported in the xylem sap approximated to saturation at over

0.1 µM Cd in the nutrient solution. However, in Lemsi the amount of Cd translocated was always from 2- to 3-fold higher than in Manel at the Cd concentrations tested.

In both the cultivars the amounts of Cd translocated in two hours into the xylem sap on a per plant basis were linearly related (r^2 = 0.998 and 0.992 for Manel and Lemsi, respectively) to the concentration reached by the metal in the shoots at the end of the treatment period (Fig. 18). The presence of Cd in the nutrient solution affected the xylem translocation of Zn differently in the two cultivars. As shown in Figure 19A, in the case of Manel, as the concentration of Cd in the nutrient solution increased, the concentration of Zn in the root tended to decrease, while at the same time the amount of Zn translocated in the xylem increased (Fig. 19B) as well as its concentration in the shoot (Fig. 19C). Conversely, in Lemsi as the concentration of Cd in the nutrient solution increased the concentration of Zn in the root tended to increase (Fig. 19A), while the amount of Zn translocated in the xylem decreased (Fig. 19B), as well as its concentration in the shoot(Fig. 19C).

Mills et al., (2012) demonstrated that the gene *HvHMA2* codifies in barley a plasmamembrane-localized protein expressed in cotyledon, shoot and root, mediating the energized efflux of Cd and Zn from the cells. Recently (Nocito et al., 2011) it has been suggested that *OsHMA2* the rice ortholog of *HvHMA2*, plays a role in the xylem loading of free Cd^{2+} ions. Assuming an analogous function for HvHMA2 in barley roots, the level of the transcript of this gene was evaluated in the roots of Manel and Lemsi plants grown in the presence of 1 µM Cd in the nutrient solution and compared with the level detectable in the roots of control plants. RT-PCR analysis carried out on root cDNA extracted from Manel and Lemsi roots showed that the transcript levels of the *HvHMA2* gene did not change in Cd-exposed with respect to control plants and were comparable between Manel and Lemsi (Fig. 16).

Effects of Cd exposure on organic acids metabolism

Similarly to the findings from plants exposed for 30 d at 25 µM Cd, the treatments for 10 d in the presence of relatively low metal concentrations in the nutrient solution also influenced the levels of malate and citrate in the roots and shoots of both the barley cultivars (Fig. 21). The progressive accumulation of Cd in the roots is accompanied by a reduction in the levels of both malate and citrate in the root and on the contrary, also by a progressive increase of the levels of both the carboxylates in the shoot. This effect was more evident in Lemsi than in Manel.

The amounts of organic acid loaded into the xylem sap in 2 h showed that (Fig. 22) in the control plants the amounts of malate, as well as those of citrate, loaded into the xylem were markedly higher in Lemsi (about 2.2 and 3.0 fold, respectively) with respect to Manel. Although this difference, in general the effects of Cd treatments were almost similar in both the cultivars. In detail, moving from 0.01 µM Cd to 1 µM Cd in the nutrient solution: a) the malate translocation was enhanced about 4.8- and 5.22-fold in Manel and Lemsi, respectively; b) the citrate translocation was enhanced about 1.5- and 2-fold, respectively.

Cd and Zn concentration in the flag leaves, husks and grains of Manel and Lemsi.

When Manel and Lemsi were grown up to ripening on a soil containing about 2.0 mg kg^{-1} total Cd and 1.2 mg kg^{-1} EDTA-extractable Cd (Tab. 9), the metal was detectable at the highest concentration in the flag leaves and then at progressively lower concentrations in the husks and in the whole grains (Tab. 10).

Consistently with the observations made on the shoots of plants grown in hydroponic conditions, the levels of Cd in the flag leaves and in the husks were significantly higher, about double, in cv. Lemsi compared with cv. Manel; the grain Cd concentration was also more than 3.5 fold higher in the former cultivar than in the latter. However, in both the cultivars the concentrations in the grains of the toxic element was higher than the official maximum allowable limit (0.1 mg kg^{-1}) established for cereals by the FAO/WHO's Codex Alimentarius Commission.

Section II
Discussion

In the second part of the research programme the physiological and molecular reasons for the contrasting behavior shown by the barley cultivars Manel and Lemsi in accumulating and tolerating Cd were investigated by exposing the plants to a broad range of relatively low Cd concentrations (from 0.01 to 1 µM), simulating the actual Cd concentrations experienced by roots in contaminated agricultural field soils.

The growth of both the cultivars was not visibly affected by the exposure of the plants to the relatively low Cd concentrations adopted, with the exception of 1µM Cd, which slightly reduced the root biomass production but only in cv. Lemsi (Fig. 10). Other than on the growth, the presence of 0.01-1µCd in the nutrient solutions had no consequences on the photosynthetic parameters, or on the leaf water transpiration of both the barley cultivars (Fig. 11). Although there was no visible sign of toxicity, Cd was accumulated by the plants. In particular, the differences between the two cultivars observed under the severe stress conditions adopted in the first part of the research programme, concerning the uptake of the metal and its distribution within the plants, were confirmed. Indeed, at each Cd concentration the levels of Cd in the shoots of Lemsi were significantly higher than in Manel despite the fact that the concentrations of the metal in the roots were not, or only slightly, different at 0.01 µM between the two cultivars (Fig. 12).

The experimental situation outlined seems to mimic well what happens in the field when crops are grown on agricultural soils which are weakly polluted with Cd, namely, the metal is dangerously accumulated in the plants without any obvious symptoms that signal its presence.
Many studies have evaluated the potential of Cd uptake in an attempt to explain the differences in shoot Cd accumulation between ecotypes, cultivars and crop relatives (Zhao et al., 2002). Manel and Lemsi roots showed very different Cd uptake kinetic properties (Fig. 13 and Tab. 7). In both the cultivars, the concentration-dependent kinetics of Cd influx showed a saturable component and a linear component. The linear component could be due to ^{113}Cd ions that remain bound to cell walls after desorption. For divalent cations, such as Cd^{2+}, it is very difficult to completely remove metals adsorbed by the cell walls without causing significant efflux of the ions from the symplasm (Reid et al., 1996; Cohen et al., 1998). However, in this study ^{114}Cd had been utilised in the desorption procedure as proposed by Mori et al. (2009); they showed that this procedure removed about 90% of the Cd adsorbed on the cell walls of *O. sativa*. The concentration dependence of Cd uptake from external solutions measured over short periods into either excised roots or intact plants generally follows the sum of a single Michaelis–Menten component plus a linear component (see Table 1 in Lux et al., 2010). The linear component is often attributed to

tight Cd binding to cell walls, but it is not possible exclude the idea that it represents an apoplasmic Cd flux toward the xylem (White, 2001; White et al., 2002; Broadley et al., 2007). Since the two linear components of Cd influx in Lemsi and Manel roots were largely different (compare the *a* values in Table 7) it is possible to hypothesize that at least part of the difference in the Cd root uptake activity of Lemsi and Manel is due to differences in the Cd cell wall binding characteristics of the two varieties and/or to different cultivar's roots' histological properties differently influencing the apoplasmic Cd flux across the root tissues (Lux et al., 2010). The results obtained from the cellular Cd fractioning experiment (Table 8), without excluding that histological differences between the roots of the two cultivar may exist, strongly suggest that the more evident linear component of Cd uptake kinetic observed in Lemsi with respect to Manel could be, at least partially, due to differences in the number of Cd-binding sites in the root cell wall of the two cultivars. Indeed, the Cd acid soluble fraction contributed with a higher percentage to the total amount of Cd in the root of Lemsi compared with that of Manel (Table 8).

It is generally accepted that the saturable component of Cd-uptake kinetic represents the true Cd transport across the plasma membrane (Lux et al., 2010). The Lemsi cultivar clearly showed a marked saturable component in the low concentration range, that was much less evident in Manel (Fig. 13). Judging from the K_m values obtained, included in the range of 20-1000 nM reported for several species (Lux et al., 2010), the saturable component has a high affinity for Cd. However, the K_m values in the two cultivars appeared to be similar (Table 7). The main difference between the two cultivars was in V_{max}, with the value obtained for cv. Lemsi being 2.8 times larger than that for Manel (Table 7). This implies that Lemsi may have on the root cell membranes a higher density of a Cd transporting system than Manel, or that the transport system is more active in the former than in the latter barley cultivar.

The identity of the protein mediating the uptake of Cd from the soil solution into the root cells is not yet completely clarified. Pedas et al. (2008) have identified and characterized in barley a gene encoding a plasma membrane-localized metal transport protein able to transport Mn^{2+}. The gene has been designated as *HvIRT1* (for Iron-Regulated Transporter 1) because it belongs to the ZIP gene family. *HvIRT1* has a high similarity to rice *OsIRT1* and when expressed in yeast shows an ability to transport, in addition to Mn^{2+} , also Fe^{2+}/Fe^{3+}, Zn^{2+}, and Cd^{2+} with relatively high affinity. Although increasing evidence suggests that some plasma membrane-localized transporters belonging to the Nramp protein family are involved in the root uptake of Cd in plants and particularly in rice (Takahashi et al., 2011; Sasaki et al., 2012), in barley no information about

this possibility exists. Thus, to date HvIRT1 is the sole Cd transporter known to mediate Cd uptake in the root of barley. It could be interesting in the future to compare sequences of HvIRT1, as well as its expression pattern under Cd stress in Manel and Lemsi since it could perhaps explain the observed differences. Moreover, as both Fe and Mn deficiency up-regulated *HvIRT1* (Pedas et al., 2008), and since Cd induces an Fe-deficient condition more evident in Lemsi than in Manel, (Fig. 20) it could be speculated that the presence of Cd in the nutrient solution in turn may amplify its uptake by further increasing the level of the *HvIRT1* transcript to a larger extend in Lemsi than in Manel.

The Cd distribution pattern between root and shoot, as percentage of the total amount of metal absorbed per plant, largely varies in Lemsi as a function of the concentration of the metal in the nutrient solution, while in contrast it remains essentially constant in Manel (Table 6).

Different mechanisms involving metal chelation and sequestration pathways (Rauser, 1995; Clemens, 2006), as well as a wide range of transport proteins belonging to various families (Williams et al., 2000; Hall and Williams, 2003; Colangelo and Guerinot, 2006; Grotz and Guerinot 2006; Ueno et al., 2010) have been described as being implicated in root metal-ion homeostasis. Among these, the Cd detoxification mechanism based on the synthesis of PCs and the activity of metal transporters sequestering Cd into the vacuoles would seem to be the major players acting in a complex 'firewall system' which retains Cd in the roots and limits its translocation to the shoots (Nocito et al., 2011).

A large number of plants , as well as other organisms, respond to Cd, rather than to other metals, by producing phytochelatins (PCs) (Rauser, 1995; Zenk, 1996; Cobbett and Goldsbrough, 2002). Such thiol compounds have largely been shown to be involved in Cd chelation and vacuolar sequestration (Clemens, 2006; Nocito et al., 2007). In our conditions, in both the cultivars, under conditions of increasing Cd exposure, the levels of NPTs in the roots progressively increased, whereas concomitantly those of GSH decreased (Fig. 14). Since GSH represents the main NPT in non Cd-stressed root cells, it can reasonably be supposed that the recorded increase in the level of NPTs was ascribable to the synthesis of PCs which conversely represent the most abundant class of NPTs in Cd-stressed root cells (Nocito et al., 2002, 2007). As shown in Figure 14F, at each Cd concentration tested, the levels of the NPTs, assumed to be PC-like compounds, are always significantly higher in the roots of Manel than in those of Lemsi. The differences were more evident at the lowest concentration (about +100%), and then, although it was maintained, it tended to be reduced at the highest Cd concentration of the nutrient solution. This difference can

explain the very low root Cd retention capacity shown by Lemsi in comparison with Manel (Table 6).

It is well known that the biosynthesis of PCs may be regulated in several ways, i.e. through a direct control of PCS level and activity or, indirectly, by a fine tuning of biosynthetic pathways leading to GSH production (Clemens, 2006; Nocito et al., 2007). In the future, it could be interesting to compare the transcriptional activation of the recently identify *HvPCS* barley gene (Kaznina et al., 2012), codifying for the enzyme phytochelatin synthase, in Cd-exposed cvs Manel and Lemsi plants. The transcriptional activation under Cd exposure of the rice gene *OsPCS* has been recently reported (Nocito et al., 2011). However, it should be borne in mind that the induction of PCs biosynthesis might also be due to post-translational regulation of PCS [do you mean PC's?]activity as a response to Cd accumulation in the root cells (i.e. on activation by metal-ions and/or metal-GSH complexes; Vatamaniuk et al., 2000).

The distribution of Cd in the plants differs between Manel and Lemsi cultivars. When grown with chronic, low concentrations of Cd in the range 0.01-1 µM the percentages of the metal retained in the roots of Lemsi were in the range 55-77%, whereas they never was lower than 80% in Manel (Table 6). The differences in root retention capacity are also evident when considering the results of short period exposure. Indeed, (Fig. 8A and B; Fig. 9) the higher translocation observed in Lemsi is coherent with the lower root retention capacity that it showed with respect to Manel: about 80% and about 93%, respectively, already after 24h exposure. These values remained almost constant in both the cultivars in the successive 72h.

The distribution of Cd among the fractions composing the total amount of the metal in the root of the plants treated with 1µM Cd was also different between the two cultivars (Table 8). In particular, approximately 60% of the total Cd taken up by Manel was found tightly bound to the anions (i.e. PCs) sub-fraction of the buffer soluble fraction, whereas this percentage in Lemsi was significantly lower (about 38%). As a consequence, though the total amount of Cd in the buffer soluble fraction is similar in the two cultivars, the total amount of the cationic Cd fraction (i.e. free Cd^{2+} available for translocation to the shoot; see below) is more than double in Lemsi with respect to Manel. The higher levels of PC-like NPT compounds observed in Manel with respect to Lemsi (Fig. 14F) may provide the explanation of the higher amount of Cd present in the anionic sub-fraction in the former cultivar.

In conclusion, this group of results suggests that, similarly with those observed among other plant species (Akhter et al., 2012), a different amount of activation of the PCs system can be the reason for the different root retention capacity observed between barley cvs Manel and Lemsi.

PC-independent mechanisms of vacuolar sequestration are central to the tolerance of plants to high levels of divalent cation metals. To date, several tonoplast-localized transporters have postulated as being involved in Cd transport into the vacuole (Korenkov et al., 2007a,b). Recently, Ueno et al. (2010) described a member of the P_{1B}-ATPase transporter family, OsHMA3, as the main candidate for direct Cd sequestration into the vacuoles of rice cells. The full length sequence of the *HvHMA3* gene, the barley ortholog of *OsHMA3*, has recently been submitted to NCBI (http://www.ncbi.nlm.nih.gov/; Mills et al., 2012). Although to date there has been no reported functional characterization of this sequence, assuming that it plays a role analogous to that of *OsHMA3*, the levels of its transcript were examined in the roots of Lemsi and Manel plants grown under Cd treatments. The results obtained indicated that the level of the *HvHMA3* transcript was negatively affected in both the cultivars at a similar extent by the Cd-treatment, suggesting that a differential transcriptional regulation of this gene is not involved in the differences in Cd root retention capacity between the two barley cultivars. Nevertheless, since nucleotide polymorphisms occurring in *OsHMA3* account for differences in Cd root retention among rice cultivars (Ueno et al., 2010) we cannot exclude the idea that this could be the case in the two barley cultivars considered here.

The fraction of buffer-soluble Cd not immobilized by the anion exchanger may have come from Cd ions in free cytosolic form (Cd^{2+}) and/or aspecifically bound with organic or inorganic ligands to form cellular complexes of relatively low thermodynamic stability. These Cd ions appear to have all the requisites to be considered the major Cd pool with relatively high mobility in the root cells and, then, potentially available for long-distance transport from roots to shoots. The amount of Cd present in this potentially mobile fraction was double in the roots of Lemsi when compared with Manel (Table 8). Actually, the amount of Cd translocated from root to shoot was significantly higher in Lemsi than in Manel (Fig. 17), coherently justifying the higher Cd concentration finally detected in the shoot of Lemsi than in that of Manel (Fig. 12A). This conclusion is further supported by the strong correlation existing in both Manel and Lemsi between the amount of Cd translocated in time from the root to the shoot and the Cd concentrations measured in the shoot (Fig. 18).

The mechanisms that control Cd translocation have not yet been completely clarified. In lettuce and barley (Akhter and Macfie, 2012), rice (Uraguchi et al., 2009) and maize (Florijn and Beusichem, 1993), as well as in other species, increased translocation of Cd to the shoots could not be explained by greater volumes of water transpired. This seems to be the case in our experimental conditions for barley, too. Indeed, no significant differences in water transpiration activity were observed between Lemsi and Manel, neither in control nor in Cd-treated plants (Fig. 11).

Higher translocation of Cd from the roots is reasonably related (apart from lower retention of Cd in the roots), to higher xylem loading activity. Since the majority of Cd is present in the xylem as the free Cd ion (Cd^{2+}), it is very probable that the transport system playing a pivotal role in its xylem loading mediates the transmembrane efflux of Cd^{2+} from the xylem parenchyma cells. Good candidate proteins for Cd^{2+} xylem loading in rice are the products of *OsHMA2* and *OsHMA4* genes, orthologs of *AtHMA2* and *AtHMA4*, the main genes controlling Cd systemic allocation in *Arabidopsis* (Mills et al., 2005; Eren and Argüello 2004; Verret et al., 2005; Wong and Cobbett, 2009). Recently, Nocito et al. (2011) demonstrated that *OsHMA2* codifies for a Zn^{2+}/Cd^{2+} ATPase involved in Cd translocation, mediating Cd efflux from yeast cells. Although additional evidence is required to fully support this conclusion, the preliminary functional analysis of *OsHMA2* in yeast by Nocito et al. (2011) and a recent mutant analysis (Sato-Nagasawa et al., 2012) confirms the involvement of this protein in Cd xylem-loading in rice. Recently, *HvHMA2* a barley P_{1B}-ATPase from the Zn/Cd/Pb (P_{1B-2}) sub-group (Williams and Mills, 2005; Zorrig et al., 2011) has been cloned and functionally analysed (Mills et al., 2012). Heterologous expression in *Saccharomyces cerevisiae* demonstrated that *HvHMA2* functions as aZn and Cd pump. Moreover, *HvHMA2* expression suppresses the Zn-deficient phenotype of the Arabidopsis *hma2hma4* mutant indicating that HvHMA2 functions as a Zn pump *in planta* and could play a role in root to shoot Zn, and Cd, transport (Mills et al., 2012). This group of findings suggested to us to compare the expression pattern of *HvHMA2* in cvs Manel and Lemsi under Cd-exposure. The results of the RT-PCR analysis showed that in both the cultivars Cd treatment did not induce any change in the level of the *HVHMA2* comparing control and 1 µM treated plants and, especially, that no obvious quantitative differences in the transcripts level exist between Manel and Lemsi (Fig. 16). Without excluding the possibility that nucleotide polymorphisms could determine differences in the HVHMA2 activity between Manel and Lemsi, (a hypothesis presently under investigation) the results here reported suggest that the difference observed between Manel and Lemsi in Cd root-to-shoot translocation

cannot be attributed to differences in the expression level of the transport system putatively involved in the Cd^{2+} xylem loading.

The different Cd shoot accumulation pointed out between Manel and Lemsi can be firstly attributed to substantial differences in the root uptake activity of the metal due to a higher density in Lemsi than in Manel of the transport system/s mediating the Cd influx into the roots. Moreover, a more limited efficiency in chelating the larger amount of Cd taken up and thus immobilizing it in the root was observed in Lemsi with respect to Manel, and this seems to be the second character distinguishing the two barley cultivars. . Due to the higher uptake and to the lower Cd chelating activity, the transport system with the task of extruding Cd^{2+} from the root xylem parenchyma cells towards the xylem vessels has in Lemsi a greater amount of available substrate (i.e free Cd^{2+}) than in Manel.

The limited efficiency of the root Cd chelating system in Lemsi might be due to particular kinetic and/or thermodynamic bottlenecks along the metabolic pathways leading to the synthesis of PCs or, possibly, of Cd chelating thiol compounds. A future comparison in Manel and Lemsi of the structural properties of the genes involved in PCs biosynthesis, as well as of the regulation of their expression, could be a promising future line of enquiry for identifying at molecular level the reason for the observed differences. In this regard it is important to take into account that reductive sulfur metabolism plays a central role in regulating PCs biosynthesis. It is well known that, due to the additional sink of sulfur established by the Cd-induced PCs synthesis, regulative steps of the reductive sulfur pathways were stimulated at transcriptional level (Lee and Leustek, 1999; Nocito et al., 2002; Nocito et al., 2006). The expected increase of the level of total S in Lemsi and Manel root under Cd-treatment is not clearly evident in our experimental conditions (Fig. 15). This behavior, particularly in Lemsi which was committed to face the higher Cd uptake from the external solution, could limit Cd root retention. Thus, in future it will be interesting to compare between Manel and Lemsi under Cd stress the properties and the expression pattern of genes codifying for proteins involved in S acquisition and reductive metabolism such as high-affinity sulfate transporters, ATP sulfurylase, *O*-Acetyl serine transferase, and Glutatione synthetase.

For the same reasons first discussed in relation to the experiments at high Cd concentrations (25 µM) it is not possible to exclude that, also when plants are exposed to relatively low Cd concentrations, malate and citrate can play direct roles in Cd root-to-shoot translocation (Fig. 22). The behaviors of the levels of these two carboxylates in the roots and the

shoots of the Cd treated plants seem to be related, as proposed in the first part of the thesis, to the activation of the anaplerotic metabolism leading to the synthesis of PCs (Nocito et al., 2008).
Very interesting to consider are the apparent competition/antagonism phenomena observed between Cd and the other mineral microelements (i.e. Zn, Mn and Fe) concerning their translocation and accumulation in the shoot (Figs. 19 and 20). However, especially regarding the effect of the presence of Cd on Zn root-to-shoot translocation and shoot accumulation, in the absence of a specific investigation, only some speculative interpretations can be proposed. Comparing what happens in the two barley cultivars, it seem reasonable to hypothesize that the root systems involved in Cd and Zn absorption, chelation, compartmentalisation and/or xylem loading in Manel are endowed with selectivity characteristics for Cd and Zn different from those of the systems operating in Lemsi. Further experiments specifically aimed to investigate antagonisms/competition phenomena between Cd and Zn in Manel and Lemsi, as well as to compare between the two cultivars the structure of the genes involved in Cd/Zn root uptake, root retention and/or root-to-shoot translocation are interesting perspectives emerging from the investigation carried out in this thesis.

Several studies have demonstrated that in rice roots Cd uptake and root-to-shoot Cd translocation via the xylem are key determinants of variation in grain Cd accumulation. The translocation is mostly a function of xylem loading activity and Cd retention in roots, in turn due to chelating molecules such as phytochelatins and/or to its sequestration in the vacuole. Once in the shoot Cd reaches the developing grain via the phloem (Tanaka et al., 2007), either following remobilization from leaves or directly after root uptake, xylem loading, and rapid accumulation at the shoot base (Rodda et al., 2011). Thus remobilisation from leaves only partially accounts for the total Cd accumulated in the grain, whereas xylem-to-phloem transfer, which is suggested to occur in the nodes (Fujimaki et al., 2010), seems to account for the prominent fraction. The context described justifies the correlation that often, if not always, is found between the accumulation of Cd in the shoot of young plants and those then observed at maturity in their grain. The existence of this correlation, apart from opening interesting perspectives in the study of the S involved in Cd distribution within plants, could prove very useful in genetic programmes aimed to develop crop genotypes which will be able to limit the accumulation of Cd in their edible parts. The above correlation is valid in the case of barley cvs Lemsi and Manel. Indeed, when plants were grown on Cd contaminated soil (Table 10) the level of Cd in the grain, as well the Cd level in flag leaf and husk, is higher in the former cultivar than in the latter. This means that Cd reallocation from the

shoot, and in particular from the flag leaf, to the spike during grain filling did not involve mechanisms able to override the differences imposed by the differential Cd root uptake and root-to-shoot translocation described for the two cultivars.

An additional practically useful output from the research is the identification of a barley cultivar (Manel), which is among some the most important cultivated in Tunisa, as being usable for a safe harvest in case of Cd contaminated soil. The levels of Cd found in the grains of both the cultivars in experiments using soil (Table 10) were very high and exceeded the official maximum allowable limit (0.1 mg kg^{-1}) established for cereals by the FAO/WHO's Codex Alimentarius Commission. However, it is important to stress that the soil utilized in this experiment had a particularly high Cd concentration (Table 9) not frequent in agricultural soil, although still lower than the concentrations reported for some agricultural soils close to the Jebel Hallouf-Sidi Bouaouane mining district in Tunisia (Chakroun et al., 2010).

Lemsi tended to accumulate in grains more Zn than Manel (Table 10). This expected behavior is very interesting in view of future potential biofortification programmes aimed at improving the content of this important nutritional microelement in cereal staple foods, such as barley, that usually contain low levels of Zn.

Conclusions

In summary, the results of this research can be listed as follows:

- *Among the six Tunisian barley cultivars compared, a large variability in the sensitiveness to Cd exists.*

- *The concentration of Cd in the roots did not significantly differ among the six barley cultivars analysed, whereas wide differences were evident in the shoots where cvs Lemsi and Manel showed the highest and the lowest values, respectively.*

- *In the six barley cvs analysed a close linear correlation exists between the concentrations of Cd in the xylem sap and those measured in the shoots.*

  a) Transpiration fluxes were not very different between Manel and Lemsi.
  b) Among the six barley cvs the concentrations of Cd in the xylem sap of Lemsi and Manel were the highest and the lowest, respectively.

- *Substantial differences exist between the Manel and Lemsi cultivars in terms of Cd influx across the root plasma membranes.*
  a) The transport system/s involved show a higher density (V_{max}) of the transport/s system on the root plasmamembrane in Lemsi than in Manel.
  b) The transport system/s involved show in Manel and Lemsi a similar affinity for Cd (K_m).
  c) Are the transport systems different or are they differently regulated?

- *The two cultivars differ in the induction of the PC system*
  a) Although total Cd concentration is not different in the roots of the two cvs, the amount of PCs in Lemsi was lower than in Manel.
  b) A higher concentration of free Cd is evident in the roots of Lemsi than in those of Manel.
  c) The RR capacity is lower in Lemsi than in Manel.

- *Cd root-to shoot translocation is higher in Lemsi than in Manel*
  a) The difference is due to a higher free Cd concentration in the roots of Lemsi.
  b) No differential involvement of *HvHMA2* and/or *HvHMA3* in Cd xylem loading or in Cd vacuolar retention could be found.
  c) In barley, malate and citrate do not seem to be involved in the root-to-shoot Cd translocation.
  d) Are other transport systems involved?

- *In barley, differences in the capacity to exclude Cd from the grain could be predicted by comparisons at the plantlet stage.*

Tables

Table 1. Cadmium amount and root retention capacity in six barley cultivars grown for 30 d in the presence of 25 µM Cd

	Cd amount (µg plant^{-1})			
	Shoot	Root	Plant	RR (%)
Manel	22.0^{a} ± 1.8	209.1^{a} ± 4.2	231.1^{bc} ± 4.6	90.5^{a} ± 2
Rihane	25.4^{ab} ± 1.5	193.4^{b} ± 3.8	218.8^{ab} ± 4.1	88.4^{ab} ± 2
Martin	36.6^{c} ± 1.1	168.5^{c} ± 2.5	205.1^{a} ± 2.7	82.1^{bc} ± 2
Souihli	34.0^{bc} ± 4.3	183.2^{d} ± 2.0	217.2^{ab} ± 4.7	84.3^{bc} ± 1
Roho	30.8^{b} ± 1.6	206.6^{a} ± 6.2	237.4^{bc} ± 6.4	87.0^{abc} ± 3
Lemsi	55.1^{d} ± 0.7	190.2^{b} ± 3.8	245.3^{c} ± 3.9	77.5^{c} ± 2

Data are the mean ± SE of three experiments run in three experiment eight plants each (n= 24). In each column figures with different letters are significantly different (p < 0.05).

Table 2. Fluorescence parameters of dark-adapted leaves of Manel and Lemsi control and Cd-treated plants.

	Manel		Lemsi	
	-	25 μM Cd	-	25 μM Cd
F_0	131.0 ± 3.9	122.2 ± 0.8	120.6 ± 1.2	115.0 ± 4.5
F_m	587.2 ± 19	583.0 ± 6	582.0 ± 18	539.4 ± 28
$F_v = F_m - F_0$	456.2 ± 19	460.7 ± 6.2	461.3 ± 18	424.2 ± 28
F_v/F_m	0.77	0.79	0.79	0.78

Data are the mean ± SE of three experiments run in three experiment eight plants each (n= 24). No significant differences resulted between C-treated and control plants at $P < 0.05$.

Table 3. Levels of TBARS, protease activity and GSH in the root and shoot of six barley cultivars grown for 30 d in the presence of 25 µM Cd

	TBARs (nmol g^{-1} DW)		Protease activity (U g^{-1} DW)		GSH (µmol g^{-1} DW)	
	-	25 µM Cd	-	25 µM Cd	-	25 µM Cd
Root						
Manel	32.2 ± 6.2	96.0* ± 2.8	33.9 ± 3.2	45.4* ± 1.0	1.17 ± .06	0.85* ± .01
Rihane	46.0 ± 2.3	152.9* ± 1.0	25.0 ± 1.4	49.7* ± 2.0	1.19 ± .03	0.77* ± .05
Martin	45.2 ± 1.5	94.3* ± 7.4	40.7 ± 1.0	71.5* ± 2.7	1.41 ± .02	0.79 ± .01
Souihli	57.3 ± 0.7	138.4* ± 9.1	26.5 ± 0.6	88.3* ± 1.4	1.92 ± .02	1.06* ± .01
Roho	90.4 ± 2.0	306.1* ± 9.4	30.0 ± 1.1	61.5* ± 1.3	2.26 ± .10	1.53* ± .07
Lemsi	81.9 ± 2.9	271.2* ± 6.8	24.2 ± 0.3	84.2* ± 1.1	2.07 ± .05	1.04* ± .01
Shoot						
Manel	33.9 ± 6.8	111.2* ± 3.7	255.3 ± 3.6	490.1* ± 7.5	1.61 ± .02	1.27* ± .02
Rihane	27.6 ± 0.9	131.5* ± 4.9	290.9 ± 13.3	604.0* ± 12.8	1.11 ± .01	0.80* ± .02
Martin	48.5 ± 0.7	194.5* ± 0.4	279.3 ± 15.2	459.4* ± 7.9	1.25 ± .03	1.03* ± .02
Souihli	45.2 ± 4.0	222.9* ± 4.5	375.4 ± 14.3	640.6* ± 10.0	1.07 ± .02	0.86* ± .02
Roho	48.4 ± 1.3	191.8* ± 2.5	393.8 ± 18.2	790.2* ± 4.7	1.18 ± .01	0.82* ± .01
Lemsi	40.7 ± 0.7	262.3* ± 0.7	381.6 ± 7.8	728.5* ± 4.7	1.18 ± .01	0.83* ± .04

Data are the mean ± SE of three experiments run in three experiment eight plants each (n= 24). Values indicated by asterisks are different from control at $P \leq 0.05$

Table 4. Concentrations of malate and citrate in the shoots and the roots of Manel and Lemsi cultivars grown for 30 d in the presence of 25 μM Cd.

	Malate (μmol g^{-1} DW)		Citrate (μmol g^{-1} DW)	
	-	25 μM Cd	-	25 μM Cd
Root				
Manel	178.0 ± 3.6	153.8 ± 4.6*	184.9 ± 8.0	140.5 ± 5.6*
Lemsi	303.2 ± 5.3	80.9 ± 0.5*	467.7 ± 12.3	235.6 ± 2.7*
Shoot				
Manel	46.2 ± 0.7	98.9 ± 1.8*	122.1 ± 2.9	149.2 ± 1.8*
Lemsi	36.6 ± 0.7	119.3 ± 1.9*	80.1 ± 1.7	141.3 ± 1.0*

Data are the mean ± SE of two experiments run in quadruplicate (n= 8). Values indicated by asterisks are different from control at $P \leq 0.05$

Table 5. Anaplerotic enzyme activities in the root and shoot of Manel and Lemsi plants grown in the presence of 25 µM Cd in the nutrient solution.

	PEPC (nmol min^{-1} mg^{-1} protein)		MDH (µmol min^{-1} mg^{-1} protein)	
	-	25 µM Cd	-	25 µM Cd
Root				
Manel	14.2 ± 1.0	13.1 ± 0.6	18.3 ± 0.4	18.6 ± 5.6
Lemsi	12.1 ± 0.4	15.7 ± 0.5*	14.2 ± 1.2	20.2 ± 0.7*
Shoot				
Manel	6.2 ± 0.2	5.6 ± 0.4	4.4 ± 0.9	4.8 ± 0.6
Lemsi	4.8 ± 0.2	5.2 ± 1.9	8.1 ± 1.1	7.6 ± 1.0

PEPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase. Data are the means ± SE of three experiments run in triplicate (n=9). Values indicated by asterisks are different from control at $P \leq 0.05$

Table 6. Cd accumulation and partition in barley plants. Plants were exposed to different Cd concentrations (0.01, 0.1 and 1 µM) for 10 d.

	Manel			Lemsi		
	0.01	0.1	1	0.01	0.1	1
Root (µg plant^{-1})	1.2* ± 0.1	13.6 ± 1.8	32.7 ± 3.2	2.5 ± 0.2	15.1 ± 1.1	40.4 ± 5.1
Shoot (µg plant^{-1})	0.2* ± .04	2.8 ± 0.4	5.8 ± 1.1	1.7 ± .06	4.9 ± 0.6	12.1 ± 0.7
Plant (µg plant^{-1})	1.4* ± 0.2	16.4± 2.3	38.5± 4.0	4.2 ± 0.2	20.0 ± 2.2	52.5 ± 6.4
Root retention (%)	82.8	85.3	84.9	58.8	75.9	77.0

Data are the mean ± SE of three experiments run in quadruplicate (n= 12). Figures with asterisks are significantly different ($p < 0.05$).

Table 7. Kinetic parameters of the influx of Cd into the barley root.

	K_m (µM)	V_{max} (nmol h^{-1} g^{-1} FW)	a (nmol h^{-1} g^{-1} FW μM^{-1})
Manel	1.38 ± 0.22	27.2 ± 3.2	2.1 ± 0.2
Lemsi	0.47 ± 0.12	76.9 ± 4.1	15.1 ± 0.4

The kinetic parameters are evaluated by the best fitting of the equation $V_{Cd} = V_{max}$ [Cd]/K_m+ [Cd]) + a[Cd] that adds a linear component to the Michaelis–Menten model. Results (±SE) derived from two experiments with four different samples for each concentration.

Table 8. Fractioning of Cd ions retained in the roots of Manel and Lemsi plants grown in the presence of 1 µM Cd in the nutrient solution.

		Cd content (µg g^{-1} DW)	
		Manel	Lemsi
Buffer soluble			
	Cationic	84.9 ± 6.5 (13.6%)	176.0*± 10.8 (29.0%)
	Anionic	377.1 ± 13.7 (60.2%)	230.2*± 21.2 (37.9%)
Acid soluble		127.7 ± 13.1 (20.4%)	172.5*± 11.1 (28.4%)
Ash		36.2 ± 2.7 (5.8%)	28.2 ± 1.9 (4.7%)
Total		625.9	606.9

Plants were exposed to 1 µM $CdCl_2$ for 10 days. Cd retained by roots was extracted with buffer and acid using the sequential procedure described in Materials and Methods section. Data are means and SE of three experiments, each performed with eight plants (n=3). Asterisk indicate the existence of significant differences between the two cultivars.

Table 9. Physico-chemical characterization of the soil utilized for the growth of barley plants

pH_{H2O}	5.70
pH_{KCl}	4.83
Organic carbon	13.5 g kg^{-1}
C.E.C	10.1 $cmol^{(+)}$ kg^{-1}
Exchangeable K^{+}	158.0 mg kg^{-1}
Exchangeable Mg^{2+}	129.0 mg kg^{-1}
Exchangeable Ca^{2+}	1540.0 mg kg^{-1}
E.C	612.0 µS cm^{-1}
Sand	42.2 %
Silt	45.3 %
Clay	12.5 %
Total Cd^{2+}	1.97 mg kg^{-1}
Total Cu^{2+}	119.61 mg kg^{-1}
Total Mn^{2+}	249.74 mg kg^{-1}
Total Zn^{2+}	402.83 mg kg^{-1}
Total Mg^{2+}	132.30 mg kg^{-1}
Bioavailable Cd^{2+}	1.12 mg kg^{-1}
Bioavailable Zn^{2+}	58.54 mg kg^{-1}
$[Cd^{2+}]$ in soil solution	0.098 µM

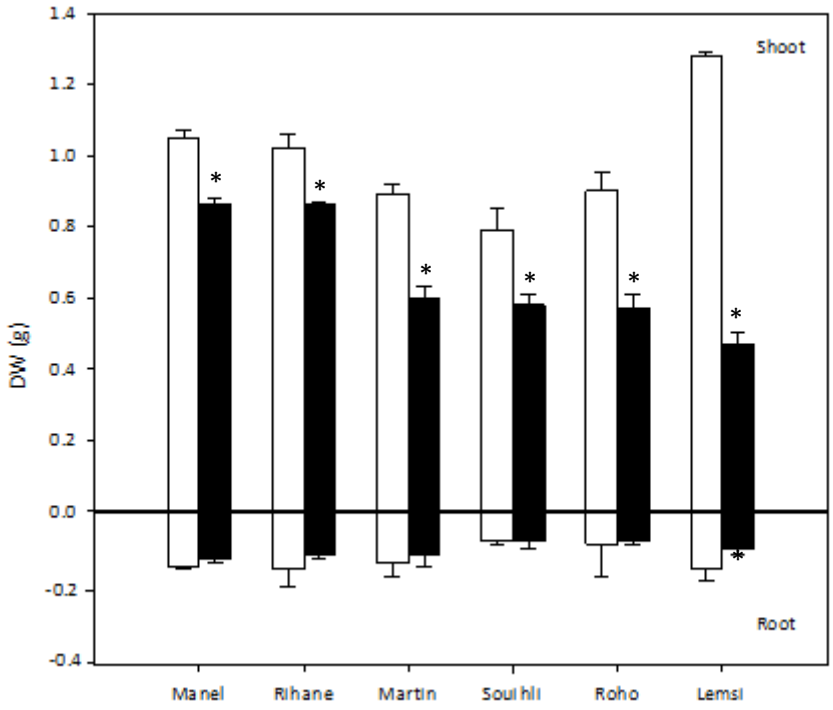
Table 10. Levels of Cd in the flag leaf, husk and grain of Manel and Lemsi barley cultivars grown on soil contaminated with the metal.

	Cd ($\mu g\ g^{-1}$ DW)			Zn ($\mu g\ g^{-1}$ DW)		
	Flag leaf	Husk	Grain	Flag leaf	Husk	Grain
Manel	$0.43^{a} \pm .05$	$0.30^{a} \pm .07$	$0.15^{a} \pm .02$	$119^{a} \pm 8.3$	$140^{a} \pm 11$	82 ± 4
Lemsi	$0.95^{b} \pm .06$	$0.62^{b} \pm 0.3$	$0.53^{b} \pm .03$	$79^{b} \pm 5.0$	$107^{b} \pm 6$	102 ± 7

Data are the means ± SE of three samples per pots. In each pots (total 4) were grown 5 plants**.** Different letters indicate significant difference between the two cultivars at $P \leq 0.05$.

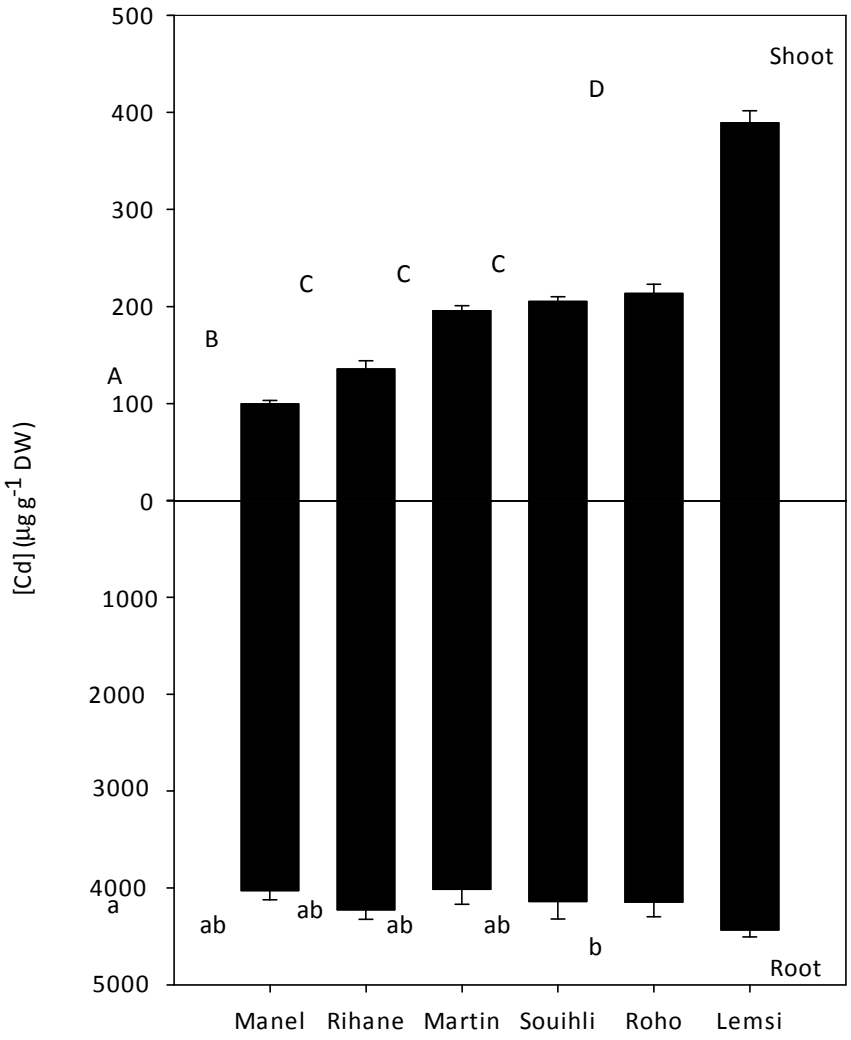
Figures

Figure 1. Effect of the presence of 25 µM Cd in the nutrient solution on the shoot and root biomass of barley plants of six Tunisian cultivars.



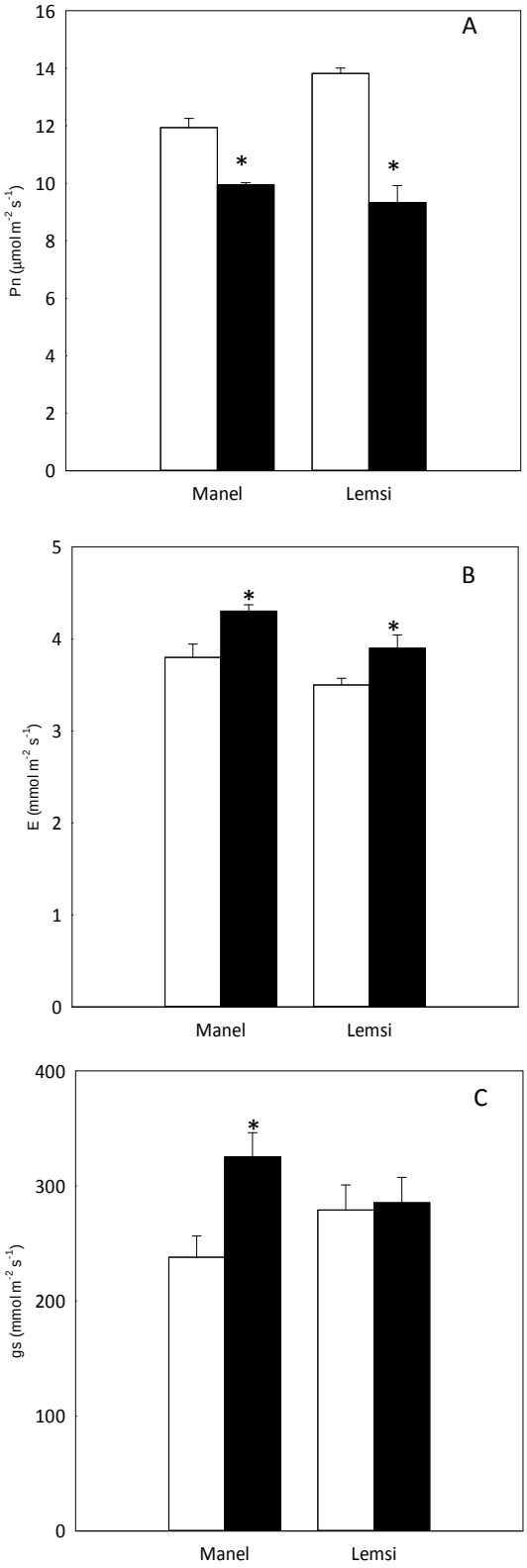
Plants were grown for 30 d in a complete nutrient solution supplemented (■ or not (□ with 25 µM $CdCl_2$. Data points and error bars are means and SE of three experiments each with at least ten plants *per* variety (*n=30*). Asterisks indicate significant differences between Cd-treated and control plants ($P < 0.05$).

Figure 2 . Cadmium concentration in the root and shoot of sx cvs of barley plants grown in the presence of 25 µM $CdCl_2$ in the nutrient solution.



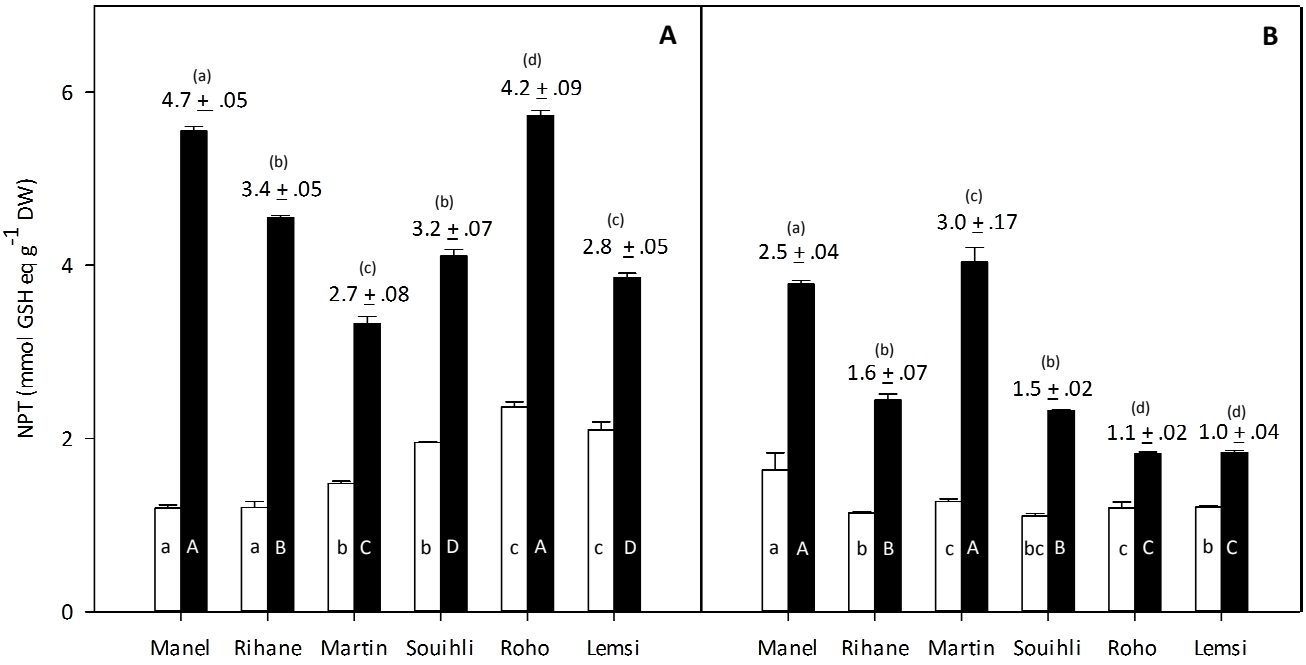
Data points and error bars are means and SE of three experiments each with at least ten plants per variety (*n*=30). Bars marked with same letter are not significantly different at $P \leq 0.05$.

Figure 3. Leaf gas-exchange parameters in barley cvs Manel and Lemsi plants grown in the presence of 25 µM Cd in the nutrient solution.



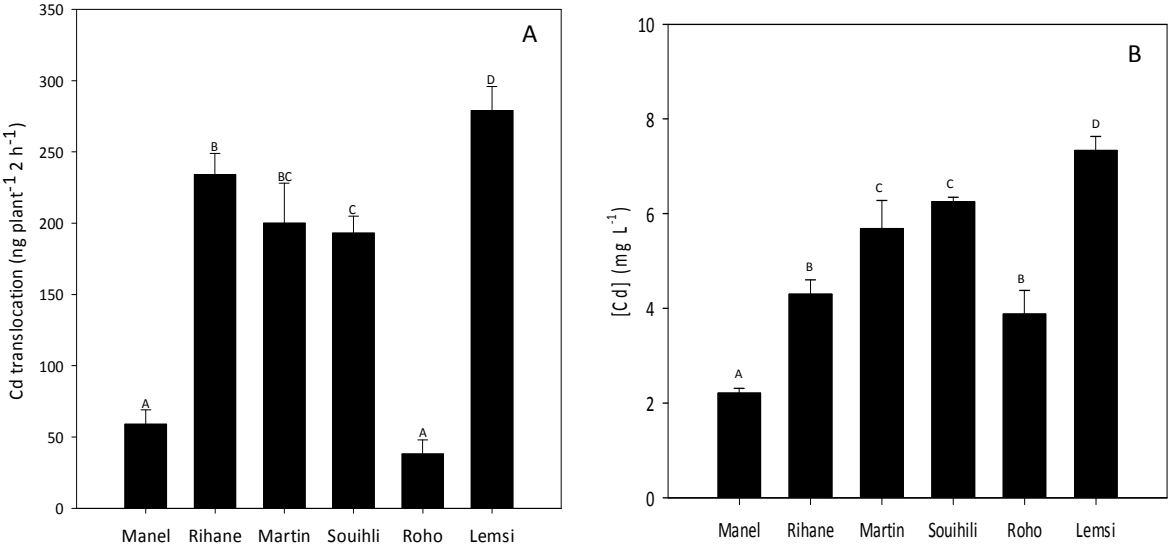
Data are the mean ± SE of three experiments with at least eight plant each (*n*= 24). Asterisks indicate significant differences between Cd-treated and control plants ($P \leq 0.05$).

Figure 4. Total nonprotein thiols in the root (A) and in the shoots (B) of barley plants grown in the presence of 25 µM $CdCl_2$ in the nutrient solution.



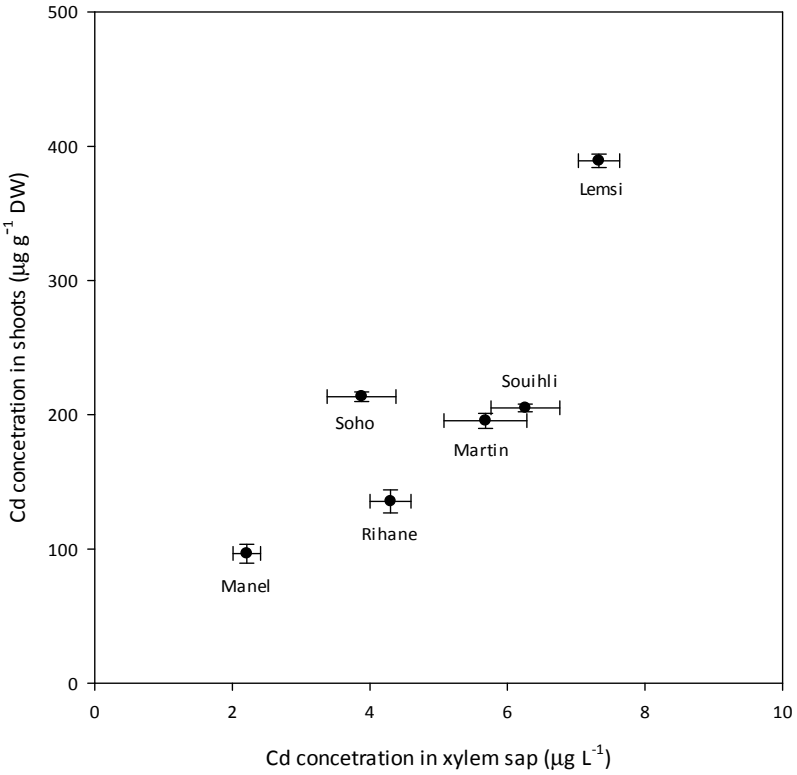
Pants were grown for 30 d in a complete nutrient solution supplemented (■ or not (□) with 25 µM $CdCl_2$. Data points and error bars are means and SE of two experiments each with at least eight plants *per* variety (*n=16*). For each cultivars the NTPs value in the treated plants is significantly higher than in the control plants at $P \leq 0.05$. Bars marked with same letter are not significantly different at $P < 0.05$. Figures inside the graphics represent the level of PC-like compounds computed by subtracting the level of GSH to total NPTs; data are the means ± SE of two experiments each with at least eight plants *per* variety (*n=16*); figures marked with the same letters within brackets are not significantly different at $P \leq 0.05$.

Figure 5. Concentration (A) and amounts (B) of Cd loaded and transported in the xylem sap of barley plants grown in the presence of 25 µM Cd in the nutrient solution.



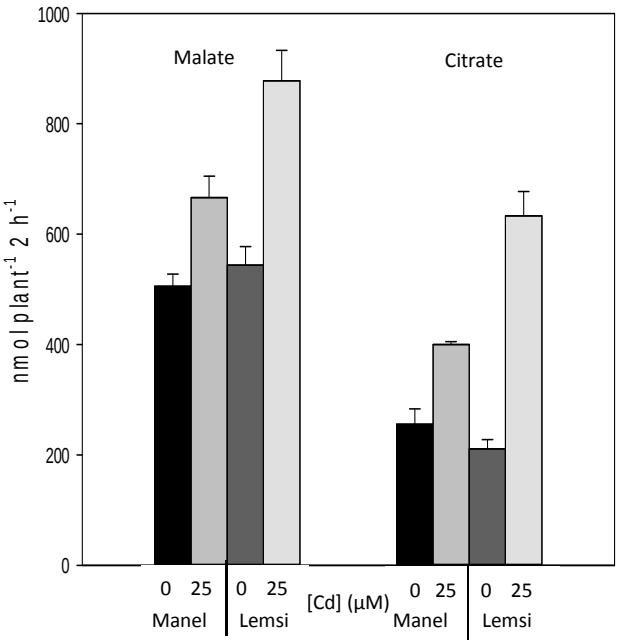
Plants were grown for 30 in a complete nutrient solution supplemented with 25 µM $CdCl_2$. At the end of the exposure period, shoots were separated from roots and the xylem sap exuded in 2 h from the cut (root side) surface was collected. Data are means and SE of two experiments each performed with 10 plants (n = 30). Bars marked with same letter are not significantly different at $P \leq 0.05$.

Figure 6. Relationship between Cd concentrations in the xylem sap and Cd concentration in shoots of barley plants



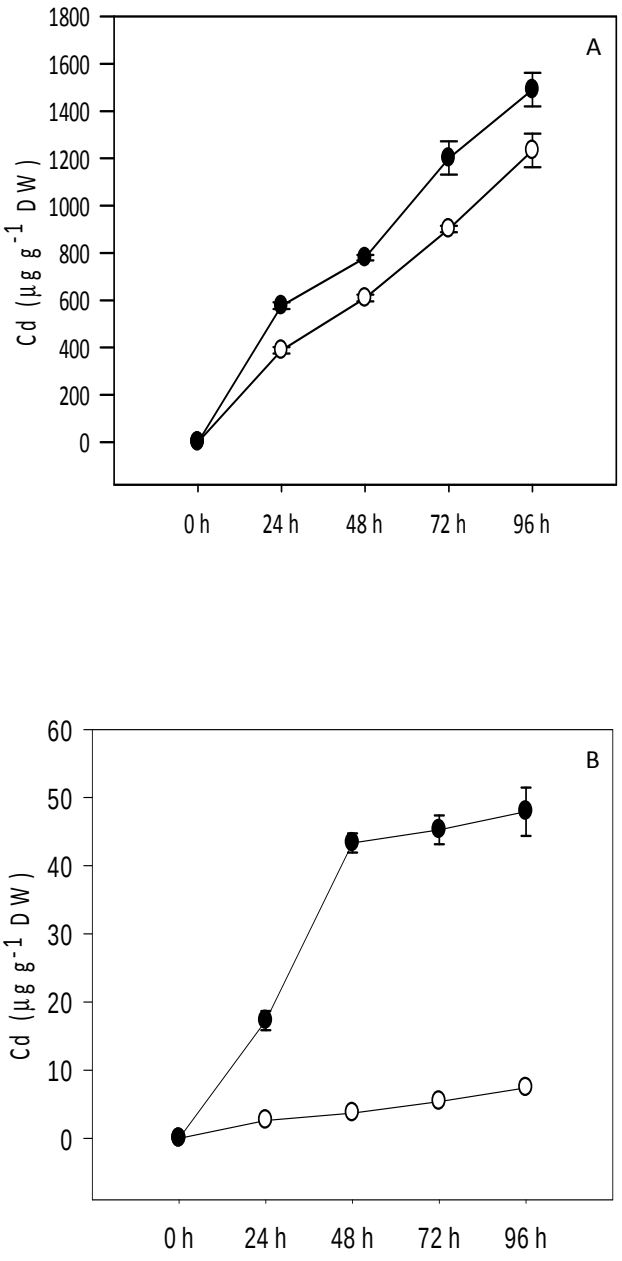
Plants were grown for 30 d in a complete nutrient solution supplemented with 25 µM $CdCl_2$. At the end of the exposure period xylem sap was collected for 2 h . Data are the means of two experiments performed with 10 plants; errors bars represent the SE of both growth and Cd concentration results

Figure 7. Root-to-shoot translocation of organic acid in Manel and Lemsi barley cultivars grown in the absence or in the presence of 25 µM Cd.



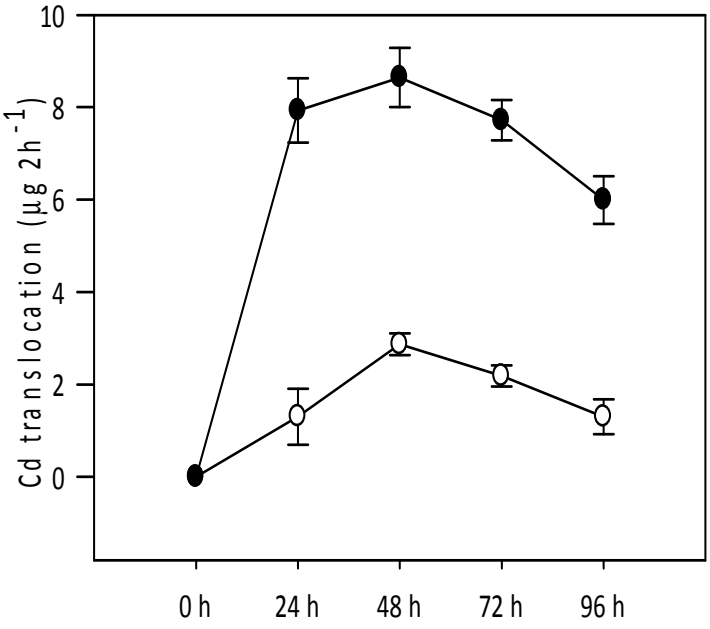
Plants were grown for 30 d in a complete nutrient solution supplemented or not with 25 µM $CdCl_2$. A the end of the exposure period xylem sap was collected for 2 h and organic acids levels were measured. Data are the mean ± SE of two experiments run in quadruplicate (*n*= 8). Asterisks indicate if in treated plants results are significantly different with respect to the controls ($P \leq 0.05$).

Figure 8. Time course of Cd accumulation in the root (A) and shoot (B) of Manel (○) and Lemsi (●) barley plants grown in the presence of at 10 µM Cd in the nutrient solution.



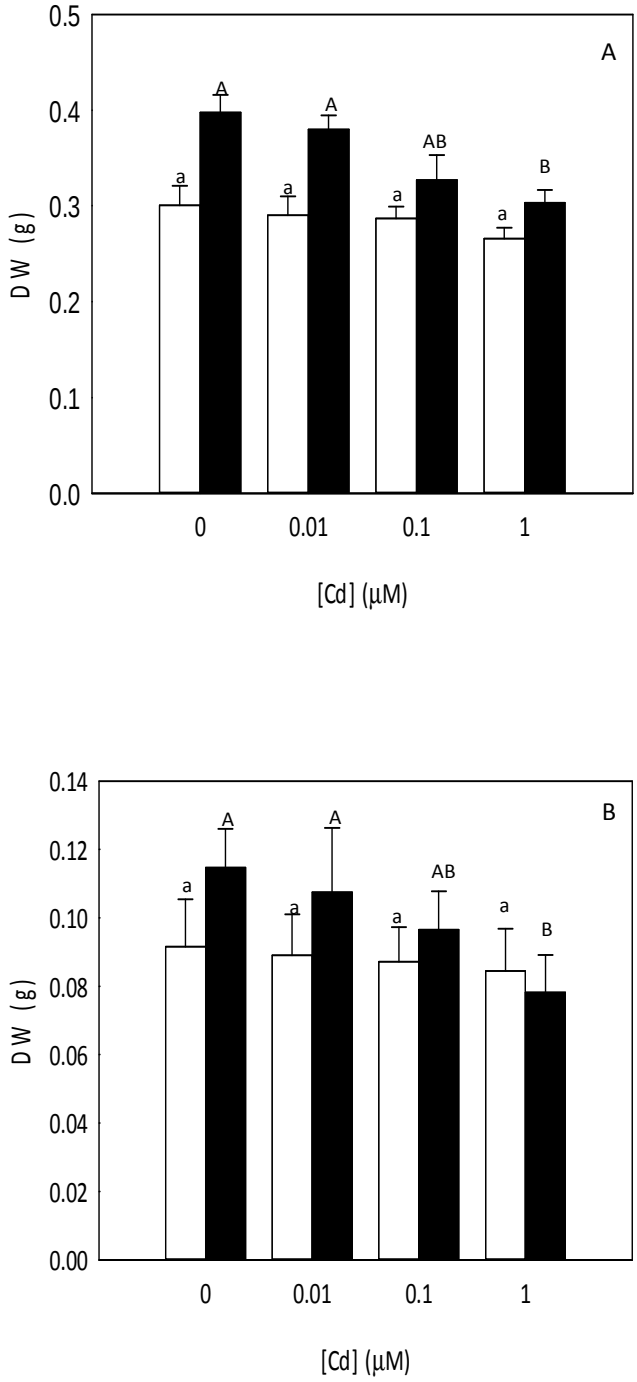
Plants were grown for 10 days in a complete nutrient in the absence of Cd. After this period the solution was supplemented with 10 µM $CdCl_2$. At different times 24h, 48h, 72h and 96h plants were harvested and their Cd content, after complete mineralization, were measured by ICP-MS technique. Data points and error bars are means and SE of two experiment run in sextuplicate (*n* =12).

Figure 9. Amounts of Cd translocated in 2h from the root to the shoot of Manel (○) and Lemsi (●) barley plants grown in the presence of 10 µM Cd in the nutrient solution.



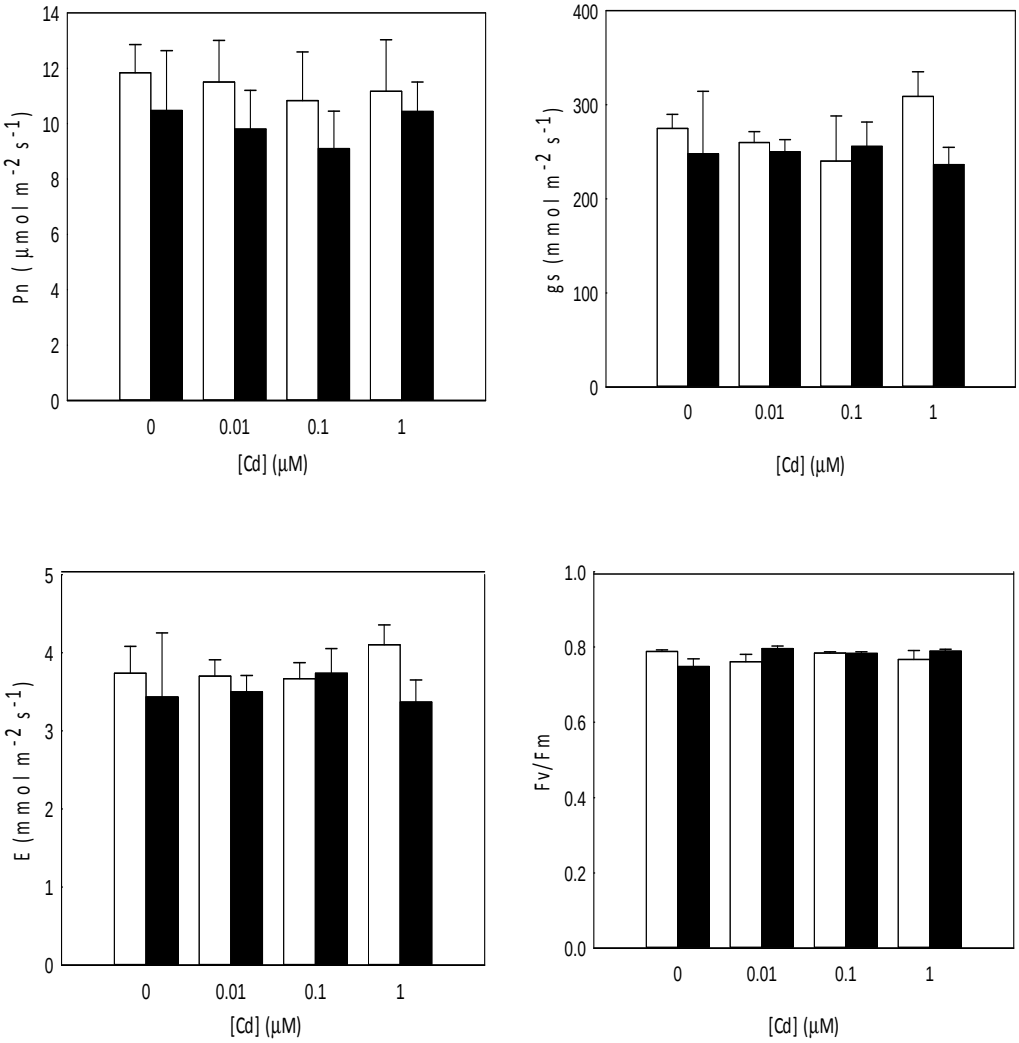
Plants were grown for 10 days in a complete nutrient then supplemented with 10 µM $CdCl_2$. At different times 24h, 48h, 72h and 96h after the addition of Cd shoots were separated from roots and the xylem sap exuded from the cut (root side) surface was collected. Data are means and SE of two experiments each performed with 8 plants (*n* = 16).

Figure 10. Effect of Cd exposure to low Cd concentrations on the growth of shoot (A) and root (B) of barley cvs Manel and Lemsi plants.



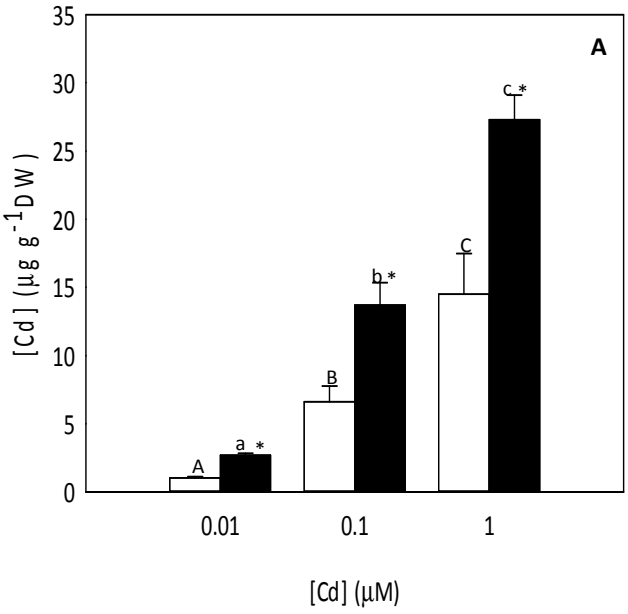
Manel (white bars) and Lemsi (black bars) plants were grown for 12 d in a complete nutrient solution in the absence of Cd. After this period the plant were grown for further 10 d in the same nutrient solution supplement or not with 0.01, 0.1 or 1 µM $CdCl_2$. Bars and error bars are means and SE of two experiments each performed with at least six plant for each variety (n=12). Bars marked with same letter are not significantly different at $P \leq 0.05$.

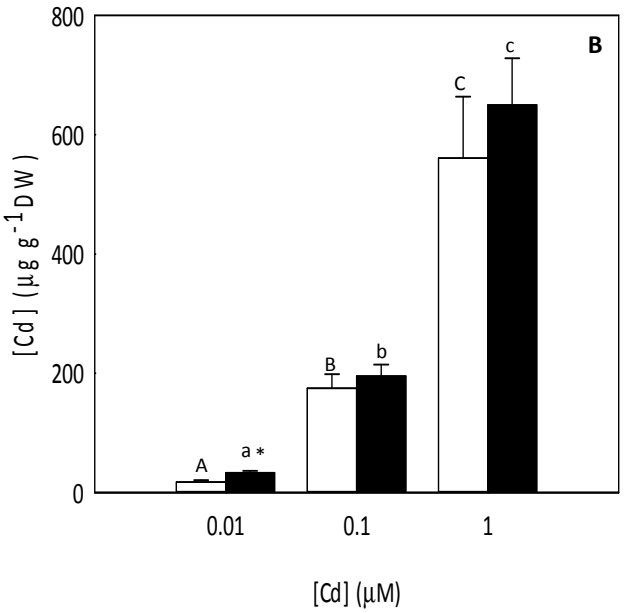
Figure 11. Effect of the presence of low Cd concentrations in the nutrient solution on gas exchange and chlorophyll parameters in barley cvs Manel and Lemsi plants.



Manel (white bars) and Lemsi (black bars) plants were grown for 12 d in a complete nutrient solution in the absence of Cd. After this period the plant were grown for further 10 d in the same nutrient solution supplement or not with 0.01, 0.1 or 1 µM $CdCl_2$. Bars and error bars are means and SE of two experiments each performed with at least six plant for each variety (n=12). Bars marked with same letter are not significantly different at $P < 0.05$.

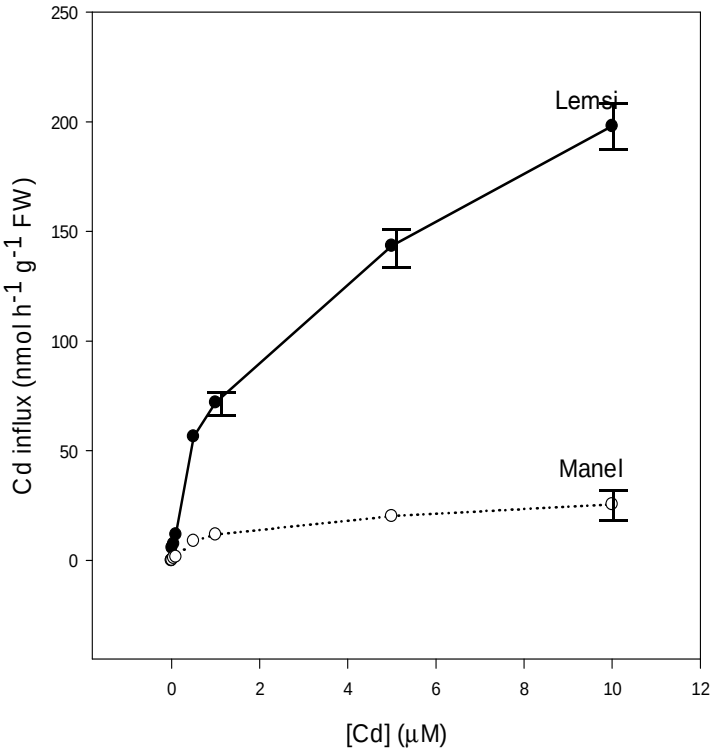
Figure 12. Cadmium accumulation in the shoot (A) and in the root (B) of barley cvs Manel and Lemsi plants grown in the presence of 0.01-1 µM Cd in the nutrient solution.





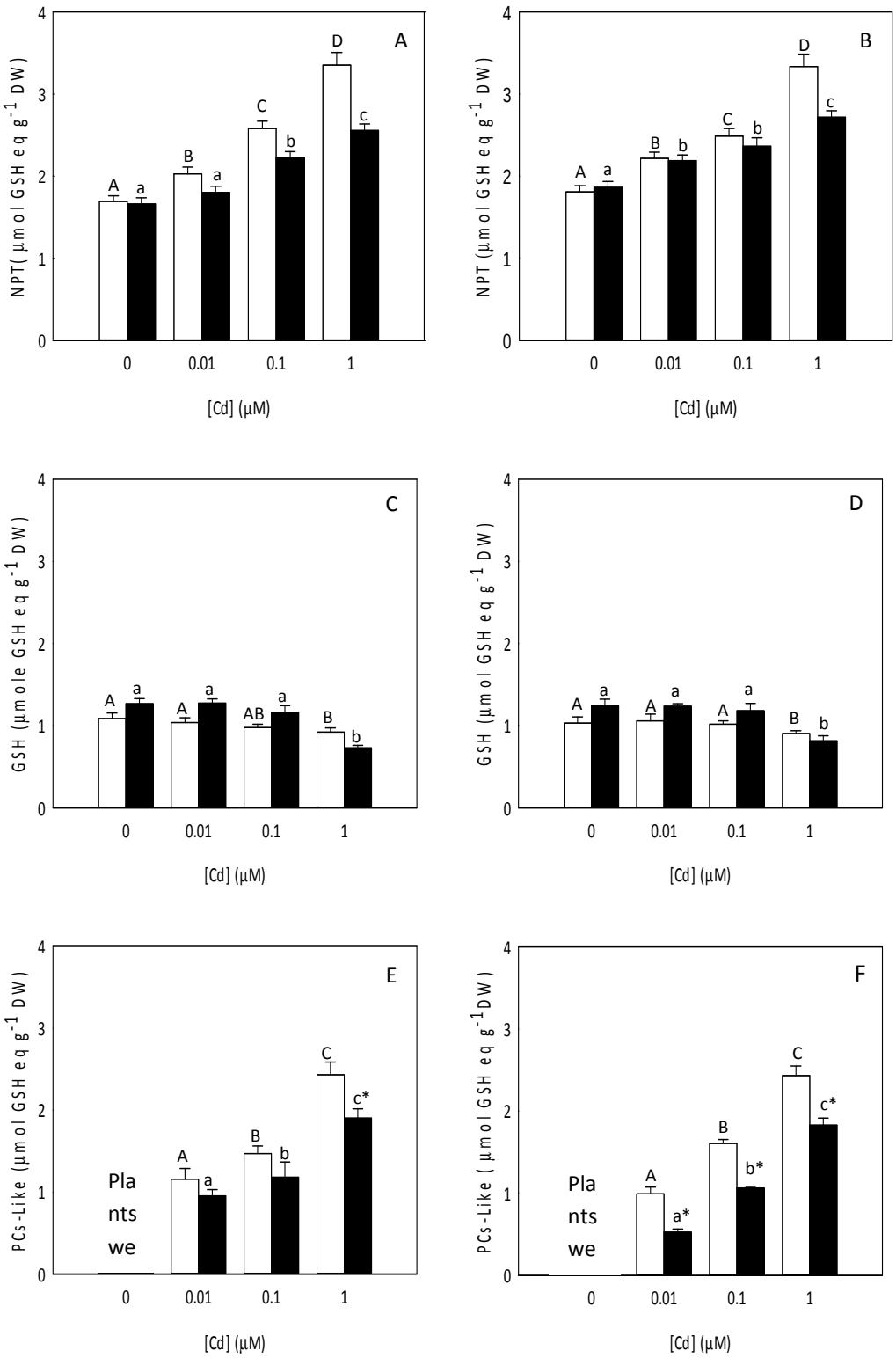
Manel (white bars) and Lemsi (black bars) plants were grown for 12 d in a complete nutrient solution in the absence of Cd. After this period the plant were grown for further 10 d in the same nutrient solution supplement or not with 0.01, 0.1 or 1 µM Cd. Plants were harvested and their Cd content were measured, after complete mineralization. Bars and error bars are means and SE of three experiments each performed with at least six plant for each variety (n = 18). Asterisks indicate the existence of significant differences ($p < 0.05$) between the two cultivars.

Figure 13. Concentration-dependent kinetics of ^{113}Cd uptake in roots of barley cvs Lemsi and Manel



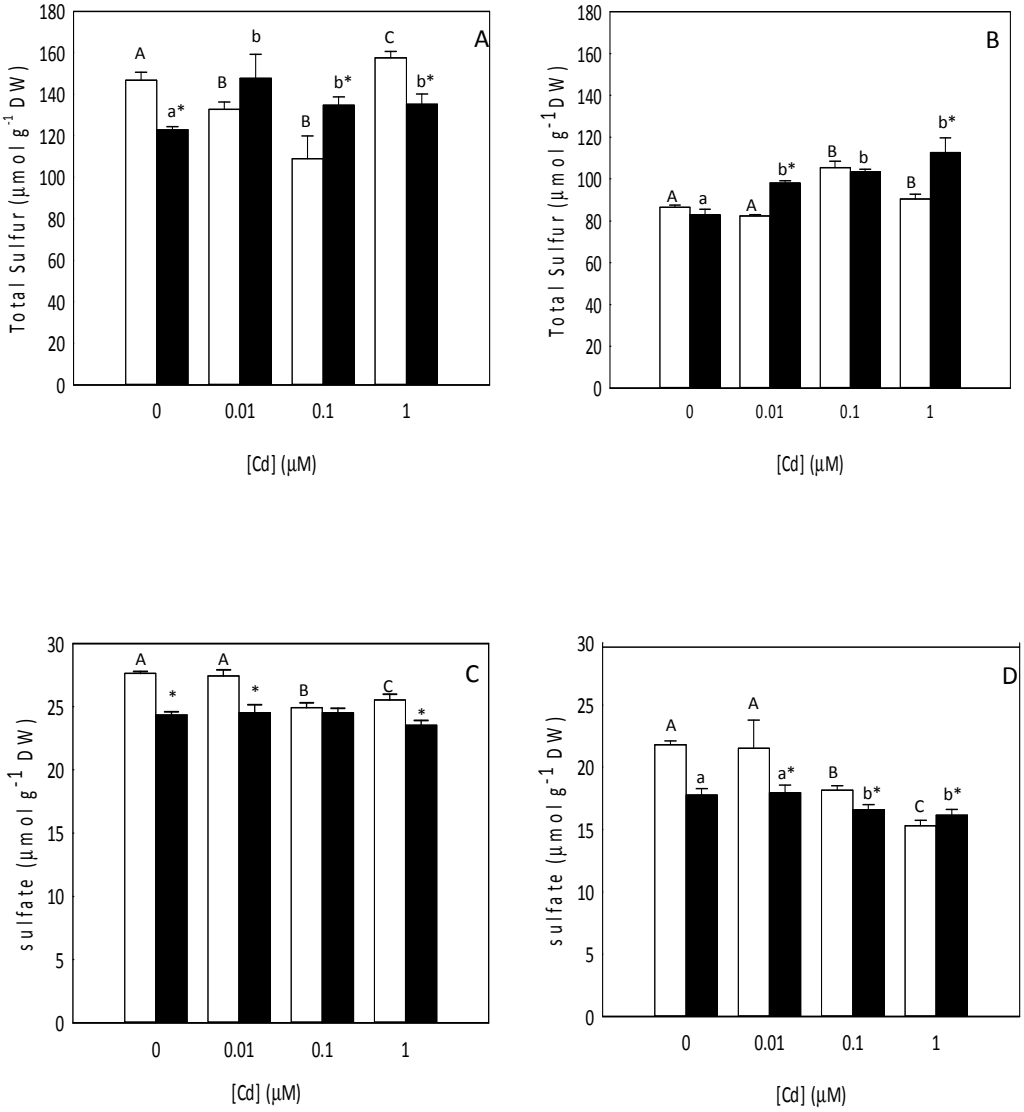
The influxes were determined incubating roots from 10-d old Manel (○) or Lemsi (●) plants grown in the absence of Cd at 0.01-10 µM ^{113}Cd. At the end of incubation time (2 h) the roots were washed twice at 4°C for 15 min with a corresponding ^{114}Cd solution. The roots were mineralized and their content in ^{113}Cd was detected by ICP-MS spectrometry. Data are the mean and SE of two experiments with four samples for each concentrations; where not present bars of SE are smaller than the data point.

Figure 14. Non-protein thiols (NPTs), GSH pool and PCs-like NTPs in shoots (A, C, E, respectively) and roots (B, D, F, respectively) of barley cvs Manel and Lemsi plants grown in the presence of increasing Cd concentrations in the nutrient solution.



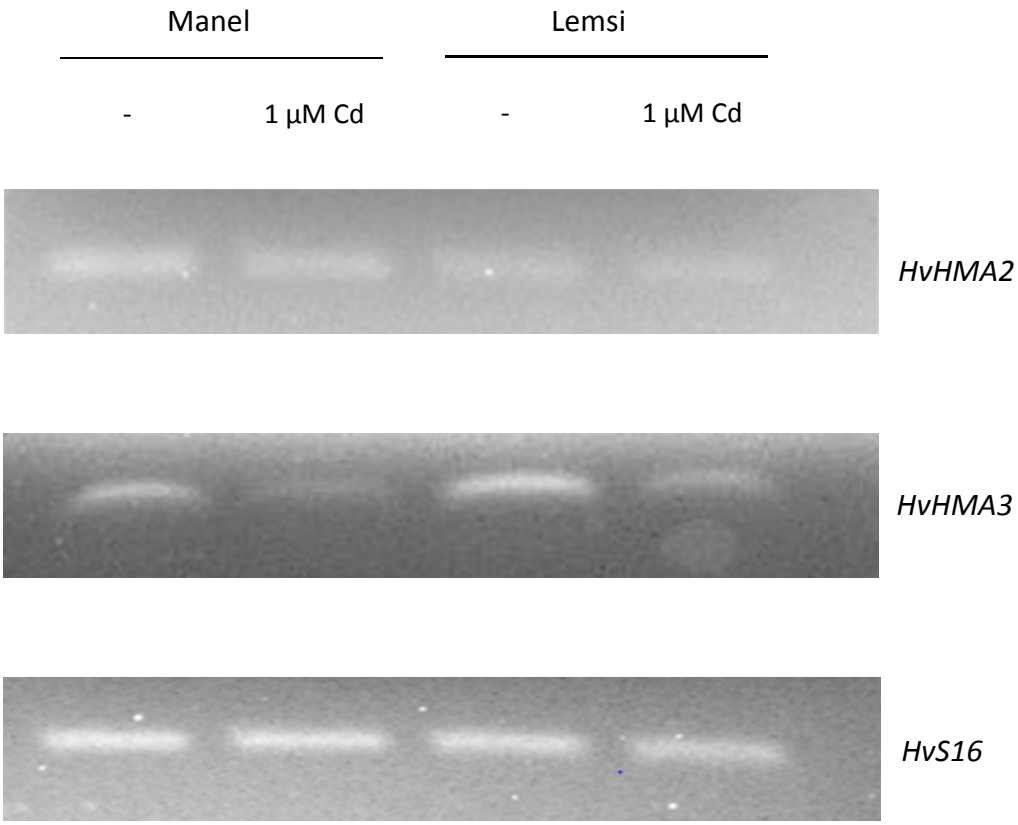
Plants of Manel (white bars) and Lemsi (black bars) cultivar were exposed to different Cd concentrations (0.01, 0.1 and 1 µM) for 10 days. NPT levels are expressed as GSH equivalent. Bars and errors bars are means and SE of three experiments run in triplicate (n = 9) at $P \leq 0.05$. Bars with different letters represent values statistically different. Asterisks indicate the existence of significant differences between the two cultivars.

Figure 15. Total sulfur and sulfate levels in shoots (A and C, respectively) and root (B and D, respectively of barley cvs Manel and Lemsi plants growth in the presence of increasing Cd concentration in the nutrients solutions.



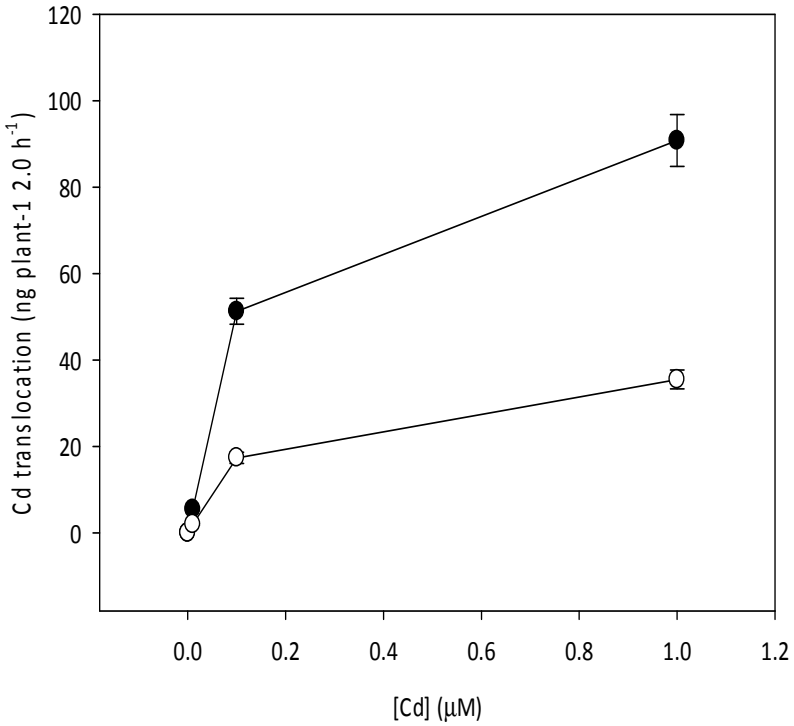
Manel (white bars) and Lemsi (dark bars) plants were exposed to different Cd concentrations (0.01, 0.1 and 1 µM) for 10 days. Bars and errors bars are means and SE of three experiments run in triplicate (n=9) (p < 0.05). Bars with different letters represent values statistically different at $P \leq 0.05$.. Asterisks indicate the existence of significant differences between the two cultivars ($P \leq 0.05$

Figure 16. RT-PCR analysis of *HvHMA2* and *HvHMA3* gene expression in the roots of barley cvs Manel and Lemsi plant grown in the presence of 1 µM Cd in the nutrient solution.



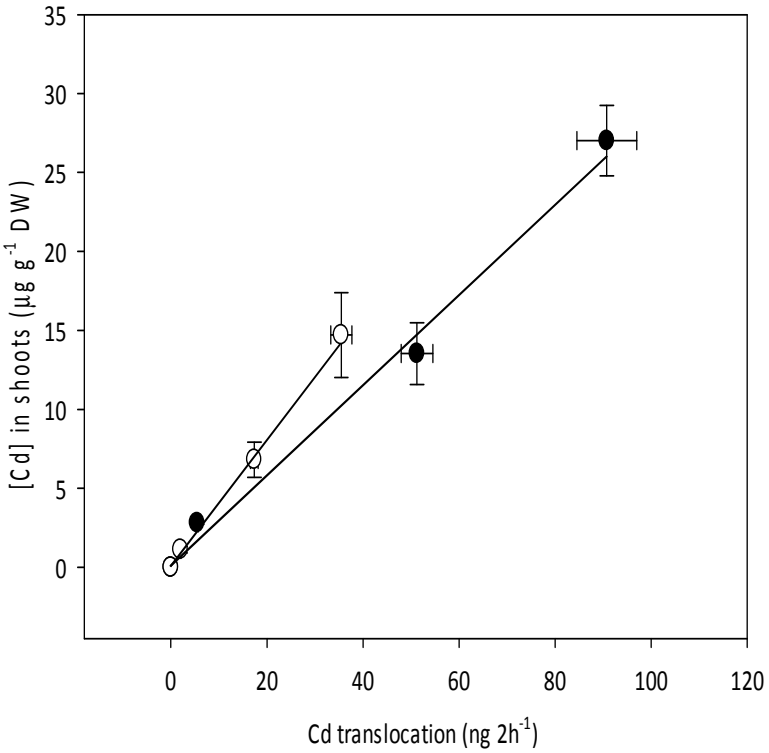
PCRs were carried out for 30 cycles where cDNAs were exponentially amplified. For *HvHMA2* and *HvHMA3 genes* primers PCR products were separated in agarose gel and stained with synergy brands (SYBR) Green I. Signals were detected using a laser scanner with 532 nm laser and 526 nm filter. *HvHMA2*, heavy metal P1B-ATPase 2; *HvHMA3*, heavy metal P1B-ATPase 3; *HvS16,* S16 ribosomal protein.

Figure 17. Analysis of Cd translocation in Manel (○) and Lemsi (●)plants grown for 10 d in the presence of increasing Cd concentrations in the nutrient solution. Cadmium ions loaded and transported in the xylem sap during 2 h.



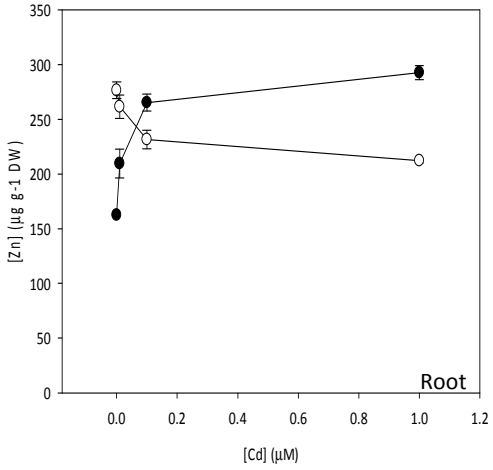
At the end of the exposure period, shoots were separated from roots and the xylem sap exuded from the cut (root side) surface was collected. Data are means and SE of two experiment each performed with 16 plants (*n*= 32).

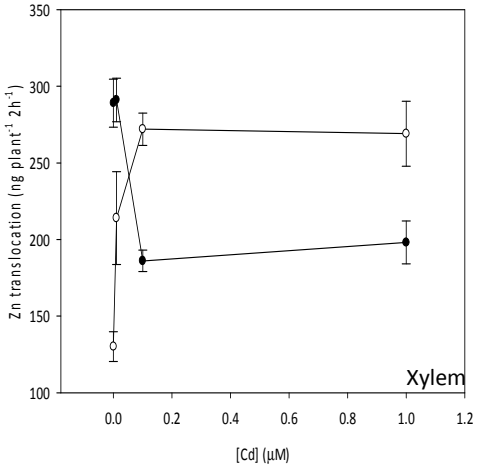
Figure 18. Relationships between Cd ions loaded in the ylem sap and Cd concentration in the shoots of barley cvs Manel and Lemsi

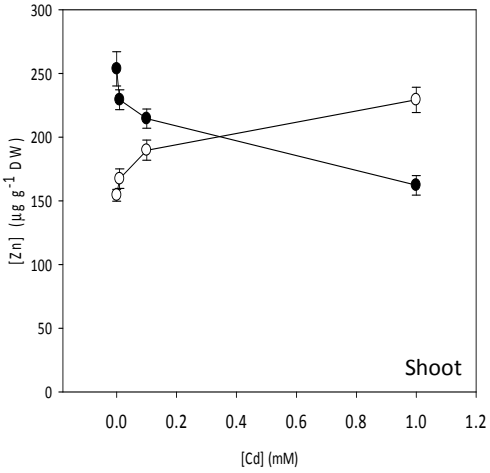


Data points and SE from Fig. 10 and Fig.15. Manel (○) r^2: 0.998; Lemsi (●) r^2: =0.992 .

Figure 19. Zinc level in the root and shoot and amount of Zn loaded and transported during 2 h in the xylem of Manel (○) and Lemsi (●) barley plants grown for 10 d in the presence of increasing Cd concentrations in the nutrient solution.

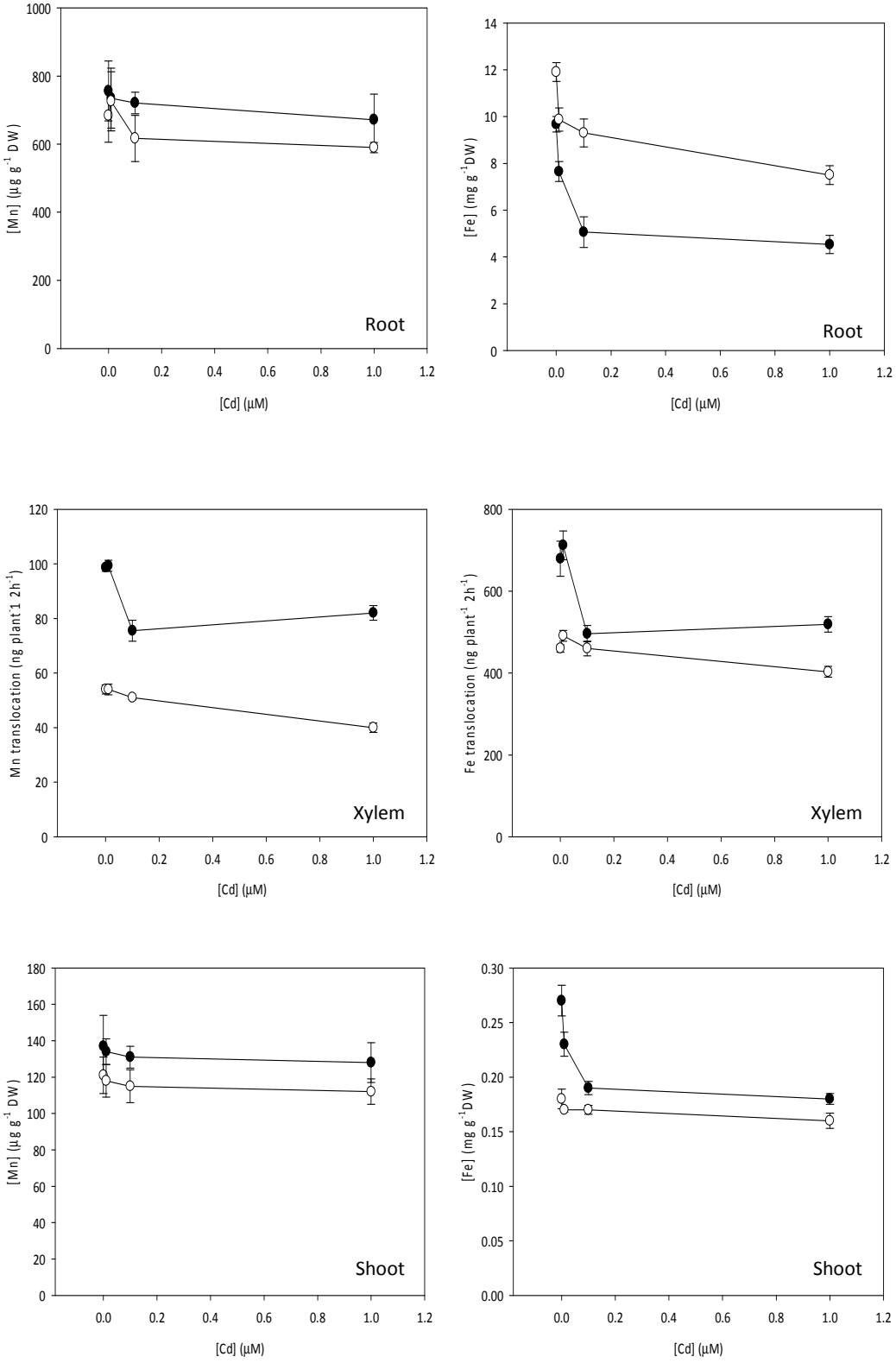






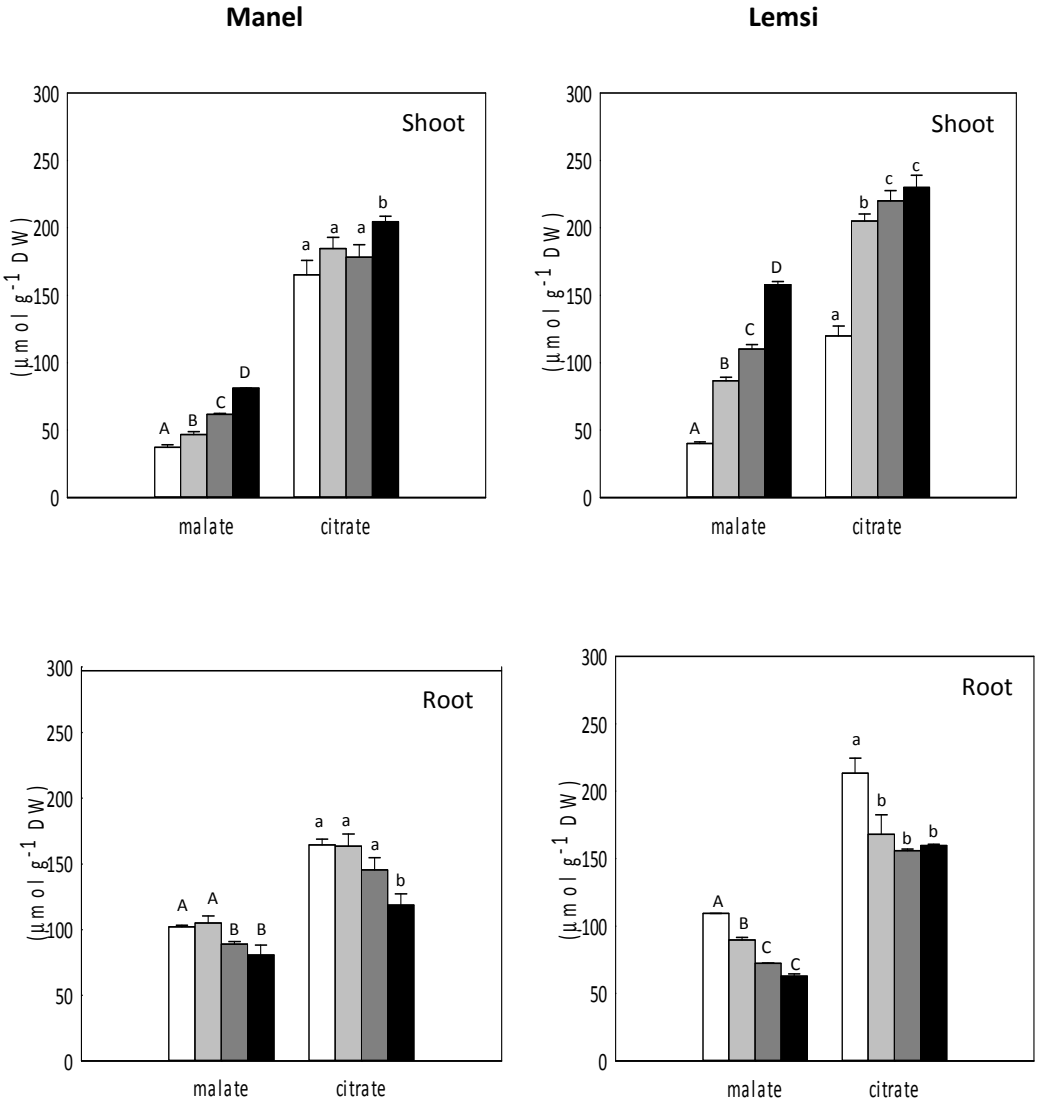
Plants were harvested and root and shoot Zn content were measured, after complete mineralization. At the end of the exposure period, shoots were separated from roots and the xylem sap exuded from the cut (root side) surface was collected. Data are means and SE of two experiment each performed with 16 plants (*n*= 32).

Figure 20. Micronutrients (Mn and Fe) levels in the root and shoot and their amount loaded and transported during 2 h in the xylem of Manel (○) and Lemsi (●) barley plants grown for 10 d in the presence of increasing Cd concentrations in the nutrient solution.



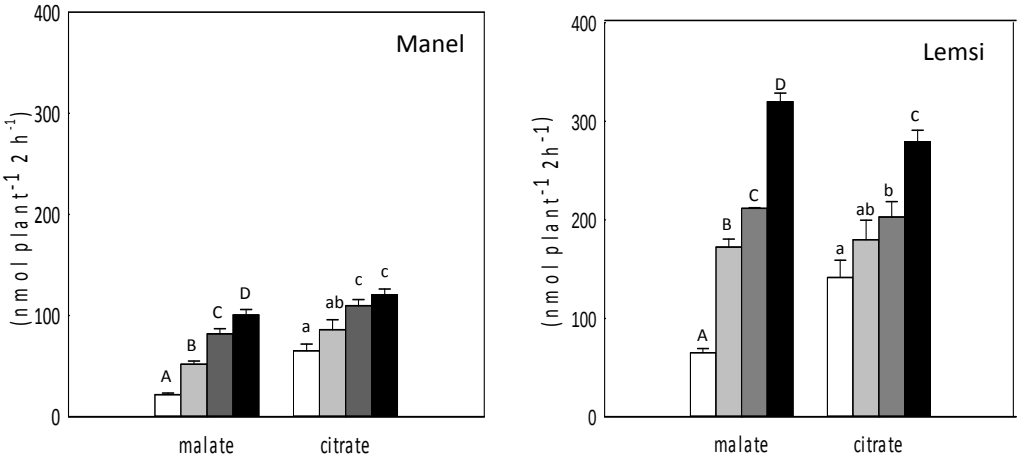
Plants were harvested and root and shoot Mn and Fe content were measured, after complete mineralization. At the end of the exposure period, shoots were separated from roots and the xylem sap exuded from the cut (root side) surface was collected. Data are means and SE of two experiment each performed with 16 plants (*n*= 32).

Figure 21. Concentrations of malate and citrate in the shoots and the roots of Manel and Lemsi barley cultivars grown for 10 d in the presence of 0-1 µM Cd.



Data are the mean ± SE of two experiments run in quadruplicate (*n*= 8). Bars with different letters represent values statistically different at $P \leq 0.05$.

Figure 22. Concentrations of malate and citrate in the shoots and the roots of Manel and Lemsi barley cultivars grown for 10 d in the presence of 0-1 µM Cd.



Data are the mean ± SE of two experiments run in quadruplicate (*n*= 8). Bars with different letters represent values statistically different at $P \leq 0.05$.

Annexes

Hordeum vulgare heavy metal transporter (HMA2) mRNA, complete cds

(http://www.ncbi.nlm.nih.gov/nuccore/295881651)

AUTHORS *Mills,R.F., Peaston,K.A., Runions,J. and Williams,L.E.*

TITLE *HvHMA2, a P(1B)-ATPase from Barley, Is Highly Conserved among Cereals and Functions in Zn and Cd Transport*

JOURNAL *PLoS ONE 7 (8), E42640 (2012)*

LOCUS GU177852 3163 bp mRNA linear PLN 15-AUG-2012

DEFINITION Hordeum vulgare heavy metal transporter (HMA2) mRNA, complete cds.

ACCESSION GU177852

VERSION GU177852.1 GI:295881651

SOURCE Hordeum vulgare

ORGANISM Hordeum vulgare

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEPclade; Pooideae; Triticeae; Hordeum.

Translation

"MAAPAPAAAGKLEKSYFDVLGICCPSEVPLVEKLLEPLAGVHKVTVVVPSRTVIVLHDAAAISQAQIVRALNGARLEASVRA
YGGAGQSKVTNKWPSPYVLVCGVLLVVSLFEHFWRPLKWFAVAGAAAGLPPIILRSVAALRRRTMDVNILMLIAVAGAIAL
KDYSEAGFIVFLFTIAEWLETRACGKATAGMSSLMSMAPQNAVLAETGQVVATQDVKINTVIAVKAGEIVPIDGVVVDGRS
EVDESTLTGESFPVSKQADSQVWAGTLNIDGYIAVRTTAMADNSAVAKMARLVEEAQNNRSSTQRLIDTCAKYYTPAVIF
MSAAVAVIPVCLKARNLKHWFELALVLLVSACPCALVLSTPVATFCALLRAARTGLLIKGGDVLESLASIKVAAFDKTGTITRG
EFSVEEFQTVGERVSKQQLLYWVSSIESRSSHPMAAALVGYAQSNSVEPKSENVAEFQMYPGEGIYGEIGGEGVYVGNKRI
LARASCQIVPDIVEHMKGVTIGYVACNKELIGVFSLSDSCRTGSAEAIKELRSLGIKSVMLTGDSTAAATHAQNQLGNILAEV
HAELLPEDKVRIVDELKARDGPTLMIGDGMNDAPALAKADVGVSMGVSGSAVAMETSHITLMSNDIRRIPKAIKLARRTH
RTIVVNIVFSVTTKLAIVALAFAGHPLIWAAVLADVGTCLLVIMYSMLLLREKGSGKVAKKCCASSHSKKHGHRTTHHCSDG
HHHENVSTGGCVDSSAGKHSCHDHHHEHDHHKEPSNLHSVDKHGCHDHGHVHSHCKEPSSQMVTSKDVAHGHGHTH
NICNPHPAANKHDCHDHEHSHHQEPNSSHSADEHDCHGHKHCEEPTSLLCATEHACHDHDQNHEHHCCDEEKTVHVAD
THSCHDHKHEQGAADSVPELSIWIEGQSPDHREQEIQCSTEHKEEACGHHLKVKDQVPAKTDCSRGGCHGTASSKTCESK
GKNVCSSWPVGRTGVVRRCCRTRTHSCCSQSMLKLPEIIVG"

Origin

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61 ggcagaggga gggaggcagg tgagccgccc aaacaagtcg ggagccaacg agtgaacaga

121 gagagagaga acgatggcgg caccggcgcc ggcggcggcg ggaaagctgg agaagagcta

181 cttcgacgtg ctgggcatct gctgcccgtc ggaggtgccg ctggtggaga agctcctcga

241 gccgctcgcc ggcgtgcaca aggtcaccgt cgtcgtcccc tcccgtaccg tcatcgtcct

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361 ggaggcgtcg gtgagggcgt acggcggcgc cgggcagagc aaggtgacca acaaatggcc

421 gagcccctac gtgctcgtct gcggggtcct gctcgtcgtc tcgctcttcg agcacttctg

481 gcggccgctc aagtggttcg ccgtggcggg ggcggccgcc gggctgcctc ccatcattct

541 ccggagcgtc gccgcgctcc ggcgacgcac catggacgtc aacatactca tgctcatcgc

601 agttgctggg gccatagctc tcaaggacta ctccgaggct gggttcatcg tcttcctctt

661 caccatagcc gaatggctcg aaaccagggc gtgcggcaag gccactgctg ggatgtcgtc

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901 cccggtgtcc aagcaggcag actcccaggt ctgggctggc acactcaaca tagatggtta

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1081 gtactacaca cctgctgtta ttttcatgtc tgcagcagtg gcagtgatcc ctgtgtgtct

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1381 gacagttggt gagcgtgttt cgaagcaaca acttctatac tgggtttcaa gcatcgagag

1441 caggtcgagc cacccaatgg cagctgctct tgttggttat gctcaatcaa actccgtgga

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1561 aattggtgga gagggcgtat atgttgggaa caaaaggatc ttggcaaggg catcgtgtca

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1861 tctaccagaa gacaaagtga gaattgttga tgaactgaag gcaagagatg gccctacact

1921 gatgattggc gatggcatga atgatgcccc agcactggcc aaggctgatg ttggagtttc

1981 catgggcgtg tccggttcag ccgtcgcaat ggagacgagt cacattactc tgatgtcgaa

2041 tgacatccgc aggatcccaa aggctatcaa gctggccagg aggactcacc ggaccatcgt

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2161 tcatccgctt atttgggcag cagtccttgc tgatgttggt acatgcttgt tggtgatcat

2221 gtacagcatg ctgctactga gagagaaagg cagtggaaag gtggcgaaga aatgctgtgc

2281 ttcttctcac tcaaagaagc atgggcaccg aactacccac cactgctcag atggtcatca

2341 ccatgagaat gtatcaacag gcggttgcgt ggattcgtct gcaggtaagc attcttgcca

2401 tgatcatcac catgagcatg accaccacaa agagccgagc aacctgcatt ccgtagacaa

2461 gcatggctgc catgatcatg gtcatgttca tagccactgc aaagagccga gcagccagat

2521 ggtcacaagc aaggatgttg cccatggaca tggccatacc cacaacatct gcaaccctca

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2641 tagttcacat tctgccgatg agcatgattg ccatggtcac aagcactgtg aagaaccaac

2701 cagcttgctt tgtgccactg agcatgcttg ccatgaccat gaccagaacc atgagcatca

2761 ctgctgtgat gaagagaaaa cagtccatgt tgcagatacg cattcctgcc acgaccataa

2821 gcatgagcag ggtgcagctg attcagttcc agagctatcg atatggatcg agggtcaatc

2881 ccctgatcac cgtgagcagg aaattcaatg cagcacagaa cacaaagagg aagcgtgtgg

2941 gcatcacctg aaggtcaagg atcaggtccc agctaagaca gattgcagca gggggggctg

3001 tcacggtacc gcgagcagca aaacctgcga aagcaaaggt aaaaatgttt gttcaagctg

3061 gccggttggt cgcaccggag ttgtccgccg gtgttgcagg actagaacgc acagctgctg

3121 cagccaaagc atgttgaaac tacctgagat aatagtagga tga

//

Hordeum vulgare subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv2092N22

(http://www.ncbi.nlm.nih.gov/nuccore/AK369525)

AUTHORS *Matsumoto,T., Tanaka,T., Sakai,H., Amano,N., Kanamori,H.,Kurita,K., Kikuta,A., Kamiya,K., Yamamoto,M., Ikawa,H., Fujii,N.,Hori,K., Itoh,T. and Sato,K.*

TITLE *Comprehensive Sequence Analysis of 24,783 Barley Full-Length cDNA Derived from 12 Clone Libraries*

JOURNAL *Plant Physiol. 156 (1), 20-28 (2011)*

LOCUS AK369525 2729 bp mRNA linear PLN 20-MAY-2011

DEFINITION Hordeum vulgare subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv2092N22.

ACCESSION AK369525

VERSION AK369525.1 GI:326526674

KEYWORDS FLI_CDNA; CAP trapper.

SOURCE Hordeum vulgare subsp. vulgare (domesticated barley)

ORGANISM Hordeum vulgare subsp. Vulgare Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEPclade; Pooideae; Triticeae; Hordeum.

Translation

"MTGSGESYPALEASLLSDEAAASARRKWEKTYLDVLGVCCSAEVALVERLLAPLDGVRAVSVVVPSRTVVVEHDPSAVSQS
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NILMLIAVVGAVALKDYTEAGVIVFLFTTAEWLETLACTKASAGMSSLMSMIPPKAVLAETGEVVNVRDIDVGAVIAVRAGE
MVPVDGVVVDGQSEVDERSLTGESYPVPKQPLSEVWAGTLNLDGYIAVRTSALAENSTVAKMERLVEEAQQSKSKTQRLI
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RATARSLEMRRSQLAAVSPDAATKSVGKTGGDASKGCHCCHKPIKSPEHSVVINVRVDEQREGPTDATCTPAKNVEVTGL
VDASVMPASSSCVSGGGCCSREKTGRNM"

Origin

1 gaaaatcatc cctggttctg tcgttgactt attgctatct ctcctttgcc tttgtttttt

61 gctgttcgtc cgccggcgtc tccggatcga tgacgggcag cggcgagtcg tacccggcgc

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181 cgtacctgga cgtgctgggc gtgtgctgct cggcggaggt cgcgctcgtc gagcgtctgc

241 tggcgccgct cgacggcgtg agggcggtgt ccgtcgtcgt cccctcccgc accgtcgtgg

301 tcgagcacga cccctccgcc gtctcgcagt cccgtattgt gaaggtcctg aacggggcgg

361 gcctggaagc ctcggtgcga gcctacggca gcagcggggt catcggccga tggcccagcc

421 cgtacatcgt cgcctgcggc gccctcctcc tcgcatcctc cttcaggtgg ctcctgcctc

481 ccctgcagtg gctggccctg ggggcggcct gcgccggcgc tcccccgatg gttctccgag

541 ggttcgccgc cgccagcagg ctcgcgctgg acatcaacat tctcatgctt atcgctgttg

601 tcggtgccgt cgcgctcaag gactacacgg aggcaggcgt catcgtcttc ctcttcacca

661 ctgcagagtg gctcgagacc ctggcctgca ccaaggccag cgccgggatg tcgtcgttga

721 tgagcatgat cccgccgaag gcagtcctcg ccgagacggg cgaggttgtc aatgtacgcg

781 acatcgatgt cggcgccgtc atcgcggtca gagcagggga gatggtgccg gtggacggcg

841 tggttgtcga cgggcagagt gaggtcgacg aaaggagcct caccggcgag tcgtacccgg

901 tgcccaagca accgctgtcc gaggtctggg ccggcacgct caacttggac ggttacatcg

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1021 aagaggcgca gcagagcaag tccaagacgc agcggctgat cgattcctgc gccaagtact

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1561 acgggaagcg catctacgtc gggaacaaga gggtcttggc aagggcatcc tcctgtcaga

1621 cagcagttcc agaaagaatg aatggtctga aaggcgtctc gatcggctac gtgatctgcg

1681 acggggacct cgtcggggtg ttctcgctct ccgacgactg ccggaccggc gcggccgaag

1741 cgattcgaga gctggcgtcc atgggcatca gctcagtgct gctcacgggg gacagcgcgg

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1861 tcttcccgga ggacaaggtc cggctggtga gtgcggtgaa ggcgagggtt ggcccgacaa

1921 tgatggtcgg cgacggcatg aacgacgccc cggcgctggc gatggcggac gtgggcgtct

1981 ccatgggcat ctccggctcg gccgcggcca tggagaccag ccatgcgacg ctcatgtcta

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2101 ccgtaaacat ggtgtcctcg gtggccgcca aggtcgccgt cctcgcgctc gcgctcgcct

2161 ggcgcccggt gctgtgggca gcggtgctcg ccgacgtggg gacgtgcctg ctcgtcgtgc

2221 tcaacagcat gctgctgctg ggggaggggg gcggacgccg cggaaaggag gaggcgtgcc

2281 gcgccacggc taggtcgctg gagatgagaa ggtctcaact cgccgccgtt tcaccggacg

2341 ctgccactaa aagcgttgga aagacgggcg gcgacgcatc gaaaggctgc cattgttgcc

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2461 gtgaagggcc gacggacgcg acatgtacgc cggctaaaaa tgtcgaggtc accggacttg

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2581 gtgaaaaaac aggtaggaac atgtagcaag ggtagcgtgc aaggagagtt aatttgcaaa

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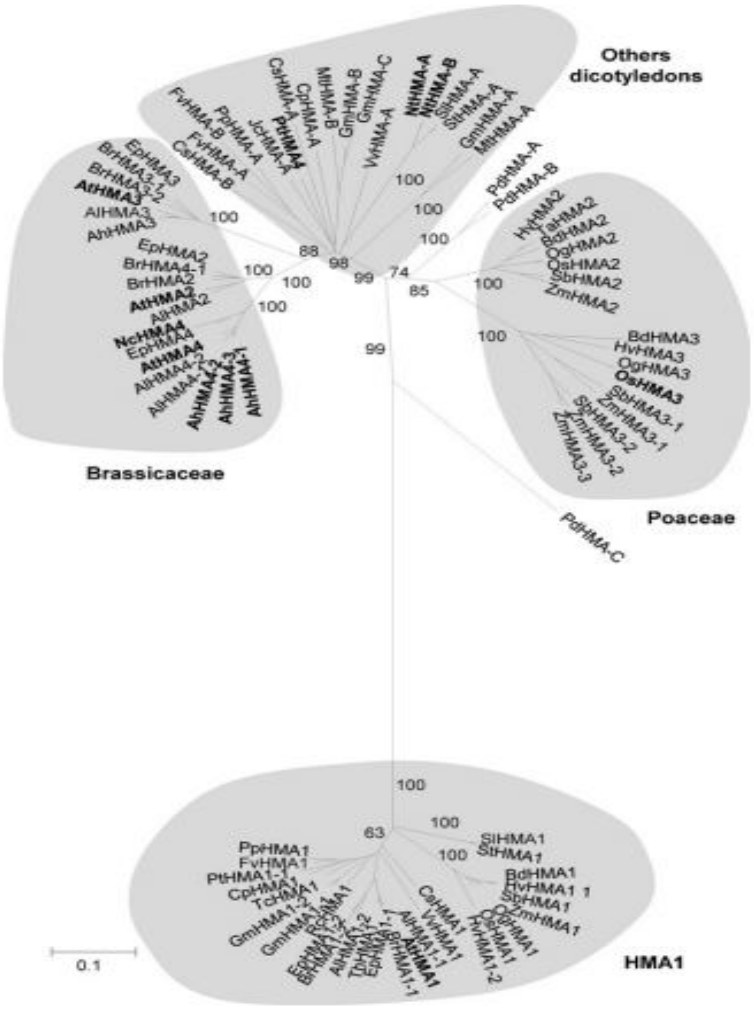

Phylogenetic analysis of the plant Zn/Cd/Pb/Co P1B -ATPase family showing families of P1B-ATPases in barley (*Hordeum vulgare* L.) adopted from Zorrig et al. (2011).

Invitrogen Custom Primers

Primer 1:

Primer Name: Hvhma2Dir

Sequence (5' to 3')GGC AAG AGA TGG CCC TAC ACT G

Molecular weight (µg/µmole)	6770.4
Micromolar Extinction Coeff (OD/µmol)	246.7
Purity	Desalted
Tm (1M Na^+)	75
Tm (50 mM Ma^+)	53
% GC	59
Primer number:	E3314H07 (H07)
Primer length:	22
Scale of synthesis:	50 nmol
µg per OD:	27.4
nmoles per OD:	4.1
OD'^{s}	10.10
$µg'^{s*}$	277.18
nmoles	40.9
coupling Eff.	99%

Primer 2:

Primer Name: Hvhma2Rev

Sequence (5' to 3')GGC AAG AAT GCT TAC CTG CAG ACG

Molecular weight (µg/µmole)	7387.8
Micromolar Extinction Coeff (OD/µmol)	271.3
Purity	Desalted
Tm (1M Na^+)	76
Tm (50 mM Ma^+)	54
% GC	54
Primer number:	E3314H08 (H08)
Primer length:	24
Scale of synthesis:	50 nmol
µg per OD:	27.2
nmoles per OD:	3.7
OD'^s	13.30
$µg'^{s*}$	362.17
nmoles	49.1
coupling Eff.	99%

Primer 3:

Primer Name: Hvhma3Dir

Sequence (5' to 3')CGA GCC TTC TGT CCG ACG AAG C

Molecular weight (µg/µmole)	6697.4
Micromolar Extinction Coeff (OD/µmol)	228.4
Purity	Desalted
Tm (1M Na^+)	77
Tm (50 mM Ma^+)	55
% GC	64
Primer number:	E3314H09 (H09)
Primer length:	22
Scale of synthesis:	50 nmol
µg per OD:	29.3
nmoles per OD:	4.4
OD'^s	9.50
$µg'^{s*}$	278.57
nmoles	41.6
coupling Eff.	99%

Primer 4:

Primer Name: Hvhma3Rev

Sequence (5' to 3')AGG ACC TTC ACA ATA CGG GAC TGC

Molecular weight (µg/µmole)	7347.8
Micromolar Extinction Coeff (OD/µmol)	266.9
Purity	Desalted
Tm (1M Na^+)	76
Tm (50 mM Ma^+)	54
% GC	54
Primer number:	E3314H10 (H10)
Primer length:	24
Scale of synthesis:	50 nmol
µg per OD:	27.5
nmoles per OD:	3.8
OD'^s	11.20
$µg'^{s*}$	308.34
nmoles	42.0
coupling Eff.	99%

Primer 5:

Primer Name: HvS162Dir

Sequence (5' to 3')GCT CCC GCT TCA AGG ACA TCG A

Molecular weight (µg/µmole)	6681.4
Micromolar Extinction Coeff (OD/µmol)	231.9
Purity	Desalted
Tm (1M Na^+)	75
Tm (50 mM Ma^+)	53
% GC	59
Primer number:	E3314H11 (H11)
Primer length:	22
Scale of synthesis:	50 nmol
µg per OD:	28.8
nmoles per OD:	4.3
OD'^s	10.30
$µg'^{s*}$	296.76
nmoles	44.4
coupling Eff	99%

Primer 6:

Primer Name: HvS162Rev

Sequence (5' to 3')AAC TTC TTG GGC TCG CAG CGA C

Molecular weight (µg/µmole)	6712.4
Micromolar Extinction Coeff (OD/µmol)	230.3
Purity	Desalted
Tm (1M Na^+)	75
Tm (50 mM Ma^+)	53
% GC	59
Primer number:	E3314H12 (H12)
Primer length:	22
Scale of synthesis:	50 nmol
µg per OD:	29.2
nmoles per OD:	4.3
OD'^s	9.50
$µg'^{s*}$	276.89
nmoles	41.2
coupling Eff	99%

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