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SYSTEMIC ALLOCATION OF TRACE ELEMENTS IN RICE

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EXTENDED ABSTRACT

Several studies have shown that Cd, a non-essential and toxic metal, is taken up from soil and translocated in a root-to-shoot direction through transporters of essential elements such as Zn, suggesting that the two metal ions may compete for the same transporter protein on a membrane. However, the movement of Zn and Cd ions across several biological membranes involves a wide range of transport systems, each characterized by a specific selectivity. Although divergent results have been obtained, they suggest that Zn-independent pathways for Cd translocation in plants could be possible.

The proteins belonging to the HMA (Heavy-Metal ATPases) family have been partially characterized as the main actors of the process of translocation of trace elements (essential or non-essential) to all organs of the plant. In particular, OsHMA2 is the main transport system so far described in rice as involved in the xylem loading of Zn and Cd, even though both its activity and function has not been unambiguously characterized.

The research carried out in this PhD project took place in this context. Indeed, the general purpose was studying the main mechanisms involved in the systemic distribution of some trace elements in rice plants. In particular the activity was aimed at better understanding the Zn and Cd translocation pathways, and was focused on studying the possible competition between the two metal ions mainly for the root-to-shoot translocation, since these processes have been seen to be crucial in determining Cd accumulation in the shoots. Specifically, the aims of this study were: (i) to investigate the effects of the possible competition between Zn and Cd on their chelation and subcellular compartmentalization at the root level, thus in reducing the amount of the two metals potentially mobile through the plant. This was done using physiological techniques aimed at isolating and quantifying thiol based Zn- and/or Cd-binding complexes; (ii) to investigate the potential inhibitory effect exerted by Zn on Cd translocation in unstressed rice plants, performing a short-term positron-emitting tracer imaging system (PETIS) experiment using ^{107}Cd as tracer; (iii) to identify genes encoding transporters involved in a putative Zn-insensitive Cd xylem loading, thus responsible for a possible Zn-independent Cd translocation pathway, by performing bioinformatic analysis. Our attention focused on the P_{1B}-type ATPase (HMA) family in order to search for orthologs of the genes codifying the transporters that in the model plant Arabidopsis were found to mediate the xylem loading of Cd; (iv) to functional characterize the transporters encoded by the abovementioned genes by heterologous expression in *Saccharomyces cerevisiae*.

A complete set of competition experiments were performed: in the first, rice plants (*O. sativa* L. ssp. *japonica* cv. Roma) were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations, in the absence or presence of a steady amount of Cd, whilst, in the second, plants were exposed for 10 days to different Cd concentrations in the presence of a steady amount of Zn. The concentrations of Zn and Cd in xylem sap, roots and shoots were evaluated by inductively coupled plasma-mass spectrometry (ICP-MS), to determine their partitioning between plant organs. The results were related to the total Zn and Cd content in root fractions obtained by a sequential extraction procedure with buffer and acid. The procedure allowed to discriminate Zn and Cd ions potentially mobile (cationic) from those retained in complexes with thiol-peptides or other soluble molecules negatively charged in the extraction buffer (anionic), or tightly adsorbed to cellular matrices or apoplast components (acid soluble and ash); so, the last three fractions should be considered not available for root-to-shoot translocation. Moreover, the systemic movement of Cd in the whole rice plants was monitored by applying to the roots fresh marked (^{107}Cd) culture solutions containing a steady amount of Cd and different concentrations of Zn in PETIS experiments.

The main results clearly indicate the lack of a fully reciprocity considering the effect of Cd on Zn accumulation, and vice versa, since the accumulation of Zn in the shoot was significantly inhibited by Cd increases in all the analyzed conditions, whereas those of Cd was only partially impaired by Zn increases. Such a finding suggests that Cd ions may use at least two distinct pathways to be translocated from the root to the shoot. The first one – shared with Zn – is probably used for Zn translocation in physiological conditions, whilst the second one appears as a Zn-independent route that Cd may preferentially use when the first pathway is saturated with Zn. Moreover, the Zn-independent pathway seems constitutively expressed in rice plants since the partial inhibitory effect exerted by Zn on Cd translocation was also observed in short-term PETIS experiments performed with unstressed plants.

Since OsHMA2 appears to play an important role in Zn/Cd root-to-shoot translocation, in this work we also contributed to elucidate some aspects related to the OsHMA2 transport activity and selectivity by comparing the inhibitory effects exerted by Zn or Cd on the growth of yeast cells expressing, or not, OsHMA2. The results indicate that OsHMA2 enhances Zn and Cd tolerance in yeast, so we can reasonably conclude that OsHMA2 may pump excess of cytosolic Zn or Cd into the apoplast and thus has all the requisites to be considered the xylem loading system potentially involved in mediating the translocation of Cd through the Zn-dependent pathway. In addition, this study represents one of the first examples of growth inhibition analysis applied to plant gene functional characterization.

In conclusion, our data provide several evidence to support the hypothesis that at least two competing pathways may be interested in mediating root-to-shoot Cd translocation in rice. The first one, prevailing at relatively low Zn concentrations, could involve OsHMA2 as Zn^{2+}/Cd^{2+} xylem loading system, while the second one appears to involve a Zn-independent system that still needs to be identified among the plethora of transporters involved in the metal homeostasis. The possible future identification of the transporter(s) responsible for the Zn-independent Cd translocation pathway(s) could allow the development of markers to select rice genotypes able to exclude Cd from the shoots. Furthermore, these activities could have important technological implications in the fields of food safety, especially in cases where the strategies used for containing Cd accumulation in the crops be founded on Zn fertilization.

INTRODUCTION

TRACE ELEMENTS

Among metal elements, copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) are micronutrients as they are essential in trace amounts for physiological processes in living organisms and therefore are a significant component of the soil–plant–food continuum (Teklić *et al.*, 2013). The essential trace elements play a wide range of critical roles in plants. Fe is a key component of haem proteins and a range of other enzymes. Cu is an integral component of certain electron transfer proteins in photosynthesis and respiration and is involved in lignification, while Mn is less redox active but is also involved in photosynthesis. Zn is non-redox-active but has a key structural and/or catalytic role in many proteins and enzymes. Other transition metals such as nickel (Ni) and molybdenum (Mo) are also essential micronutrients for plant functions. It must be emphasized that even if the most part of micronutrients play similar roles, they are not identical, so they cannot replace one another in the organism (Stiles, 1946; Marschner, 1995; Clemens, 2001, 2006; Hall and Williams, 2003; Kirkby and Römheld, 2004). When any of these metals is present in short supply, a range of deficiency symptoms can appear and growth is reduced (Marschner, 1995). However, although essential, when supplied in excess, these cations can become toxic, like heavy metals with no generally established function (e.g. cadmium, lead, arsenic, mercury, aluminum and silver). The non-essential trace elements are potentially toxic due to their reactivity with S and N atoms in amino acid side chains. They can be taken up from soil through the same transporters used for essential nutrients, and accumulated by crops. Such a process represents the main entry pathway for potentially health-threatening toxic metals into animal and human food chain. Thus, to maintain micronutrient homeostasis and to cope with the detrimental effects of non-essential metal ions, plants have developed a complex network of metal uptake, chelation, trafficking, and storage processes (Clemens, 2001; Hall, 2002; Hall and Williams, 2003).

There is a significant natural variation in the homeostatic mechanisms among crop species and in cultivars within the same species for what concern both essential and non-essential trace elements (Bell *et al.*, 1997; Clarke *et al.*, 1997). This variation results from several genetically controlled barriers that affect the abovementioned processes at different levels. Although many factors controlling these processes have been widely investigated and are now well known, the physiological basis for micronutrient use efficiency in crop plants and the processes controlling the accumulation of trace elements in the edible portions still need to be clarified (Welch and Shuman, 1995; Welch and Graham, 1999, 2004; Gregorio, 2002; Hall and Williams, 2003). The principal factors affecting the micronutrient use efficiency are here briefly discussed below. The first and most important barrier to micronutrient absorption resides at the root-soil interface (i.e. the rhizosphere) and several factors affect the trace element bioavailability, mostly depending on soil characteristics (e.g. pH and redox potential) (Gupta *et al.*, 2008) or due to some root activities, such as: (a) proton efflux from root cells, especially considering non-graminaceous plants presenting the mechanism of Fe uptake defined as 'strategy I'; (b) root

respiration leading to the release of carbon dioxide causing soil acidification; (c) efflux of reductive compounds; (d) plasma membrane reductase activity; (e) extrusion of organic acids, non-proteic amino acids and metal chelating phytosiderophores. In particular, the efflux of organic acids characterized by low molecular weight induces changes in the soil characteristics (mainly pH and redox potential) which, in turn, may affect metal solubility and mobility. However, the contribution of such compounds to the uptake of non-essential elements still need to be investigated in details (Prasad, 1995; Welch and Graham, 2004; Clemens, 2006). In fact, the total micronutrient content in soils is not related to that potentially available to the plant (Gupta *et al.*, 2008); then, the bioavailability of a trace element is defined as the fraction of the nutrient that is absorbed and subsequently utilized for physiological functions (Fairweather-Tait and Hurrell, 1996). Moreover, absorption mechanisms (e.g. transporters and ion channels), located in the root-cell plasma membrane, must be sufficiently active and specific enough to allow the accumulation of micronutrients once they enter the apoplasm of root cells from the rhizosphere. Then, the nutrients must be efficiently translocated from the root to the shoot and accumulated in edible plant organs. Finally, micronutrients must be bioavailable to people that eat these plant portions since plants are the main source of micronutrients for animals and humans, especially staple food crops (Fairweather-Tait and Hurrell, 1996; Welch and Graham, 2004).

Today, over three billion people worldwide are currently micronutrient (i.e. iron, iodine and zinc) malnourished ('Hidden Hunger') and the numbers are increasing (Gibson, 1994; Welch and Graham, 2004). Almost two-thirds of the deaths of children are associated with nutritional deficiencies, many from micronutrients deficiencies. This widespread issue results in poor health, increased rates of chronic diseases (coronary heart disease, cancer, stroke and diabetes), permanent impairment of cognitive abilities of infants born to micronutrient-deficient mothers and finally in high rates of mortality (Welch and Graham, 1999; Caballero, 2002). Most of people afflicted are dependent on staple crops for their sustenance. In fact the cereals, rice in particular, contain inherently low amounts of micronutrients and are eaten primarily after milling that removes most of the trace elements that cereals contain (Welch and Graham, 1999; Gregorio, 2002).

The staple crops can be "biofortified", meaning that the bioavailability of a micronutrient is increased using plant breeding and/or transgenic strategies, improving significantly the amount of these nutrients consumed by the world's poor without negatively impacting crop productivity (Bouis, 1996, 2000; Welch and Graham, 1999, 2004; Graham *et al.*, 2001, 2007). Other important goals of the research are enhancing substances (e.g. ascorbic acid and S-containing amino acids) that promote micronutrient bioavailability or decreasing the level of antinutrients (substances that reduce the bioavailability of trace metals to humans, e.g. phytate and polyphenolics) and limiting the detrimental effects of non-essential elements (e.g. Cd, As and Pb) on plants that have direct consequences on human health.

For the reasons abovementioned, agriculture needs a new paradigm based on food systems approaches aimed not only at productivity and sustainability, but also at empowering people and insuring balanced and adequate nutrition and improved health for all (Graham and Welch, 1996; Welch and Graham, 1999; Gregorio, 2002; Graham *et al.*, 2007).

ZINC HOMEOSTASIS

Zinc (Zn) is a micronutrient essential for growth and development of all organisms (Broadley *et al.*, 2007; Alloway, 2009). In plants Zn exists only as Zn(II) and does not take part in oxidoreduction reactions (Marschner, 1995). The non redox active property of Zn combined with its geometry makes it fundamental for an array of cellular processes (Berg and Shi, 1996; Broadley *et al.*, 2007; Palmer and Guerinot, 2009). In plants, Zn plays a key role as a structural constituent or regulatory cofactor of a wide range of enzymes (more than 300) (Vallee and Auld, 1990; Coleman, 1998) and proteins in many important biochemical pathways and these are mainly concerned with: (i) integrity of biomembranes; (ii) RNA and DNA metabolism; (iii) carbohydrate metabolism, both in photosynthesis and in the conversion of sugars to starch; (ii) protein metabolism; (iv) cell division; (v) auxin metabolism; (vi) pollen formation (Marschner, 1995; Cakmak, 2000; Alloway, 2007, 2009). Therefore, the relevance of these processes for cell metabolism makes clear that Zn uptake, homeostasis, and allocation to the different plant organs and cellular organelles need to be tightly regulated, in order to provide the required amount of Zn and to prevent the toxic effects caused by its excess. Homeostatic mechanisms include control of uptake, intracellular binding to metal chelators, efflux from the cell and sequestration into vacuoles (Clemens, 2001; Hall and Williams, 2003; Ishimaru *et al.*, 2011).

ZINC IN THE SOIL

In soil, Zn is present in various forms coming from different inputs. The first one is the chemical and physical weathering of parent rocks. Other natural inputs arise because of atmospheric (e.g. volcanoes, forest fires, and surface dusts) and biotic (e.g. decomposition, leaching/washoff from leaf surfaces) processes (Broadley *et al.*, 2007; Ishimaru *et al.*, 2011). Moreover, different human activities have influenced Zn inputs to soils such as mining and smelting activities (Nriagu, 1996). Other anthropogenic inputs of Zn to soils include fossil fuel combustion, phosphatic fertilizers, limestone, manure, sewage sludge, other agrochemicals, particles from galvanized (Zn-plated) surfaces and rubber mulches (Chaney, 1993; Alloway, 1995).

Zn in soil occurs in three primary fractions: (a) water-soluble Zn (including Zn^{2+} and soluble organic fractions); (b) adsorbed and exchangeable Zn in the colloidal fraction (associated with clay particles, humic compounds and Al and Fe hydroxides) and (c) insoluble Zn complexes and minerals (Barrow,

1993; Alloway, 1995). Zn is mobile at slightly acidic conditions and is immobilized in alkaline soils (Broadley *et al.*, 2007; Gupta *et al.*, 2008). The distribution of Zn between soil fractions is determined by soil-specific precipitation, complexation and adsorption reactions. Many factors determine soil Zn distribution and then inducing Zn deficiency including pH, soil type and moisture, mineral and clay types and contents, diffusion and mass flow rates, weathering rates, organic matter, soil biota and plant uptake (Hacisalihoglu and Kochian, 2003; Rashid and Ryan, 2004). The soluble Zn fraction consists for up to 50% of Zn^{2+}, which is the dominant form available for plants (Hacisalihoglu and Kochian, 2003).

ZINC DEFICIENCY

Zn deficiency appears the most widespread and frequent micronutrient deficiency problem in crops worldwide, resulting in severe losses in yield and nutritional quality (Graham and Welch 1996; Cakmak, 2000, 2002; Alloway, 2007). A wide range of crops are affected by Zn deficiency, including mainly cereals (i.e. rice, barley, wheat and maize), fodder crops, pulses, bush and tree fruits, nuts, vegetables and non-food crops, such as cotton and tobacco (Welch and Graham, 2004; Grotz and Guerinot, 2006). In particular, it is estimated that nearly half of the soils on which cereals are grown have levels of available Zn low enough to cause Zn deficiency (Graham and Welch 1996; Cakmak *et al.*, 1999; Alloway, 2007). This causes cereals to be inherently low in grain Zn concentrations to meet daily requirement of humans thus causing Zn deficiency symptoms and pathologies, especially in developing countries where cereal grains, especially wheat and rice, contribute to about 70% of the daily calorie intake (Cakmak, 2008). Zn deficiency in humans is a major nutritional and health problem in developing countries, especially among young children. It affects, on average, one-third of the world's population, ranging from 4 to 73% in different countries (Hotz and Brown, 2004; Alloway, 2007; Black *et al.*, 2008; Wessells and Brown, 2012).

Concerning rice, Zn deficiency is widespread on neutral to alkaline-calcareous soils which contain more than 1% organic matter and incidence of the deficiency appears more closely related to Zn availability than to total Zn content (Forno *et al.*, 1975). Moreover, flooding and submergence determine a decrease in available Zn due to pH changes and the formation of insoluble Zn compounds (Alloway, 2009).

There are a relatively small number of different types of symptoms which are found to occur in crops suffering from Zn deficiency. These may occur at varying degrees of severity and in various combinations in different plant species. The main type of visible symptoms on both old and new leaves are: chlorosis, necrotic spots, 'bronzing' and 'rosetting'. Other symptoms are: stunting of plants, dwarf leaves ('little leaves') and malformed leaves (e.g. 'goblet' leaves) (Marschner, 1995; Hacisalihoglu and Kochian, 2003; Alloway, 2007, 2009; Broadley *et al.*, 2007). Moreover, deficiency of Zn causes low

fertility (Yamaji *et al.*, 2013) and plants show a high susceptibility to environmental stress factors such as drought stress and pathogenic infections (Alloway, 2007).

The prevention of Zn deficiency can be obtained through increasing Zn concentration in grains ('biofortification') through two possible strategies: agronomic and genetic. The first one uses soil-applied Zn fertilizers or, less effective for rice, foliar sprays containing Zn (Broadley *et al.*, 2007), but is not always successful due to agronomic, economic and environmental factors (Graham and Rengel, 1993; Hacisalihoglu and Kochian, 2003). In the long-term, it appears more promising and cost-effective the genetic biofortification through breeding new varieties of crops (e.g. rice), accumulating higher Zn concentrations in grains or other edible parts. This will benefit whole population, especially those living in rural areas where it is more difficult to ensure that everybody has access to Zn-supplemented diets (Ruel and Bouis, 1998; Graham *et al.*, 1999; Welch and Graham, 1999, 2004). Furthermore, this strategy would reduce fertilizer inputs and protect the environment as well (Hacisalihoglu and Kochian, 2003).

ZINC UPTAKE BY PLANT ROOTS

Zn is taken up from soil solution by roots primarily as Zn^{2+}, but also potentially complexed with organic ligands. Then, Zn is translocated from root-to-shoot through the xylem. The Zn uptake into excised roots and intact plants is dependent from the Zn external concentration ($[Zn]_{ext}$) following often the sum of one or more Michaelis-Menten functions, each defined by a V_{max} and an affinity constant K_m, plus a linear term, k ($V/[Zn]_{ext}$). Some kinetic studies report a Michaelis-Menten function with a K_m of 1.5-50 μM, and, occasionally, additional Michaelis-Menten functions with higher K_m values (Wheal and Rengel, 1997; Hacisalihoglu *et al.*, 2001). In many plant species, like sugarcane, rice and tomato, K_m and V_{max} differ between Zn-efficient and Zn-inefficient genotypes (Broadley *et al.*, 2007). In rice, Zn uptake efficiency also correlates with exudation rates of low molecular weight organic anions and a substantial proportion of the phenotypic variation in Zn uptake efficiency is under genetic control (Hoffland *et al.*, 2006; Wissuwa *et al.*, 2006). Moreover, the linear term present in the Zn influx function seems due to the accumulation of Zn strongly bound to cell walls (Lasat *et al.*, 1996; Hart *et al.*, 1998, 2002).

Higher plants acquire Zn from the rhizosphere through a number of transporters which are strictly regulated. Plant genomes contain several gene families involved in the transport of divalent micronutrients, including Zn (Maser *et al.*, 2001). The selectivity of these transporters determines whether other divalent cations are imported at the same time as Zn (Ramesh *et al.*, 2003) and some of them have broad substrate specificity (Korshunova *et al.*, 1999). Ionic selectivity is particularly important for plant Zn transporters in root cells. In soils that contain contaminants such as toxic heavy metal like cadmium (Cd), Zn transport mechanisms may allow for Cd entry into whole plants

(Holmgren *et al.*, 1993; Zhao *et al.*, 2002). Cd can be potentially lethal to plants and its entry into the food chain may result in human toxicity (Lasat *et al.*, 2000; Pence *et al.*, 2000).

Several members of the Zn-regulated transporters and the Fe-regulated transporter-like protein (ZIP) gene family (Guerinot, 2000) have been characterized and shown to be involved in metal uptake and transport in plants (Eide *et al.*, 1996; Korshunova *et al.*, 1999; Vert *et al.*, 2001, 2002; Connolly *et al.*, 2002). Moreover, their functional expression in yeast is an helpful tool to determine their substrate specificity. Yeast Zrt1 and Zrt2 are high- and low-affinity Zn uptake transporters, respectively (Eide, 1998; Guerinot, 2000) and AtZIP1 and AtZIP3 from *Arabidopsis thaliana* expressed in the Zn-uptake mutant (*zrt1zrt2*) of the yeast *Saccharomyces cerevisiae* restore Zn uptake; indeed, they have been proposed to play a role in Zn transport. In particular, AtZIP1 and AtZIP3 are expressed in roots in response to Zn deficiency, suggesting that they transport Zn from the soil to the plant (Grotz *et al.*, 1998; Guerinot, 2000). Further characterization of homologs from several plant species supported the proposed role of ZIP transporters in Zn nutrition. In rice, for instance, many ZIP transporters have been identified. The Zn deficiency induces the expression of some ZIPs including OsZIP1 and OsZIP3 that seem important for Zn uptake from soil. They are also responsible for Zn homeostasis in shoots (Ramesh *et al.*, 2003). A member of the ZIP family, TcZNT1, from the Zn/Cd-hyperaccumulating plant *Thlaspi caerulescens*, was shown to mediate high-affinity Zn uptake and low-affinity Cd uptake following heterologous expression in yeast (Pence *et al.*, 2000). Another member of this family, GmZIP1, has been identified in soybean. By functional complementation of the *zrt1zrt2* yeast cells, GmZIP1 was found to be highly selective for Zn, while yeast Zn uptake was inhibited by Cd. GmZIP1 was specifically expressed in the nodules and not in roots, stems or leaves, and the protein was localized to the peribacteroid membrane, indicating a possible role in the symbiosis (Moreau *et al.*, 2002).

Graminaceous plants specifically secrete mugineic acid family phytosiderophores (MAs) (Marschner, 1995) that play a role in Fe and Zn uptake and translocation (Takagi, 1976; Welch and Shuman, 1995; Suzuki *et al.*, 2006, 2008). In fact, only these plants can synthesize the nicotianamine aminotransferase (NAAT), a critical enzyme in the biosynthetic pathway of MAs that catalyzes the aminotransfer of nicotianamine (NA), an essential intermediate in the production of MAs (Mori and Nishizawa, 1987; Shojima *et al.*, 1990). Although MAs are produced only in graminaceous plants, NA has been found in all plants investigated to date (Takahashi *et al.*, 2003). In particular, physiological and molecular studies have indicated that one of the principal metal chelators inside the plant is NA (Hell and Stephan, 2003; Takahashi *et al.*, 2003). Unlike MAs, NA is not secreted and is thought to play a role in the internal transport of Fe and other metals, like Zn. NA also might function as scavenger to protect cells from oxidative stress (von Wirén *et al.*, 1999). However, the precise roles of NA in higher plants remain unclear. The synthesis and secretion of MAs seem increased under Zn and Fe deficiency in wheat and barley (Cakmak *et al.*, 1994; Walter *et al.*, 1994; Suzuki *et al.*, 2006). Moreover, a recent

study suggested that deoxymugineic acid (DMA) can increase Zn deficiency tolerance in rice (Widodo *et al.*, 2010). In addition, a modelling study proposed a strong correlation between (DMA) secretion and rooting density, and suggested a role of DMA for Zn absorption in rice (Ptashnyk *et al.*, 2011). Thus, DMA and NA are suggested to play a significant role in plant Zn uptake and mobilization.

ZINC ROOT-TO-SHOOT TRANSLOCATION AND ZINC INTRACELLULAR MOVEMENTS

Once inside the plant, metals must reach the tissues in which they are required. Developing tissues with low transpiration especially request higher Zn concentration for the active cell division and growth (Marschner, 1995), but the molecular mechanisms are still unknown (Yamaji *et al.*, 2013).

Once within the root epidermal and cortical cells after the uptake from the soil, Zn can reach the root xylem both through symplastic and apoplastic fluxes and must be actively loaded into the xylem and transported by the transpiration stream to shoot tissue (Curie *et al.*, 2009). Proper loading and unloading of the vasculature is essential for metal transport in the plant (Palmer and Guerinot, 2009). In *Arabidopsis thaliana*, Zn is effluxed into the xylem for root-to-shoot translocation by the heavy metal transporters AtHMA2 (Heavy-Metal ATPase) and AtHMA4, members of the P_{1B}-type ATPase family (Hussain *et al.*, 2004). AhHMA4 was also identified as the major responsible for shoot Zn hyperaccumulation in the hyperaccumulator *Arabidopsis halleri* (Becher *et al.*, 2004; Weber *et al.*, 2004), resulting from a triplication of the gene and changes in the regulation of their expression (Hanikenne *et al.*, 2008). In the monocots rice (Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012), barley (Mills *et al.*, 2012) and *Triticum aestivum* (Tan *et al.*, 2013) HMA2 has been recently identified as responsible for Zn xylem loading. The P_{1B}-type ATPase family will be further discussed. Other metal transporters seem to be implicated in the Zn systemic movement. In particular, OsZIP4, OsZIP5 and OsZIP8 appear involved in Zn root-to-shoot translocation, and OsZIP4 and OsZIP8 seem also particularly important for Zn transport to seed (Ishimaru *et al.*, 2005; Bashir *et al.*, 2012). Also AtZIP4, expressed in both roots and shoots, is suggested as involved in the transport of Zn intracellularly or between plant tissues (Grotz *et al.*, 1998; Guerinot, 2000). Furthermore, a recent paper (Song *et al.*, 2010) proposed a Zn transport pathway independent from AtHMA2 and AtHMA4 in *A. thaliana*. The responsible for that pathway is suggested to be the AtPCR2 (Plant Cadmium Resistance 2). This plasma membrane protein seems implicated in the detoxification of Zn and in the translocation of Zn in a root-to-shoot direction. These roles are possible because AtPCR2 is expressed in epidermal cells and in the xylem of young roots, and in epidermal cells of fully developed roots.

Once transported to the proper tissue, Zn has to be distributed to the different organelles to ensure sufficient levels to the necessary compartments. It has been suggested that the majority of Zn that is not associated with proteins is bound to various compounds, such as metal chaperones, NA, glutathione, or organic acids (Takahashi *et al.*, 2003; Krämer *et al.*, 2007; Palmgren *et al.*, 2008). Zn is

exported from the cytosol and accumulated into the vacuoles by different transporters. The great part of them belongs to the cation diffusion facilitator (CDF) transporter family whose transporters are involved in heavy metal transport, particularly of Zn, Mn and Fe. For instance, the metal tolerance proteins (MTPs) are plant members of the CDF family involved in cellular metal homeostasis (e.g. MTP1 and MTP3 are involved in Zn homeostasis) (Blaudez *et al.*, 2003; Kobae *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005; Arrivault *et al.*, 2006; Gustin *et al.*, 2009; Podar *et al.*, 2012; Yuan *et al.*, 2012). Another example of protein belonging to the CDF family involved in the Zn homeostasis is the *Oryza sativa* Zn transporter 1 (OZT1), a vacuolar Zn transporter recently characterized (Lan *et al.*, 2013). Other transporters seem to be involved in the vacuolar sequestration of Zn, such as ZIF1 (Zinc-induced facilitator 1), a member of the major facilitator superfamily (MFS) (Haydon and Cobbett, 2007) and the *Arabidopsis* MHX, a Mg^{2+}/H^{+} exchanger that functions as an electrogenic exchanger of protons with Mg^{2+} and Zn^{2+} ions (Shaul *et al.*, 1999). Finally, the overexpression of AtHMA3 (Heavy-Metal ATPase) improved plant tolerance to Zn, Cd, cobalt (Co) and lead (Pb), suggesting a role in the detoxification of different heavy metals, by participating in their vacuolar sequestration (Morel *et al.*, 2009). The transporters responsible for Zn remobilization from the vacuole are not yet identified. Furthermore, Zn is most likely transported in mitochondria by a ZIP, but no ZIP transporters have been assigned to this function yet (Palmer and Guerinot, 2009).

CADMIUM AS A POTENTIAL RISK FOR FOOD SAFETY

Some trace elements (e.g. Fe, Zn, Mn and Cu) are essential to all organisms; other trace metals, such as Cd, Pb, chromium (Cr), mercury (Hg) and the metalloid arsenic (As) are biologically non-essential and potentially toxic. They can enter plants using the same transporters used for essential nutrients uptake (Clemens, 2001, 2006; Mendoza-Cózatl *et al.*, 2011). These heavy metals are important environmental pollutants, particularly in areas where there is high anthropogenic pressure. Their presence in the atmosphere, soil and water – even in trace concentrations – can cause serious health problems to all organisms (Sanità di Toppi and Gabbrielli, 1999). In particular, Cd pollution has become a global environmental problem (Zhang *et al.*, 2013) and is of great concern in the environment because of its toxicity to animals and humans, and its relative mobility in the soil-plant system (McLaughlin and Singh, 1999; Kirkham, 2006).

CADMIUM IN THE SOIL

Cd is a toxic trace element with a great chemical similarity to Zn, with which it is commonly associated in natural geological settings. Cd(II) is the most common valence of Cd in natural environments (Traina, 1999) and occurs in the soil solution mainly as Cd^{2+}, but also as Cd-chelates (Tudoreanu and

Phillips, 2004). The Cd concentrations range from 0.04 to 0.32 μM in non-polluted soil solutions, and from 0.32 to about 1 μM in soil solutions considered as polluted to a moderate level (Wagner, 1993). Cd occurs in the soil either naturally or through anthropogenic activities. In the first case, natural mineral outcrops can be enriched in Cd through the weathering of Cd-rich parent materials. The release of Cd in the soil due to anthropogenic activities has increased over the last decades since it has kept pace with the rising consumption of Cd by the industry. The most important sources of Cd which contaminate soils derive from fly-ash caused by smelting, refining and burning of fossil fuels, and from atmospheric deposition, urban refuse and sludge, agricultural and animal wastes (including fertilizers) (Alloway and Steinnes, 1999; Kirkham, 2006). The degree to which higher plants are able to take up Cd depends on its concentration in the soil and its bioavailability, affected by several factors mostly depending on soil characteristics. Cd concentration increases with clay proportion and availability is inversely related to soil pH and increases in oxidative condition. Moreover, soil organic matter has high sorption affinity for Cd, making Cd non-available (Prasad, 1995; Kirkham, 2006). Furthermore, Cd competes with nutrients (e.g. K, Ca, Mg, Fe, Mn, Cu, Zn and Ni) for plant uptake through the same transmembrane carriers (Sanità di Toppi and Gabbrielli, 1999). Therefore, the real bioavailable Cd concentration in soil strictly depends on the speciation processes it undergoes when introduced in the soil medium, as well as on the concentration and stability of the ligands it can be complexed to. Cd, compared to other metals including Cu, Pb, Hg, Fe and Al, tends to be more mobile and thus more available to plants (Prasad, 1995; Alloway and Steinnes, 1999). Finally, Cd bioavailability is deeply affected by plants, especially in the rhizospheric soil, due to different root activities like those abovementioned discussing on trace elements (i.e. root exudates) (Fairweather-Tait and Hurrell, 1996; Zhu *et al.*, 1999; Welch and Graham, 2004; Clemens, 2006). Cd released to the environment enters in the food chain when it is taken up by roots and translocated to the edible portion of the plant, get bioconcentrated and can become dangerous to all kinds of organisms (Kawada and Suzuki, 1998).

CADMIUM TOXICITY ON PLANTS

The symptoms of Cd toxicity have been studied in several plant systems and under various conditions, mainly with applications of extremely high Cd^{2+} concentrations. Nevertheless, the consequences of acute Cd stress are well documented, the most of Cd toxicity bases are still not completely understood. Under long-term exposure to Cd almost all physiological processes are affected. Visible effects of exposure to high Cd^{2+} doses are leaf roll and chlorosis and growth inhibition, both of stems and roots (Prasad, 1995; Clemens, 2006). Many aspects of root anatomy are also altered, appearing to be species- and tissue-specific (Lunáčková *et al.*, 2003; Seregin *et al.*, 2004; Ďurčeková *et al.*, 2007; Maksimović *et al.*, 2007; Seregin and Kozhevnikova, 2008). Cd has high affinity for sulfydryls and thus leads to protein misfolding, sulfur metabolism and membrane damages (Hall, 2002). Cd causes oxidative stress, even

though Cd is not directly involved in the production of reactive oxygen species (ROS), for example enhancing the lipid peroxidation (Clemens, 2006) and decreasing the activity of different anti-oxidative enzymes (e.g. glutathione reductase) (Gallego *et al.*, 1996). Cd inhibits also the activity of other several enzymes (e.g. Rubisco) (Van Assche and Clijsters, 1990) and it significantly reduces the normal H^+/K^+ exchange (Obata *et al.*, 1996). Cd inhibits photosynthesis and chlorophyll biosynthesis inhibiting many enzymes (e.g. Fe^{3+} reductase) (Alcantara *et al.*, 1994), the photosystem II, and also the photosystem I, even though to a lesser extent (Siedlecka and Baszynsky, 1993; Seidlecka and Krupa, 1996), and leading to the degeneration of the fine structure of chloroplasts (Krupa *et al.*, 1993). Furthermore, Cd can interfere with homeostatic pathways for essential metals (Roth *et al.*, 2006) because of the chemical similarity between Cd^{2+} and functionally active ions located in active sites of enzymes and signaling components. Thus, Cd^{2+} ions displace divalent cations, such as Zn^{2+} and Fe^{2+}, from structural proteins and enzymes causing the release of "free" ions which might trigger oxidative injuries (DalCorso *et al.*, 2008). Likewise, Ca^{2+} binding proteins such as calmodulin might well be prime intracellular binding sites of Cd^{2+} and such binding will most likely be detrimental to cellular signaling cascades (Clemens, 2006). Finally, Cd inhibits the oxidative mitochondrial phosphorylation (Kessler and Brand, 1995) and, mimicking Ca^{2+} ions, Cd enters stomatal guard-cell and activates the opening of the plasma membrane anion and K^+_{out} channels. As more ions leave the cell, water follows and turgor is lost with stomatal pore closure (Barceló and Poschenrieder, 1990; Costa and Morel, 1994) leading to, combined with the degradation of the xylem cells, a decreased tolerance of plants to water stress (Prasad, 1995).

CADMIUM UPTAKE BY PLANT ROOTS

Higher plants can take up Cd, depending on its availability and concentration, form soil or water. In fact, only a fraction of the total Cd is available for plant uptake (Clemens, 2006). The concentration dependence of Cd uptake from hydroponic 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. The linear component is often attributed to tight Cd binding to cell walls, but it could also represent an apoplasmic Cd flux to the xylem (White, 2001; White *et al.*, 2002; Broadley *et al.*, 2007). Estimates of the K_m value for 'high-affinity' Cd uptake commonly fall between 20-1000 nM. Generally Cd uptake by plant roots is inhibited by Ca^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} or Mn^{2+} in the rhizosphere solution, due to a competition for uptake (Cataldo *et al.*, 1983; Costa and Morel, 1993, 1994; Cohen *et al.*, 1998; Lombi *et al.*, 2002; Hart *et al.*, 2002; Zhao *et al.*, 2002; Berkelaar and Hale, 2003; Han *et al.*, 2006; Zhao *et al.*, 2006). It was assumed that for Cd, being a non-essential element, there would be no specific uptake mechanisms. Instead, Cd, and other non-essential metal ions, would enter plant cells via uptake systems for essential cations (Cosio *et al.*, 2004; Roth *et al.*, 2006; Papoyan *et al.*, 2007). In plants many transporters for essential divalent cations have a Cd^{2+} uptake activity. It has been shown that Cd can

enter root cells through ZIP transporters. AtIRT1, a ZIP family transporter for Fe, Zn and Mn also mediates Cd uptake in *Arabidopsis thaliana*, whose expression is also induced by Fe deficiency (Cohen *et al.*, 1998; Connolly *et al.*, 2002; Vert *et al.*, 2002). Also the rice OsIRT1 and OsIRT2 have an influx activity of Cd as well as Fe in yeast, suggesting a role in Cd uptake especially upon re-aeration of soil after flooding, when Fe is less available and OsIRTs result induced (Ishimaru *et al.*, 2006; Nakanishi *et al.*, 2006). Likewise, TcZNT1/TcZIP4 present in the Cd/Zn hyperaccumulator *Thlaspi caerulescens* mediates the uptake of both Zn and Cd when expressed in yeast (Pence *et al.*, 2000). Competitive interaction between Cd and Zn in the uptake processes has been proved in non-accumulator plants (Cataldo *et al.*, 1983), including crop species (Hart *et al.*, 2002, 2005) and also in the Zn-hyperaccumulator *Arabidopsis halleri*, in which Zn treatment caused inhibition of both short-term Cd influx and long-term Cd accumulation (Pence *et al.*, 2000; Bert *et al.*, 2003; Zhao *et al.*, 2006; Ueno *et al.*, 2008). Moreover, transporters of the Nramps (Natural Resistance Associated Macrophage Proteins) family are also known to mediate Cd transport. In *A. thaliana*, AtNramp1 functions as a high-affinity transporter for Mn uptake under Mn deficiency (Cailliatte *et al.*, 2010) and showed transport activity in yeast for Fe, Mn, and Cd (Curie *et al.*, 2000; Thomine *et al.*, 2000). In rice, OsNramp5 has recently been identified as the major responsible for Mn and Cd uptake, and seems to contribute also at the uptake of Fe (Ishimaru *et al.*, 2012; Sasaki *et al.*, 2012). Moreover, OsNramp1 showed transport activity for Fe and Cd in yeast but not Mn (Curie *et al.*, 2000; Takahashi *et al.*, 2011). OsNramp1 is suggested to be involved in cellular Cd uptake under Fe deficiency and Cd transport within the plant, but the exact role of OsNramp1 in rice is still unknown (Takahashi *et al.*, 2011). The transporter LCT1 (Low-affinity Cation Transporter 1), a non-selective transmembrane transporter for Na, K (Schachtman *et al.*, 1997; Amtmann *et al.*, 2001) and for Ca, also appeared to mediate Cd transport to the yeast cell (Clemens *et al.*, 1998). However, its subcellular localization *in planta* is still to be determined. Due to the similarity to Ca^{2+}, Cd^{2+} can also enter root cells through 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 and Broadley, 2003; White, 2005). It is important to note that this type of transport is particularly significant in case of relatively low Cd concentrations, which is the most widespread condition in agricultural contaminated soils. Finally, in addition to the free ion form, Cd might be taken up from soil as Cd-chelates through YSL (Yellow-Stripe 1-Like) proteins (Curie *et al.*, 2009). YSLs can also mediate the uptake of Fe, Zn, Mn and Cu complexed with organic compounds, such as phytosiderophores or non-proteic amino acids (Curie *et al.*, 2001; Colangelo and Guerinot, 2006).

CADMIUM DETOXIFICATION

Once inside root system, plants respond to Cd toxicity with a 'firewall system' that includes immobilization, exclusion, chelation and compartmentalization of the metal ions, as well as the expression of more general stress response mechanisms involving ethylene and stress proteins (Sanità di Toppi and Gabbrielli, 1999). Cd can firstly be immobilized by adsorbtion to the negative charges present on the cell walls. This portion of Cd is tightly bound to the apoplastic component and is unlikely to be released in the cytosol (Nishizono *et al.*, 1989). The most recurrent general mechanism for Cd detoxification in plants is the metal chelation by a ligand in the cytosol and, in some cases, the subsequent compartmentalization of the ligand-metal complex (Song *et al.*, 2014). The best-characterized heavy metal-binding ligands in plants are the phytochelatins (PCs) and metallothioneins (MTs). MTs are Cys-rich polypeptides encoded by a family of genes. In contrast, PC is a family of Cys-rich peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2-11; Rauser, 1995; Zenk, 1996; Cobbett and Goldsbrough, 2002) enzymatically synthesized by PC synthase (PCS) from glutathione (GSH) in a transpeptidation reaction (Grill *et al.*, 1989; Rea *et al.*, 2004). Cd cellular uptake induces PC synthesis and produced PCs chelate the free Cd ions by forming the low molecular weight (LMW) complexes. These are then transported into vacuoles, where additional sulfur (S) in form of sulfide is incorporated to generate the high molecular weight (HMW) PC-Cd-S^{-2} complexes. Thus, 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 increasing Cd tolerance of the organism (Ortiz *et al.*, 1992, 1995; Rauser and Meuwly, 1995; Rauser, 2003; Clemens, 2006). Moreover, Cd can also be aspecifically transported across the tonoplast in form of free ion through antiporter mechanisms actuated by divalent cation/H^+ transporters (CAX) (e.g. Ca^{2+}/H^+ transporters) (Salt and Wagner, 1993; Ortiz *et al.*, 1995; Martinoia *et al.*, 2007), due to the already mentioned similarities between Ca and Cd ions. Recently, a transporter belonging to the P_{1B}-type ATPase class, HMA3, has been proved to mediate Cd vacuolar sequestration both in Arabidopsis (Morel *et al.*, 2009) and rice (Ueno *et al.*, 2009a, 2009b, 2010, 2011). OsHMA3 appears constitutively expressed in rice roots and highly selective for Cd sequestration into the root vacuoles. Interestingly, a single amino acid mutation in this protein from the high Cd-accumulating cultivar resulted in a complete loss of activity of this transporter, which – failing in its putative function of root firewall – both reduced Cd root retention and promoted root-to-shoot Cd translocation (Ueno *et al.*, 2010; Miyadate *et al.*, 2011; Satoh-Nagasawa *et al.*, 2013). Finally, Lan and coworkers (2013) characterized a new vacuolar Zn transporter in rice (*Oryza sativa* Zn Transporter 1 - OZT1) belonging to the CDF family suggesting its involvement in Zn, Cd or other heavy metals transport and homeostasis in plant. The efficiency of all these processes may contribute to the natural variation in Cd partitioning between roots and shoots observed in crop

species, as only Cd ions escaping these detoxification pathways may be potentially available for root-to-shoot translocation via the xylem (Nocito *et al.*, 2011).

CADMIUM ROOT-TO-SHOOT TRANSLOCATION

Once within the root, Cd can reach the xylem either by radial symplastic or extracellular apoplasmic pathway and then must be actively loaded into the xylem vessels to be translocated to the shoots (Colangelo and Guerinot, 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 (Salt *et al.*, 1995; Hart *et al.*, 2006) and Cd in the xylem sap is predominantly present in the free ionic form and only small amounts is complexed with citrate, malate and histidine in the hyperaccumulator *A. halleri* (Ueno *et al.,* 2008). In contrast, indirect evidence in Arabidopsis showed that small amounts of PCs undergo long-distance transport in a root-to-shoot direction (Gong *et al.*, 2003). However, the significance of this mechanism in Cd translocation still remains a controversial issue, especially in relation to the possible localization of Cd-PCs complexes into the xylem vessels, since several studies failed to detect these complexes in the xylem sap of different species (Salt *et al.*, 1995; Mendoza-Cózatl *et al.*, 2008; Ueno *et al.*, 2008). Moreover, other experimental evidence strongly suggested phloem as the major vascular system for long-distance source to sink transport of Cd as Cd-PCs and Cd-GSH complexes (Mendoza-Cózatl *et al.*, 2008).

It is generally accepted that Cd ions compete with Zn ions not only for plant uptake (Cataldo *et al.*, 1983; Hart *et al.*, 2002, 2005) but also for accumulation in the shoot, as they probably use the same transport systems to be loaded into the xylem. For instance, Hart and coworkers (2005) found that, under Zn deficiency, the low grain Cd trait in wheat may be connected with decreased Zn accumulation in grains. Moreover, a positive and strong correlation between Cd and Zn concentrations in the shoot has been observed in 69 rice varieties, where the root-to-shoot Cd translocation via the xylem has been proved to be the major and common physiological process determining Cd accumulation in shoots and grains of rice (Uraguchi *et al.*, 2009).

In the last decade some members of the P_{1B}-type ATPase family that cluster with the Zn/Cd/Pb/Co subgroup (Axelsen and Palmgren, 2001; Baxter *et al.*, 2003; Williams and Mills, 2005) have been identified as responsible for Cd and Zn xylem loading. In particular, AtHMA2 and AtHMA4 play primary roles in root-to-shoot Zn translocation in Arabidopsis (Hussain *et al.*, 2004) and several reports also suggest their involvement in Cd transport (Mills *et al.*, 2003, 2005; Eren and Argüello, 2004; Verret *et al.*, 2005; Wong and Cobbett, 2009; Wong *et al.*, 2009). Both AtHMA2 and AtHMA4 are localized on the plasma membrane and the genes encoding them result predominantly expressed in the vascular bundles (Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Verret *et al.*, 2005), suggesting their function as efflux pumps to extrude the excess of the metals into the apoplast. The xylem loading of

both Zn and Cd ions itself could be intended as a detoxification system as it would allow the plant to translocate the excess of metals to the highly vacuolated cells in the shoot where they can be sequestered into the vacuolar compartment (Hussain *et al.*, 2004). Interestingly, the *hma2hma4 A. thaliana* double mutant shows a near-complete abolition of root-to-shoot Cd translocation (Wong and Cobbett, 2009), whereas decreasing HMA4 transcript levels by RNA interference in Zn hyperaccumulator *A. halleri* resulted in enhanced Cd root retention capacity (Hanikenne *et al.*, 2008). The role of HMA4 in Cd and Zn movement has also been confirmed in one other hyperaccumulator species, *Thlaspi caerulescens*, where HMA4 was seen to be involved in the xylem loading of both Zn and Cd (Papoyan and Kochian, 2004). Recently, OsHMA2, a rice Zn^{2+}/Cd^{2+}ATPase mainly localized in roots vascular bundles, has been characterized as responsible for Zn and Cd xylem loading (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012). Moreover, Satoh-Nagasawa and coworkers (2012) found in three Tos17 insertion rice mutants translocation ratios of Zn and Cd lower than in the wild type, suggesting that OsHMA2 is a major transporter of Zn and Cd from roots to shoots. More recently, the barley HMA2 (Mills *et al.*, 2012) and *Triticum aestivum* HMA2 (Tan *et al.*, 2013) have been characterized. Their heterologous expression in yeast demonstrated that HvHMA2 and TaHMA2 function as Zn and Cd pumps. TaHMA2 overexpression also improved root-to-shoot Zn and Cd translocation, especially when expressed in rice. These findings suggest that both HvHMA2 and TaHMA2 are responsible for root-to-shoot Zn and Cd translocation. The P_{1B}-type ATPase family will be further discussed. Finally, Yuan and collaborators (2012) reported that rice MTP1, belonging to the CDF protein family and localized at the plasma membrane, is necessary for efficient translocation of Zn, Cd and other heavy metals, and maintenance of ion homeostasis in plant.

However, divergent results have been obtained. For instance, contrasting effects of Zn application on Cd accumulation have been reported and they may depend on the several factors, not clearly understood yet, that interact both in the soil and within the plant (Christensen, 1987; Abdel-Sabour *et al.*, 1988; Oliver *et al.*, 1994; Choudhary *et al.*, 1995; Grant and Bailey, 1998; Hart *et al.*, 2005). In particular, Christensen (1987) reported that Zn addition can displace Cd from soil adsorption sites so that Zn fertilization might lead to increased Cd uptake by plants because of increased Cd availability in the soil solution. By contrast, other studies have shown that the addition of Zn to soils can reduce Cd accumulation in the shoots (Abdel-Sabour *et al.*, 1988; Oliver *et al.*, 1994; Choudhary *et al.*, 1995; Grant and Bailey, 1998). In particular, applications of low rates of Zn fertilizer (up to 5.0 kg Zn ha^{-1}) were found to markedly decrease the Cd concentration in wheat grain grown in areas of marginal to severe Zn deficiency. No further significant decreases in Cd concentration in grain occurred at higher rates of applied Zn (Oliver *et al.*, 1994). Taken together, all these results highlight that Zn and Cd have similar chemical properties, but also seem to suggest that the two metal ions shared only a part of their systemic movement pathways, that still need to be identified and fully understood.

CADMIUM EXPOSURE AND RISKS FOR HUMAN HEALTH

When taken up by plants, Cd concentrates along the food chain and ultimately accumulates in the body of people eating contaminated foods. The most salient toxicological property of Cd is its exceptionally long half-life in the human body (more than 20 years). In addition, Cd is also a highly toxic metal usually at doses that are much lower than most toxic metals (Järup *et al.*, 1998; Bernard, 2004; Nordberg *et al.*, 2007). For these reasons, Cd results one of the most potentially toxic substances for human health, constituting a big issue in terms of food safety (ATSDR, 2008; Nordberg, 2009), and environmental exposure to Cd should be reduced. Therefore, in order to ensure a high level of protection of consumers, both the EU and the Codex Alimentarius Commission of the Food and Agriculture Organization/World Health Organization (FAO/WHO) fixed the official maximum allowable limits of Cd concentration in foodstuffs (COMMISSION REGULATION (EC) No 629/2008, 2008; CODEX STAN 193-1995, 2009). In particular, the European Food and Safety Authority (EFSA) and the US Agency for Toxic Substances and Disease Registry (ATSDR) have reduced the provisional tolerable weekly intakes (PTWI) of Cd from 7 to 2.5 μg kg^{-1} body weight (b.w.) (EFSA Panel on Contaminants in the Food Chain – CONTAM –, 2011). The present levels of Cd intake of most European adult populations are far below the limit recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), below of PTWI, and below those of many populations worldwide as well as those of some European subgroups such as children and vegetarians (EFSA, 2011).

Primary chronic exposure sources of Cd for the general population include food and tobacco smoking. Cd 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 Cd dietary exposure. The highest concentrations of Cd (10 - 100 ppm) are found in offals as well as in some species of fish, mussels and oysters, especially when caught in polluted seas. Consumption of staple foods such as rice and wheat also significantly contributes to human exposure. The amounts of Cd ingested daily with food in most countries are in the range of 10 to 20 μg per day. 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 (UNEP, 2008; FAO/WHO, 2010; EFSA, 2012). Moreover, in the industry, Cd is hazardous both by inhalation and ingestion and can cause acute and chronic intoxications.

Once absorbed, Cd irreversibly accumulates in the body and concentrates in the liver and even more in the kidneys, which can contain up to 50% of the total body burden of Cd in general population, but causes damages also to the skeletal system. Only a very low amount of Cd (0.005 - 0.01%) is eliminated via the urine (Bernard, 1986, 2004, 2008; Kobayashi *et al.*, 2002; Bhattacharyya, 2009). The first well documented case of Cd poisoning occurred in the Jinzu River basin of the Toyama

Prefecture in Japan since 1910s, but generally recognized since 1950s. The locals named that disease "*itai-itai byō*" characterized by softening of the bones and kidneys failure. It was established that the Cd poisoning derived from Cd released into rivers by mining companies and then accumulated in rice grains (Kobayashi *et al.*, 2002, 2009).

STRATEGIES FOR REDUCING CADMIUM ACCUMULATION IN PLANTS

Although nowadays the emissions of Cd in the environment are decreasing due to legislation and technological improvements, it is still important to reduce the accumulation of Cd in the edible plant organs which would be better achieved combining soil management practices and genetic approach (Grant *et al.*, 2008). Concerning soil management, a number of strategies are available to reduce Cd contamination, like liming (Bolan *et al.*, 2003; Holm *et al.*, 2003), the application of organic matter (Grant *et al.*, 1999) or the addition of Zn to soil, which is particularly well-expressed under conditions of Zn deficiency (Oliver *et al.*, 1994; Choudhary *et al.*, 1995). The water management is another factor deeply affecting Cd availability in soil: in rice cultivation, for instance, flooded conditions are desirable in order to reduce Cd accumulation in the grain (Cattani *et al.*, 2008). Other techniques could include soil dressing, electronic thermodynamic remediation and on-site soil washing/clean up (Mulligan *et al.*, 2001; Murakami *et al.*, 2007; Makino *et al.*, 2008). Anyway, interventions on soil are neither always feasible nor cost-effective, thus do not solve the problem of Cd accumulation in plants grown especially on low contaminated soils.

More promising seems the plant breeding to select for genetically low-Cd concentration cultivars, as it is happening for rice taking advantage from the broad variability in the Cd accumulation trait observed in *japonica* (low-Cd accumulating) and *indica* (high-Cd accumulating) cultivars, as well as in hybrids (Morishita *et al.*, 1987; Arao and Ae, 2003; Arao and Ishikawa, 2006; Liu *et al.*, 2007; Ishikawa *et al.*, 2011). However, the genetic control of Cd accumulation remains poorly understood, even if in the recent years some progresses in this direction have been made. Two major QTL controlling Cd accumulation in rice have been identified on the short arm of chromosome 7: OsHMA3, responsible for Cd vacuolar sequestration and then functioning as a "firewall" to limit Cd translocation (Ueno *et al.*, 2009b, 2010; Ishikawa *et al.*, 2010), and OsNRAMP5, the major transporter responsible for Mn and Cd uptake in rice (Ishikawa *et al.*, 2012; Sasaki *et al.*, 2012). These findings constitute the preliminary step to include low-Cd trait in the selection breeding strategy for the release of varieties able to exclude Cd from the grains. The high genetic variability both in different species and in different cultivars within the same species can be also exploited for phytoextraction purpose.

Phytoextraction, a cost-effective and environmentally friendly green technology, utilizes the capacity of hyperaccumulator plants to extract heavy metals from soil. It has been proposed also for restore soil characterized by low level of Cd contamination (Krämer, 2005; McGrath *et al.*, 2006).

Nevertheless, field trials or commercial operations that demonstrate successful phytoremediation of metals have been just few so far (Robinson *et al.*, 2006; Maxted *et al.*, 2007). However, among the Cd hyperaccumulators, *S. nigrum* L., *Populus* spp., *Salix 'calodendron', Arabis paniculata* and *Salix* spp. (Wei *et al.*, 2005; French *et al.*, 2006; Maxted *et al.*, 2007; Yang *et al.*, 2014), 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 phytoextraction process (Salt *et al.*, 1998).

P_{1B}-TYPE ATPase, A CLASS OF TRANSPORTERS WITH A MAJOR ROLE IN TRACE ELEMENTS MOVEMENT THROUGH THE PLANT

BIOLOGY, STRUCTURE AND MECHANISM OF THE P-TYPE ATPases

P-type pumps are a large, ubiquitous and varied family of membrane proteins that are involved in many transport processes in virtually all living organisms. Basically, P-type pumps use ATP to maintain an ion gradient across a cell membrane. In general, P-type ATPase genes are more widespread and varied in eukaryotes than in bacteria and archea. In *Saccharomyces cerevisiae* 16 P-type ATPases have been found (Goffeau, 1998), whereas in *Arabidopsis thaliana* 46 transporters belonging to this class have been identified, pointing out their importance in vascular plants (Baxter *et al.*, 2003). All P-type ATPases are multi-domain membrane proteins with molecular masses of 70-150 kDa. Both the carboxyl and amino termini are on the cytoplasmic side of the membrane, so they all have an even number of transmembrane segments. Based on sequence homology, the P-type ATPase family can be divided into five branches, which are referred to as types 1 to 5 (Kühlbrandt, 2004).

P_{1B}-TYPE ATPases

Particularly interesting for trace elements transport is the type-1B ATPases subgroup. P_{1B}-ATPases, also known as Heavy Metal Associated (HMA) ATPases, transport heavy metals (Cu^+, Cu^{2+}, Zn^{2+}, Co^{2+}) across biological membranes (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996; Axelsen and Palmgren, 1998; Rensing *et al.*, 1999; Argüello, 2003; Williams and Mills, 2005). Due to the chemical similarities among transition metals, these pumps can aspecifically transport alternative non-physiological substrates; for instance, Cu^+-ATPases transport Ag^+ while Zn^{2+}-ATPases can transport Cd^{2+} and Pb^{2+}, causing toxicity effects to the organisms (Argüello *et al.*, 2007). Members of this class are, for instance, the bacterial metal-resistance proteins CopA (Rensing *et al.*, 2000), ZntA (Okkeri and Haltia, 1999) and CadA (Rosen, 2002) which remove toxic ions such as Cu^+, Ag^+, Zn^{2+}, Cd^{2+} or Pb^{2+} from the cell. The activity of these transporters is crucial to maintain the homeostasis of trace elements, such as Cu^+ and Zn^{2+} by balancing the activity of the ABC-type metal-uptake proteins (Nelson, 1999;

Rosen, 2002). Close homologs of bacterial P_{1B}-type ATPases have been found in *S. cerevisiae* (Goffeau, 1998), plants (Axelsen and Palmgren, 1998) and animals (Lutsenko and Petris, 2003).

MEMBRANE TOPOLOGY

When compared to other P-ATPases, P_{1B}-ATPases have a distinct structure which is characterized by a reduced number (six to eight) of transmembrane segments (TM), smaller ATP-binding domain (ATP-BD) and the presence of N- and/or C- terminal metal binding domains (MBD) in many of them. P_{1B}-ATPases also present a particular distribution of TMs with respect to the large cytoplasmic loop forming the ATP-BD, having two TMs on the C-terminal end of the ATP-BD (Fig. 1). However, in spite of the indicated differences, a common pattern is present among these metal ATPases, which is the presence of large cytoplasmic loops. These central components appear to confer their basic functionality to these enzymes, i.e. the ability to transport metals using the energy resulting from ATP hydrolysis.

The transmembrane metal binding sites (TM-MBS) of P_{1B}-ATPases are responsible for metal recognition and movement across the membrane permeability barrier. Analysis of the available genomes reveals the presence of a CPC sequence in the center of their sixth transmembrane domain (H6), or some alternative sequences (SPC, CPS, CPT, CPA, CPG, CPD) in putative P_{1B}-ATPases. This CPx or xPC motif appeared as a defining element of these enzymes that likely takes part in metal coordination during transport (Argüello *et al.*, 2007). Enzyme phosphorylation by ATP, subsequent turnover and transport, require metal binding to the TM-MBS and this is independent of metal binding from the N- and C-MBDs (Voskoboinik *et al.*, 1999; Bal *et al.*, 2001; Mitra and Sharma, 2001; Fan and Rosen, 2002; Mana-Capelli *et al.*, 2003; Mandal and Argüello, 2003). Metals activate P_{1B}-ATPases with apparent affinities ($K_{1/2}$) in the 0.1 - 3 μM range but, since most of these assays have been performed in the presence of various metal ligands (e.g. DTT, Cys and ATP), these $K_{1/2}$ values do not refer to free metal concentrations but to the total metal in the media (Okkeri and Haltia, 1999; Voskoboinik *et al.*, 1999; Sharma *et al.*, 2000; Fan and Rosen, 2002; Mandal *et al.*, 2002; Tsivkovskii *et al.*, 2002; Mana-Capelli *et al.*, 2003; Eren and Argüello, 2004). Considering the low dissociation constants for the soluble metal complexes (metal-thiolate, metal-chaperone, metal-ATP) (Martell and Smith, 2004), it can be proposed that these complexes deliver the metal directly to the TM-MBS, perhaps by a kinetically controlled ligand exchange. The question remains whether this is a plausible *in vivo* mechanism of metal delivery to the TM-MBS.

The large cytoplasmic loop between transmembrane domains H6 and H7 of P_{1B}-ATPases, referred to as ATP-binding domain (ATP-BD), encompasses the nucleotide binding (N) and the phosphorylation (P) domains. The smaller loop between H4 and H5 forms the actuator (A) domain.

The ATP-BD structure generally consists of the P- and N-domain joined by two short loops (the hinge region) (Sazinsky *et al.*, 2006). The P-domain contains the DKTGT sequence as well as a number of residues conserved in all P-ATPases that interact with the ATP γ-phosphate during binding and hydrolysis, including the aspartic acid phosphorylated during the catalytic cycle (Sørensen *et al.*, 2004). The N-domain contains the ATP-binding pocket, pointing out toward the cytosol near the P-domain and might be associated with various roles still to be clarified, including alternative regulatory mechanisms and required targeting. This and the P-domain together form the so-called phosphorylation site (Olesen *et al.*, 2007).

In the A-domain has been found the highly conserved sequence (S/T)GE(P/S) and, in the Ca^{2+}-ATPase, the interactions of this segment with the P-domain during enzyme phosphorylation/dephosphorylation appears critical since it drives the rotation of the A-domain with a subsequent rearrangement of TMs (Toyoshima and Nomura, 2002; Olesen *et al.*, 2004; Toyoshima and Inesi, 2004; Toyoshima *et al.*, 2004). This rearrangement, in turn, leads to metal deocclusion and release. Although the different disposition of TMs across the P_{1B}-ATPase class might require different transmembrane movements, the structural similarities suggest an equivalent mechanism for metal release.

Most P_{1B}-ATPases have various types of cytoplasmic metal binding domains (MBD) located either in the N-term (N-MBD) or C-term (C-MBD). The N-MBDs observed in Cu^{+}-ATPases and some bacterial Zn^{2+}-ATPases are 60-70 amino acids domains and contain a highly conserved CxxC metal binding sequence (Rensing *et al.*, 1999; Arnesano *et al.*, 2002; Lutsenko *et al.*, 2003). *In vitro*, these N-MBDs can bind both monovalent and divalent cations including Cu^{+}, Cu^{2+}, Zn^{2+} and Cd^{2+} (DiDonato *et al.*, 1997; Lutsenko *et al.*, 1997; Jensen *et al.*, 1999; Liu *et al.*, 2005). Moreover, plant Zn^{2+}-ATPases possess N-MBDs with a unique conserved CCxxE sequence (Eren *et al.*, 2007). Although the N-MBD is required for maximum enzyme turnover rate, it would not influence the metal binding to the TM-MBS and the resulting transport selectivity. In other words, it might not be essential for the transport of the metal but plays an important role in the post-translational regulation of the enzyme, maybe controlling the conformational changes the transporter goes through during the catalytic cycle, determining the phosphorylation/dephosphorylation processes that are the rate limiting step of the transport mechanism (Eren *et al.*, 2007). Plant Zn^{2+}-ATPases also present long C-term containing numerous His and Cys. These can have various lengths and generally present two different patterns: numerous Cys but no (or few) His residues (e.g. AtHMA3) or, alternatively, His- and Cys-rich C-MBD (e.g. AtHMA2 and AtHMA4) (Argüello *et al.*, 2007). As observed for the N-term, also the C-term plays a role in metal coordination due to the functional groups contained in its sequence; so, probably the C-term is required for the maximum turnover rate but it does not affect the interaction of metals with the transport sites, as already postulated for N-MBDs (Eren *et al.*, 2006).

CATALYTIC MECHANISM

P_{1B}-ATPases, like all P-ATPases, transport metals across biological membranes following the classical E1/E2 Albers-Post catalytic cycle (Fig. 2), well studied especially in P_2-ATPases (Na^+/K^+-, Ca^{2+}-, and H^+/K^+-ATPases) (MacLennan *et al.*, 1997; Kaplan, 2002). In simple terms, in the E1 state, the metal ion (M) binds to its high-affinity site in the TM-MBD, which is accessible from the cytoplasm. The ion binding itself causes the P-domain to move into the E1 conformation. As a result, the key Asp residue within the P-domain can be phosphorylated by Mg^{2+}-ATP, which is delivered to the phosphorylation site by the N-domain. In the E1P state, the Asp is phosphorylated, and is able to transfer the phosphoryl group back to ADP. In the rate-limiting E1P to E2P transition, the P-domain reorientates from its E1 to its E2 position, while the A-domain rotates to get in contact with the phosphorylation site, apparently protecting the phosphoryl group against hydrolysis, and ADP dissociates. The A-domain rotation seems also involved in the shutting off the cytoplasmic ion-access channel, preventing the binding of additional metal ions. The P-domain movement then disrupts the high-affinity metal binding site (TM-MBS) so the ion is released to the outside (extracellular/lumenal side) through an exit channel. The TM-MBS is now available to bind a proton (H^+) from the outside with high affinity. The hydrolysis of the phosphorylated Asp results in the E2 state. Mg^{2+} and inorganic phosphate (P_i) dissociate so the enzyme reverts to the E1 state, in which H^+ is released into the cell, and another cycle can begin (Kühlbrandt, 2004).

Because of their central role in cellular metabolism, the mechanism of action of P-ATPases needs to be tightly controlled on a short enough timescale to respond to cellular and external stimuli as well as to stress signals. Regulation is achieved at several different levels. P_{1B}-ATPases are regulated by domains that are situated within to the main chain of the enzyme. Moreover, plants have been found to have N- or C-terminal binding regulatory domains (Eren *et al.*, 2006; Eren *et al.*, 2007; Wong *et al.*, 2009; Mills *et al.*, 2010).

Transport experiments clearly indicated that P_{1B}-ATPases drive metal efflux from the cytoplasmic compartment to the outside (Rensing *et al.*, 1997; Voskoboinik *et al.*, 1998; Fan and Rosen, 2002; Mana-Capelli *et al.*, 2003; Eren and Argüello, 2004). However, due to experimental difficulties to obtain highly active everted vesicles or, alternatively, conditions to stabilize the metal occluded within the binding site (E1P state), for most P_{1B}-type ATPases the correct stoichiometry of the transport has not been established yet. Liu and coworkers (2006) provided evidence of the binding of 1 Zn^{2+} ion per ATPase to the TM-MBS of *E. coli* ZntA in the absence of other substrates. Even though it could be argued whether the metal binding site of the TM-MBS was fully occupied, this study is of great interest as it is the first analyzing isolated metal binding to the TM-MBS.

DISTRIBUTION AND PHYSIOLOGICAL ROLES

Unlike other P-ATPases subfamilies, P_{1B}-ATPases are present in all life kingdoms. P_{1B}-ATPases were first identified and partially characterized in bacteria, in which they maintain metal homeostasis, particularly those of Cu and Zn (Argüello *et al.*, 2007). This has been demonstrated by gene knockout studies that resulted in sensitivity of bacteria to high concentrations of metals (Odermatt *et al.*, 1993; Phung *et al.*, 1994; Rensing *et al.*, 1997, 2000; Rutherford *et al.*, 1999; Tottey *et al.*, 2001). Along with these studies, complementation assays enabled an initial insight on the substrate specificity of P_{1B}-type ATPases. As mentioned above, functional and biochemical assays showed that P_{1B}-ATPases can also transport non-physiological substrates (Tab. 1).

The extremophile *Archaeoglobus fulgidus* has two P_{1B}-type ATPases, CopA and CopB, that transport Cu^{+} and Cu^{+2} respectively, suggesting the presence of a fine tuning Cu homeostasis depending on redox conditions (Mandal *et al.*, 2002; Mana-Capelli *et al.*, 2003). In humans there are two genes (ATP7A and ATP7B) encoding Cu^{+}-ATPases: mutations in these genes lead to Menkes syndrome and Wilson disease respectively, which are associated with genetic Cu transport disorders (Bull *et al.*, 1993; Vulpe *et al.*, 1993; Bull and Cox, 1994; Lutsenko *et al.*, 2003). Studies on these proteins have considerably contributed to the understanding of P_{1B}-ATPase functions.

Plants significantly differ from other organisms both in number and selectivity of their P_{1B}-type ATPases; higher plant have more P_{1B}-ATPases than other organisms (Williams and Mills, 2005). From evolution studies and phylogenetic analysis it emerged that higher plants evolved with a relatively high number of P_{1B}-ATPases in their genomes (Baxter *et al.*, 2003) and it is possible to suppose that gradually there has been a diversification of functions so that some P_{1B}-ATPases that first have a simple role in expelling metals in excess from the cell, then turned out to be involved in xylem loading of micronutrients for long-distance transport (Williams and Mills, 2005). So far, several studies have been conducted in the model plant Arabidopsis that have led to a classification of these transporters. Eight genes have been found in the dicot Arabidopsis (*AtHMA1* to *AtHMA5*, *AtHMA6/PAA1*, *AtHMA7/RAN1* and *AtHMA8/PAA2*). In the last few years the attention has also been extended to P_{1B}-ATPases of monocots: nine genes have been found in rice (*O. sativa*) (*OsHMA1* to *OsHMA9*) and ten in barley (*H. vulgare*) (*HvHMA1* to *HvHMA10*). Even though rice genome has been sequenced since 2002 (Goff *et al.*, 2002; Yu *et al.*, 2002), poor functional information are still available on P_{1B}-type ATPases of rice. A dendrogram showing the relationships between P_{1B}-ATPase in Arabidopsis and rice is reported in fig. 3. A previous comparison between P_{1B}-type ATPases of these two model species was performed by Baxter and collaborators (2003). More recently, Williams and Mills (2005) analyzed the P_{1B}-ATPases of Arabidopsis, rice, barley and two algae species. In all dendrograms six clusters can be clearly identified. This means that the common ancestor of monocots and dicots was likely to have six P_{1B}-type ATPases. This is also proved by the high degree of conservation in intron number and

position observed in Arabidopsis and rice P_{1B}-ATPases within the same cluster but not between different clusters, as well as in the type of putative metal binding domains and their location. From functional studies, mainly conducted on Arabidopsis, and the comparison of sequences from Arabidopsis and rice, it is possible to divide these pumps into two big groups based on the metal specificity: Cu/Ag ATPases (clusters 3-6) and Zn/Cd/Co/Pb ATPases (clusters 1-2) (Williams and Mills, 2005). Finally, in Tab. 1 is summarized the current knowledge on P_{1B}-type ATPases of Arabidopsis and rice, following the six clusters in which they are divided.

THE POSITRON-EMITTING TRACER IMAGING SYSTEM (PETIS) FOR STUDYING THE SYSTEMIC MOVEMENT OF TRACE ELEMENTS

Since last decades, new opportunities for studying biology have been opened up by radionuclide imaging technologies. In particular, significant advancements in the positron emission tomography (PET) technique have been done and now is possible to obtain images of molecular dynamics for quantitating physiological functions noninvasively in clinical and animal studies (Kawachi *et al.*, 2011a). However, the conventional radionuclide counting and imaging tools used in plant science (i.e. Geiger-Müller counter, NaI(Tl) scintillation detectors and autoradiography) are invasive and require calibration by statistical analysis over a large number of test plants. In addition, in many cases real-time detection apparatuses have a limited field of view (FOV), making them inadequate for studying the most important agricultural theme (Kawachi *et al.*, 2011b). A new imaging system has been developed in recent years by Uchida and coworkers (2004), the positron-emitting tracer imaging system (PETIS), which is equipped with planar-type imaging apparatus. Most of the higher plants studied in laboratory experiments are thin and small; therefore, 2-D planar images are sufficient for studying them. Radioisotopes tracers, like ^{11}C, ^{13}N, ^{15}O, ^{52}Fe, ^{52}Mn, ^{62}Zn, ^{64}Cu, and ^{107}Cd, can be produced by a cyclotron (Arakawa *et al.*, 1995; Ishioka *et al.*, 1999; Watanabe *et al.*, 2001, 2009) or can be now bought directly as commercial products (e.g. ^{65}Zn and ^{22}Na), depending on their half-life. At the moment, PETIS is one of the most powerful tools for conducting real-time imaging *in vivo* on intact plants for studying, for instance, uptake and translocation of mineral nutrients (macro- and micronutrients) (Kiyomiya *et al.*, 2001b; Ohtake *et al.*, 2001; Suzuki *et al.*, 2006, 2008; Tsukamoto *et al.*, 2006; Kawachi *et al.*, 2008; Suwa *et al.*, 2008; Ishii *et al.*, 2009; Kawachi *et al.*, 2011a, 2011b; Yoneyama *et al.*, 2011) and non-essential elements (e.g. Cd) (Fujimaki *et al.*, 2010; Ishikawa *et al.*, 2011; Hu *et al.*, 2013; Nakamura *et al.*, 2013; Yoshihara *et al.*, 2014). Moreover, it is also possible to gain information about the distribution and translocation of water (Kiyomiya *et al.*, 2001a) and photoassimilates (Matsuhashi *et al.*, 2005; Kawachi *et al.*, 2006a, 2006b).

YEAST AS A MODEL SYSTEM TO STUDY METAL IONS TRANSPORT: HETEROLOGOUS PLANT GENE EXPRESSION

Analysis of gene function is of central importance for the understanding of physiological processes. In 1978, the development of yeast transformation provided a new way to isolate eukaryotic genes (Hinnen *et al.*, 1978) and, since then, functional expression has been frequently used to prove the function of genes or to isolate new genes and has contributed a lot to the functional analysis of the gene products (Romanos *et al.*, 1992; Frommer and Ninnemann, 1995). Since 1986, yeast cells have been used as functional expression systems for membrane proteins of bacterial and animal origin. For plant genes, yeast has become the preferred expression system. This technique can be especially valuable for the analysis of plant functions for which no mutants are available and for which no screening scheme or phenotype is predictable. This approach has been most powerful in identifying genes that are otherwise difficult to define, such as integral membrane proteins. The major breakthrough in transport physiology was the isolation of carrier genes involved in the uptake and distribution of specific nutrients. The expression assay also allows the analysis of structure-function relationships (Frommer and Ninnemann, 1995).

Heterologous expression systems are based on the assumption that the basic principles of protein expression and function are similar in all organisms. The sequences of most eukaryotic proteins are well conserved (Botstein and Fink, 1988). Eukaryotic organisms share many principles of cell compartmentation, intracellular transport, and regulation, such as vesicular trafficking along the secretory pathway (Bednarek and Raikhel, 1992; Bennett and Scheller, 1993). Nevertheless, important differences exist between fungal, plant, and animal cells in terms of presence and composition of cell walls, and the presence of specialized organelles such as plastids and vacuoles. Regarding energization of secondary active transport processes at the plasma membrane, plants are similar to yeast cells because they both use proton gradients. Multicellular organisms, however, have many properties for which no equivalent exists in unicellular organisms, such as intercellular communication across cell walls and through signals transferred in the vascular system in plants (Frommer and Ninnemann, 1995).

Heterologous systems can be used if the respective function is lacking in the host. Examples of plant transporters functionally expressed in yeast are reported below. The plasma membrane *A. thaliana* H^+-ATPase AHA2 partially complemented the *S. cerevisiae* ATPase *pma1* mutation. The protein was functional, but a large proportion was trapped in the endoplasmic reticulum (ER). Removal of the C-terminal domain of AHA2 led to increased targeting to the plasma membrane and fully complemented *pma1*. The same yeast mutant was used to compare the biochemical properties of three known major H^+-ATPase isoforms (Palmgren and Christensen, 1994). Other ATPase proteins have been characterized by complementation of the *ccc2* mutant of yeast. In particular, *Brassica napus* RAN1

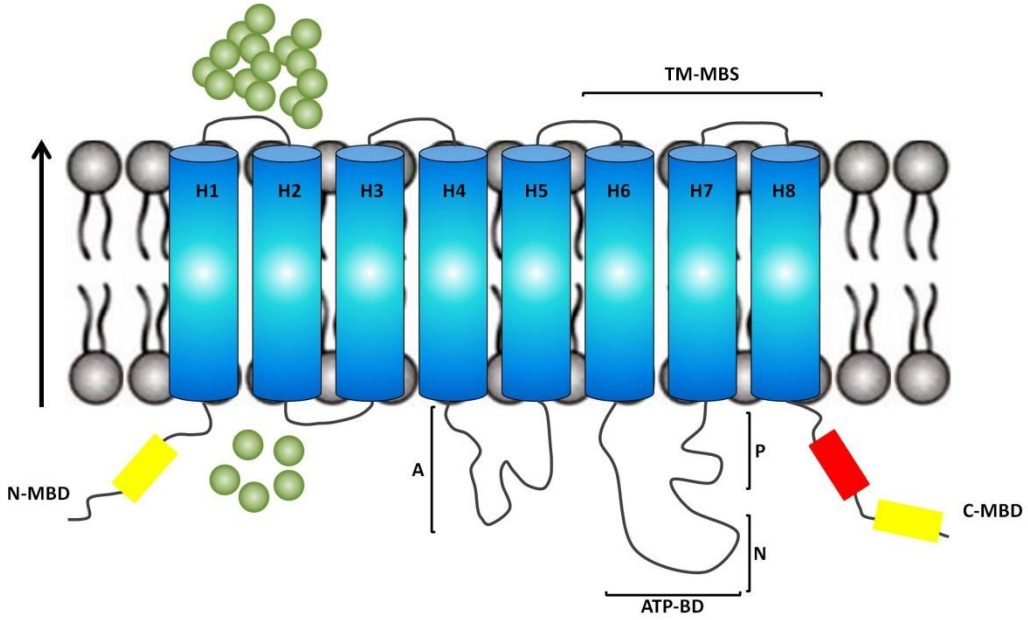
(Southron *et al.*, 2004), Arabidopsis AtHMA5 (Kobayashi *et al.*, 2008) and rice OsHMA5 (Deng *et al.*, 2013) have been identified as Cu-transporting proteins using drop test analysis.

Other membrane proteins not localized on the plasma membrane have also been functionally expressed in yeast. HMA3 P_{1B}-type ATPase of Arabidopsis (Gravot *et al.*, 2004) and rice (Ueno *et al.*, 2010), which are localized on the tonoplast, have been functionally characterized also by expression in wild type (wt) and/or mutant strain (*ycf1*) of *S. cerevisiae*, which is more sensitive to Cd than the wt. Another P_{1B}-ATPase well characterized using both wt and mutant strains, including *ycf1*, is the plasma membrane-localized rice HMA2. In particular, wt and/or *ycf1* mutant strains have been used to functional characterize OsHMA2 for Cd transport (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012; Yamaji *et al.*, 2013). Moreover, *zrc1* (Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012) and *zrt1zrt2* (Yamaji *et al.*, 2013) mutant strains have been transformed in order to prove that OsHMA2 is also able to transport Zn. In particular, *zrc1* mutant is sensitive to high Zn concentrations (Kamizono *et al.*, 1989; MacDiarmid *et al.*, 2000); conversely, *zrt1zrt2* results sensitive to low Zn concentrations (Regalla and Lyons, 2006). All the cited studies used yeast drop tests to functional characterize these proteins. However, controversial results have been sometimes obtained. Yamaji and coworkers (2013), for instance, obtained opposite results from those previously reported by Nocito *et al.* (2011) and Satoh-Nagasawa and coworkers (2012). In particular, they supposed OsHMA2 as an influx transporter instead of an efflux transporter of Zn and Cd, as hypothesized by the other research groups. A similar case is that of AtHMA4: expression of AtHMA4 in yeast resulted in hypersensitivity to excess Zn and Cd in one study (Bækgaard *et al.*, 2010), but resulted in increased tolerance to excess Zn and Cd in others (Papoyan and Kochian, 2004; Mills *et al.*, 2005; Verret *et al.*, 2005). These inconsistencies may be attributed to different yeast strains, experimental conditions, expression vector, and medium components (Yamaji *et al.*, 2013).

Some decades ago, Galgiani and Stevens (1976) developed a turbidimetric technique for studying yeast susceptibility to antimicrobial substances. This method is reproducible, inoculums independent, rapid, free from subjectivity and observer variability. For what regard plant gene functional characterization, only sometimes yeasts have been grown in liquid culture and growth rates of different transformants have been compared (Ramesh *et al.*, 2003; Gravot *et al.*, 2004; Nocito *et al.*, 2011; Li *et al.*, 2014). Finally, only in few studies (Clemens *et al.*, 1999; Li *et al.*, 2014) the yeast growth has been expressed in relation to different metal (Cd or Al) concentrations. In addition, Li and collaborators (2014) reported the growth of the different transformants as a 'relative growth', which is independent from the inoculums and the growth rate.

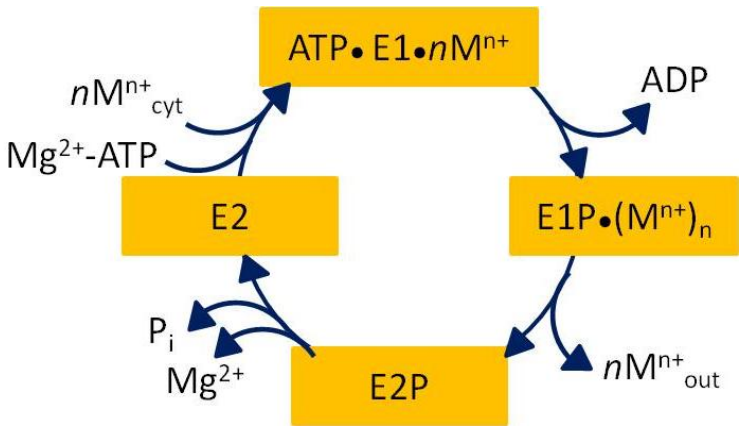
FIGURES AND TABLES

Figure 1. Schematic illustration of the topology and main domains present in P_{1B}-ATPases.



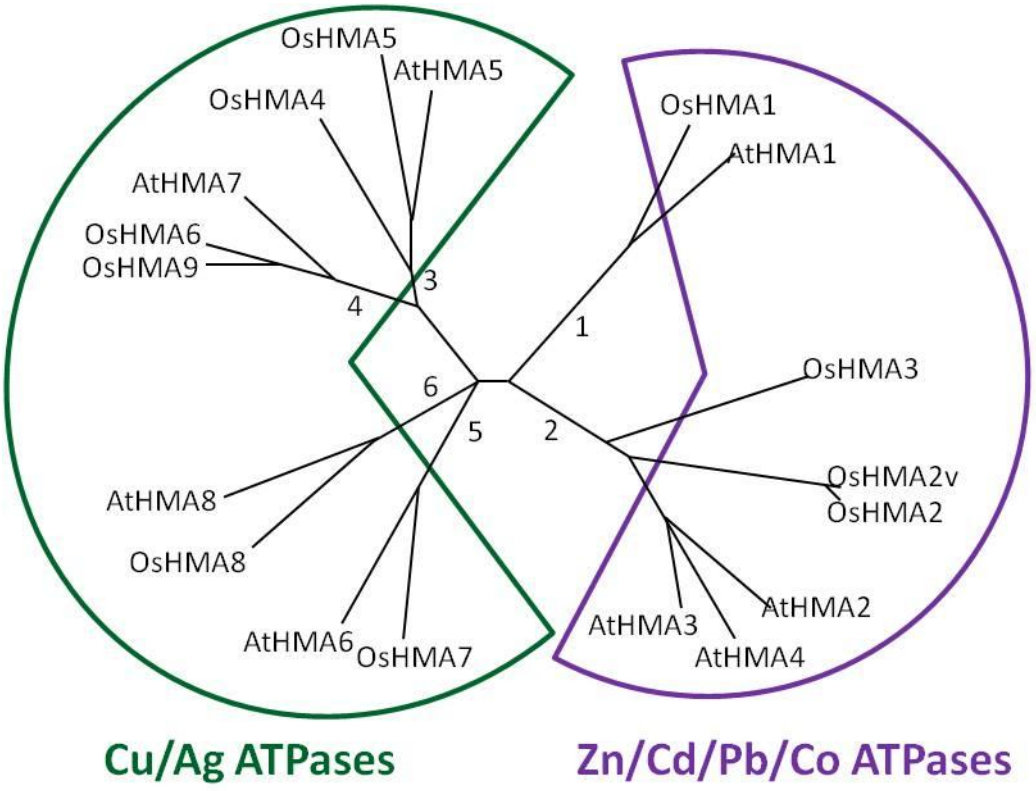
Transmembrane segments, H1 to H8, are indicated. The relative locations of the cytoplasmic actuator (A), phosphorylation (P) and nucleotide (N) domains are shown, as well as the ATP binding domain (ATP-BD). The conserved amino acids in H6, H7 and H8 form the transmembrane metal binding sites (TM-MBS); the N- and C-terminal metal binding domains (MBDs) are also reported (adapted from Argüello *et al.*, 2007).

Figure 2. P_{1B}-ATPases catalytic cycle.



E1, E2, E1P and E2P represent the basic conformations that the enzyme can assume. M^{n+} represents a metal transported by these enzymes; *n* indicates the uncertainty on the specific stoichiometry of transport. M^{n+}_{cyt} and M^{n+}_{out} represent the cytoplasmic or extracellular/luminal localization of the transported metal (adapted from Kühlbrandt, 2004; Argüello *et al.*, 2007).

Figure 3. Dendrogram showing families of P_{1B}-ATPases in Arabidopsis and rice.



The dendrogram was constructed using ClustalW (http://clustalw.ddbj.nig.ac.jp/). Accession numbers for *Arabidopsis thaliana* (UniProtKB) are: AtHMA1, Q9M3H5; AtHMA2, Q9SZW4; AtHMA3, P0CW78 (Q9SZW5); AtHMA4, O64474; AtHMA5, Q9SH30; AtHMA6, Q9SZC9 (Q3E9R8); AtHMA7, Q9S7J8; AtHMA8, B9DFX7. Accession numbers for *Oryza sativa* (Rice Genome Annotation Project, GenBank or PlantsT) are: OsHMA1, LOC_Os06g47550; OsHMA2, PlantsT 64490; OsHMA2v, which is OsHMA2 of the cultivar Volano, GenBank HQ646362; OsHMA3, LOC_Os07g12900; OsHMA4, LOC_Os02g10290; OsHMA5, LOC_Os04g46940; OsHMA6, LOC_Os02g07630; OsHMA7, LOC_Os08g37950; OsHMA8, LOC_Os03g08070; OsHMA9, LOC_Os06g45500. In brackets the accession numbers for AtHMA3 and AtHMA6 used by Nocito and coworkers and out of brackets the new accession numbers for AtHMA3 and AtHMA6 (adapted from Nocito *et al.*, 2011).

Table1. Summary of distribution and metal specificity of Arabidopsis (*A. thaliana*) and rice (*O. sativa*) P_{1B}-ATPases and the relative references.

Sub-group	Cluster	Protein	Tissue expression	Cellular localization	Metal specificity	References
Zn/Cd/Pb/Co	1	AtHMA1	Roots, shoots, flowers	Chloroplast envelope	Ca^{2+}, Zn^{2+}, Cu^{+}, Cd^{2+}, Co^{2+}	Seigneurin-Berny *et al.*, 2006; Moreno *et al.*, 2008; Kim *et al.*, 2009; Boutigny *et al.*, 2014
		OsHMA1	Roots, shoots, flowers, seeds	Chloroplast envelope	Zn^{2+}	Williams and Mills, 2005; Suzuki *et al.*, 2012
	2	AtHMA2	Vasculature of roots and shoots	Plasma membrane	Zn^{2+}, Cd^{2+}	Eren and Argüello, 2004; Hussain *et al.*, 2004; Eren *et al.*, 2006; Eren *et al.*, 2007; Wong and Cobbett, 2009; Wong *et al.*, 2009
		AtHMA4	Vasculature of roots and shoots	Plasma membrane	Zn^{2+}, Cd^{2+}, Pb^{2+}	Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Verret *et al.*, 2004, 2005; Papoyan and Kochian, 2004; Wong and Cobbett, 2009
		OsHMA2	Vasculature of roots and shoots	Plasma membrane	Zn^{2+}, Cd^{2+}	Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012; Satoh-Nagasawa *et al.*, 2013; Yamaji *et al.*, 2013
		AtHMA3	Roots, leaves	Vacuole	Cd^{2+}, Pb^{2+}, Zn^{2+}, Co^{2+}	Gravot *et al.*, 2004; Morel *et al.*, 2009; Chao *et al.*, 2012
		OsHMA3	Roots, shoots	Vacuole	Cd^{2+}	Ueno *et al.*, 2010; Miyadate *et al.*, 2011; Ueno *et al.*, 2011; Satoh-Nagasawa *et al.*, 2013
Cu/Ag	3	AtHMA5	Roots, flowers	?	Cu^{+}	Andrés-Colás *et al.*, 2006; Kobayashi *et al.*, 2008
		OsHMA4		?	?	
		OsHMA5	Vasculature of roots and shoots	Plasma membrane	Cu^{+}	Deng *et al.*, 2013
	4	AtHMA7	?	Post-Golgi compartment	Cu^{+}	Hirayama *et al.*, 1999; Woeste and Kieber, 2000; Binder *et al.*, 2010
		OsHMA6	?	?	?	
		OsHMA9	Vasculature of roots and shoots	Plasma membrane	Zn^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+}	Lee *et al.*, 2007
	5	AtHMA6	Roots, shoots	Chloroplast envelope	Cu^{2+}	Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005
		OsHMA7	?		?	
	6	AtHMA8	Shoots	Thylakoid membrane	Cu^{2+}	Abdel-Ghany *et al.*, 2005
		OsHMA8	?		?	

AIMS OF THE RESEARCH

It has been reported that Cd, a non-essential and toxic metal, is taken up from soil and translocated in a root-to-shoot direction through transporters of essential elements such as Zn, suggesting that the two metal ions may compete for the same transporter protein on a membrane. However, the movement of Zn and Cd ions across several biological membranes involves a wide range of transport systems, each characterized by a specific selectivity. Although divergent results have been obtained, they suggest that Zn-independent pathways for Cd translocation in plants could be possible.

The proteins belonging to the HMA (Heavy-Metal ATPases) family have been partially characterized as the main actors of the process of translocation of trace elements (essential or non-essential) to all organs of the plant. In particular, OsHMA2 is the main transport system so far described in rice as involved in the xylem loading of Zn and Cd, even though both its activity and function has not been unambiguously characterized. Moreover, it has been shown that root-to-shoot Cd translocation via the xylem is the major and common physiological process determining Cd accumulation in shoots and grains of rice plants.

The research carried out in this PhD project took place in this context. Indeed, the general purpose was studying the main mechanisms involved in the systemic distribution of some trace elements in rice plants. In particular the activity was aimed at better understanding the Zn and Cd translocation pathways, and was focused on studying the possible competition between Zn and Cd mainly for the root-to-shoot translocation, since these processes have been seen to be crucial in determining Cd accumulation in the shoots.

Specifically, the aims of this study were: (i) to investigate the effects of the possible competition between Zn and Cd on their chelation and subcellular compartmentalization at the root level, thus in reducing the amount of the two metals potentially mobile through the plant. This was done using physiological techniques aimed at isolating and quantifying thiol based Zn- and/or Cd-binding complexes; (ii) to investigate the potential inhibitory effect exerted by Zn on Cd translocation in unstressed rice plants, performing a short-term positron-emitting tracer imaging system (PETIS) experiment using ^{107}Cd as tracer; (iii) to identify genes encoding transporters involved in a putative Zn-insensitive Cd xylem loading, thus responsible for a possible Zn-independent Cd translocation pathway, by performing bioinformatic analysis. Our attention focused on the P_{1B}-type ATPase family in order to search for orthologs of the genes codifying the transporters that in the model plant Arabidopsis were found to mediate the xylem loading of Cd; (iv) to functional characterize the transporters encoded by the abovementioned genes by heterologous expression in *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

PLANT MATERIAL, GROWTH CONDITIONS AND SAMPLING

Rice (*Oryza sativa* L. spp. *japonica* cv. Roma) caryopses were placed on filter paper saturated with distilled water and incubated in the dark at 26 °C. Seven days later, seedlings were transplanted into 5 L plastic tanks (eight seedlings per tank) containing the following complete nutrient solution (pre-growing solution): 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 500 µM $MgSO_4$, 250 µM $NH_4H_2PO_4$, 30 µM Na_2O_3Si, 25 µM Fe-tartrate, 46 µM H_3BO_3, 9 µM $MnCl_2$, 1 µM $ZnCl_2$, 0.3 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$ (pH 6.5). Seedlings were kept for a 12-day-pre-growing period 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 400 µmol m^{-2} s^{-1}. At the end of the pre-growing period, plants were differentially exposed for a 10-day period to different concentrations of Zn (0.1, 1 and 10 µM), in the absence or presence of a steady amount of Cd (0.1 µM), or to different concentrations of Cd (0.01, 0.1 and 1 µM), in the presence of a steady amount of Zn (1 µM), by supplementing the pre-growing solution with different amounts of $ZnCl_2$ and/or $CdCl_2$. All hydroponic solutions were renewed daily to minimize nutrient depletion.

Plants were harvested and roots were washed for 10 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.

For PETIS (positron-emitting tracer imaging system) experiments, rice (*O. sativa* L. spp. *japonica* cv. Nipponbare) caryopses placed on plastic mesh floated on distilled water and incubated in the dark at 25 °C. Seven days later, seedlings were pre-grown in a one-quarter-strength Kimura B nutrient solution for 7 days and then grown in a full-strength Kimura B nutrient solution for another 7-day period. The Kimura B nutrient solution consisted of 700 µM $(NH_4)_2SO_4$, 470 µM $MgSO_4$, 370 µM $CaCl_2$, 270 µM K_2SO_4, 170 µM Na_2HPO_4, 11 mg L^{-1} Fe-citrate, 15 µM H_3BO_3, 4.6 µM $MnSO_4$, 0.16 µM $CuSO_4$, 0.15 µM $ZnSO_4$, 0.10 µM Na_2MoO_4 (pH 5.5). Plants were kept in a growth chamber maintained at 30 °C and 65% relative humidity during the 16-h light period and at 25 °C and 65% relative humidity during the 8-h dark period. Photosynthetic photon flux density was 400 µmol m^{-2} s^{-1}.

DETERMINATION OF Zn AND Cd IN ROOTS AND SHOOTS

Samples of 200 mg fresh weight (FW) were mineralized at 120 °C in 5 mL 14.4 M HNO_3, clarified with 1.5 mL 33% (w: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. Zn and Cd content was measured by inductively coupled plasma mass spectrometry (ICP-MS; Bruker Aurora M90 ICP-MS).

ANALYSIS OF ROOT-TO-SHOOT Zn AND Cd TRANSLOCATION

At the end of the exposure period, shoots were cut at 2 cm above the roots with a microtome blade. Xylem sap exuded from the lower cut surface was collected for 45 min and stored into 1.5 mL plastic vial. The amount of collected sap was determined by weighing and the concentration of Zn and Cd was measured by ICP-MS.

DETERMINATION OF NON-PROTEIN THIOLS

Roots were pulverized using mortar and pestle in liquid N_2 and stored frozen in a cryogenic tank. For total non-protein thiol (NPT) content, 400 mg of root powders were extracted in 600 µL of 1 M NaOH and 1 mg mL^{-1} $NaBH_4$, and the homogenate was centrifuged for 10 min at 13 000 *g* and 4 °C. Four hundred microliters of supernatant was collected, 66 µL of 37% HCl was added and then centrifuged again for 10 min at 13 000 *g* and 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 (pH 7.5) containing or not 0.6 mM Ellman's reagent {[5,5'-dithiobis(2-nitrobenzoic acid); DTNB]}. The samples' absorbances at 412 nm were then spectrophotometrically measured.

Zn AND Cd FRACTIONING IN RICE ROOTS

Metal fractioning was carried out essentially as described by Rauser and Meuwly (1995). Briefly, frozen root tissues (2 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 phenylmethanesulfonyl fluoride (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 resuspended 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, and the supernatant (extract 2) was collected and added to the extract 1 for freezing. Resuspension 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% (w: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 2 cm column of diethylaminoethyl cellulose (DEAE) Sephadex A-25 (GE Healthcare, Uppsala, Sweden) equilibrated

with 10 mM Tris-HCl (pH 8.6). After loading, the column was washed with 50 mL of equilibrating buffer to remove unadsorbed solutes. All the fluid passing through the anion-exchanger was collected for Zn and Cd analysis (cationic fraction). Anionic material was eluted with 6 mL of 10 mM Hepes (pH 8.0) and 1 M KCl. Five milliliters of the anionic fraction so obtained was further resolved by gel filtration on a Sephadex G-50 column (0.8 x 130 cm) equilibrated with 10 mM Hepes (pH 8.0) and 300 mM KCl. The column was developed in equilibrating buffer at 12.5 mL h^{-1} at 4 °C. The absorbance at 254 nm was recorded and fractions of about 5 mL were collected for Zn, Cd and NPT analysis. The column was calibrated by using 5 mL of 0.25% (w:v) Blue dextran 2000 and 1% (w:v) $K_3Fe(CN)_6$ to estimate void (V_0) and total volume (V_t), respectively. The partition coefficient, K_{av}, was calculated using the following equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e was the elution volume. For NPT determination, selected fractions from gel filtration were pooled in a glass tube, lyophilized, and finally analyzed as above described. The amount of Zn and Cd ions in mineralized pellets, extracts and column effluents was measured by ICP-MS.

^{107}Cd TRACER

^{107}Cd was produced as described by Fujimaki *et al.* (2010). Briefly, a silver foil was bombarded for 2 h with a 17 MeV energetic proton beam at a current of 5 μA from a cyclotron at Takasaki Ion Accelerators for Advanced Radiation Application, Japan Atomic Energy Agency. The irradiated material was dissolved in HNO_3, and then diluted in warm water. Silver was precipitated by adding to the solution a 0 to 2 M gradient of HCl. Supernatant, containing ^{107}Cd was filtered, dried and dissolved in water. Aliquots of ^{107}Cd (6.7 MBq) were added to 12.2 mL of the culture solutions [0.5 mM $CaCl_2$, 0.1 μM nonradioactive $CdCl_2$, different concentrations (0.1, 1, 10 μM) of $ZnSO_4$] used for the experiments.

PETIS IMAGING

Rice plants were transferred into appropriate 120 x 14 x 10 mm plastic vessels, containing 12 mL of full-strength Kimura B nutrient solution. Before starting the experiments, plants were acclimatized for 1 h in aerated 0.5 mM $CaCl_2$ solutions. In a typical experiment, 6 vessels – each containing one plant – were placed in the mid-plane between two opposing detector heads of the PETIS apparatus (a modified type of PPIS-4800; Hamamatsu Photonics, Japan). Detectors were focused on the plants in order to observe the culture solutions, the whole root apparatus, the shoot bases, and the proximal portions of the shoots, in a 12 x 19 cm field of view (FOV). The entire setup was installed in a growth chamber maintained at 30 °C and 65% relative humidity in continuous light at the density of 400 μmol m^{-2} s^{-1}.

PETIS experiments were started by injecting the ^{107}Cd marked culture solutions in the different plastic vessels. All the solutions were continuously stirred with gentle aeration in order to maintain an uniform composition. The surface level of the solution in the vessels was maintained by supplying fresh 0.5 mM $CaCl_2$ solutions with an appropriate solution supply system. Images of the ^{107}Cd distribution in the FOV were obtained every one minute for 24 h. The data of serial images obtained from the PETIS apparatus were analyzed for ^{107}Cd distribution in specific regions of interest (ROIs; background, culture solution, distal roots, proximal roots, shoot base, and proximal shoot) using NIH ImageJ 1.45s software (Schneider *et al.* 2012). ROIs were manually selected on the image data and the time-activity curves (time-courses of signal intensity in the ROIs) were generated and used to estimate the dynamics of Cd in the culture solution, whole root apparatus, shoot base, and proximal shoot, as described by Yoshihara *et al.* (2014).

AUTORADIOGRAPHY

At the end of the PETIS experiments, plants were dissected, fixed on paper sheets with adhesive tape, and then placed in contact with imaging plates (BAS-MS2040, GE Healthcare, Japan) in cassettes for 3 days. The imaging plates were scanned using a Bio Imaging Analyzer (Typhoon FLA 7000, GE Healthcare, Japan) to generate the autoradiographic images of ^{109}Cd in the plants. In fact, ^{109}Cd with a longer half-life (461 days) than ^{107}Cd (6.5 h) was also obtained at a minor ratio (approximately 1:3000) in the production process of ^{107}Cd.

CLONING OF *OsHMA2, OsHMA4 AND OsHMA5* cDNA

Total RNA was extracted from rice roots using TRIzol Reagent (Life Technologies) and first-strand cDNA synthesis was carried out using SuperScript III first-strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (Life Technologies), according to the manufacturer's instructions. The entire coding sequence of the heavy metal P_{1B}-ATPase 2 (*OsHMA2*), 4 (*OsHMA4*) and 5 (*OsHMA5*) was amplified by PCR using the first-strand cDNA, Pfu DNA polymerase (Promega) and the following couples of primers:

Gene	Fwd Primer Sequence (5' → 3')	Rev Primer Sequence (5' → 3')
OsHMA2	AAAAA*GGATCC*TAAATA**ATG**GCGGCGGAGGGAGGG	AAAAAGGATCCCTACTCCACTACGATCTCAGG
OsHMA4	AAAAATAAATA**ATG**GAGCAGAATGGAGAGAA	AAAAATCACACCAAATCCGGGTCAT
OsHMA5	AAAAA*GGATCC*TAAATA**ATG**GCGGCGAGCACTCGAGC	AAAAAGGATCCTCAACGGCCCAGTTTTGGGCT

The primers were designed according to *OsHMA2* sequence in PlantsT database (PlantsT 64490) and to *OsHMA4* and *OsHMA5* sequences in the MSU Rice Genome Annotation Project Database and

Resource (*OsHMA4* - LOC_Os02g10290; *OsHMA5* - LOC_Os04g46940). The forward primers included a consensus sequence for yeast translation initiation (underlined) before the ATG codon (bold) (Cigan and Donahue, 1987; Donahue and Cigan, 1988; Hamilton *et al.*, 1987). The *Eco*RI-ended *OsHMA4* cDNA was cloned into the *Eco*RI site of the yeast (*Saccharomyces cerevisiae*) expression vector pESC-URA and pESC-TRP (Stratagene) under the control of *GAL10* promoter, while the *Bam*HI-ended *OsHMA2* and *OsHMA5* cDNAs were cloned into the *Bam*HI site of the yeast expression vector pESC-URA and pESC-TRP (only *OsHMA5*) under the control of *GAL1* promoter. The identity of the PCR products was verified by sequencing both the strands.

OsHMA2, OsHMA4 AND OsHMA5 FUNCTIONAL CHARACTERIZATION IN YEAST

The yeast strain INVSc1 (*MATa his3D1 leu2 trp1-289 ura3-52*) was transformed with the chimeric OsHMA2-pESC-URA, OsHMA4-pESC-URA, OsHMA5-pESC-URA or the empty pESC-URA vector by the standard lithium acetate method (Gietz *et al.*, 1992; Yeast Protocols Handbook, Clontech). URA3 recombinant yeast cells were selected on solid synthetic minimal medium (SD) containing 2% (w:v) Glc, 6.7 g L^{-1} yeast nitrogen base without amino acids and 1.92 g L^{-1} yeast synthetic dropout media without uracil (Sigma) and then plated both on fresh solid SD and SG media. The SG medium differed from the SD one for the presence of 2% (w:v) Gal instead of Glc.

The standard lithium acetate method (Gietz *et al.*, 1992) was also used to transform the yeast mutant strain ZHY3 (*zrt1zrt2*) (*MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*) (Zhao and Eide, 1996), sensitive to low Zn concentrations, with the chimeric OsHMA4-pESC-TRP, OsHMA5-pESC-TRP or the empty pESC-TRP vector. TRP1 recombinant yeast cells were selected on solid synthetic minimal medium (SD) containing 2% (w:v) Glc, 6.7 g L^{-1} yeast nitrogen base without amino acids and 1.92 g L^{-1} yeast synthetic dropout media without tryptophan (Sigma) and then plated both on fresh solid SD and SG media. The SG medium differed from the SD one for the presence of 2% (w:v) Gal instead of Glc.

Moreover, during previous studies, the yeast strain INVSc1 was transformed with the empty pYES2.1 expression vector or the chimeric OsHMA2-pYES2.1, OsHMA4-pYES2.1, OsHMA5-pYES2.1 obtained cloning the full length coding sequences of the genes into the expression vector pYES2.1/V5-His-TOPO (pYES2.1, Life Technologies) under the control of *GAL1* promoter.

For the drop test analyzing the Cd tolerance, URA3 recombinant cells of the yeast strain INVSc1 were grown to approximately 1 A_{600} unit in the liquid SD medium, washed twice with sterile distilled water and then resuspended in water to final 0.01, 0.1 and 1, or 0.5, 0.05 and 0.005 A_{600} units. Ten microliters of each cell suspension was dropped on SG media, in order to induce gene expression, containing or not different concentration of Cd^{2+} (from 10 to 200 μM), supplemented as $CdCl_2$. Yeast cells were incubated at 28 °C for 3 or 5 days and analyzed for Cd tolerance.

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In addition, OsHMA4 and OsHMA5 were also tested for the Zn transport using a yeast mutant strain ZHY3 defective for Zn uptake. TRP1 recombinant cells were grown to approximately 1 A_{600} unit in the liquid SD medium, washed twice with sterile distilled water and then resuspended in water to final 0.01, 0.1 and 1 A_{600} units. Ten microliters of each cell suspension was dropped on SG media, in order to induce gene expression, supplemented or not with 0.2 mM $ZnCl_2$ and 0.2 mM EDTA (2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid) that chelates Zn creating a Zn-limiting condition. Yeast cells were incubated at 28 °C for 3 days and analyzed for Zn transport.

For the growth analysis, recombinant yeast cells of the strain INVSc1 carrying the chimeric OsHMA2-pESC-URA, OsHMA4-pESC-URA, OsHMA5-pESC-URA or the empty pESC-URA vector were grown – in liquid SD or SG, under continuous shaking (150 rpm), at 28°C – up to reach a mid-log phase. Yeast cells were then resuspended to a final absorbance of 0.05 A_{600} unit in fresh liquid SD or SG media and finally grown under the same condition for 6 (SD) or 27 (SG) h. For the growth inhibition analysis, yeast cells expressing OsHMA2, OsHMA4, OsHMA5 or harboring the empty pESC-URA vector were resuspended to a final absorbance of 0.01 A_{600} in fresh liquid SG media containing or not different excesses of Zn^{2+} (from 1.5 to 16.5 mM) or Cd^{2+} (from 15 to 165 μM), supplemented as $ZnCl_2$ or $CdCl_2$ respectively, and then grown for 48 h, under continuous shaking (150 rpm), at 28°C.

Yeast growth was monitored by measuring the optical density at 600 nm. The duplication times of the yeast cells were calculated by fitting the equation $A_{600}(t) = A_{600}(t_0)\ e^{kt}$ to the experimental data. The percentage of growth inhibition was calculated with respect to the growth of the yeast cells in the absence of any excess of Zn or Cd and data were fitted with a four parameter sigmoid curve using SigmaPlot for Windows version 11.0 (Systat Software, Inc., Chicago, IL, USA).

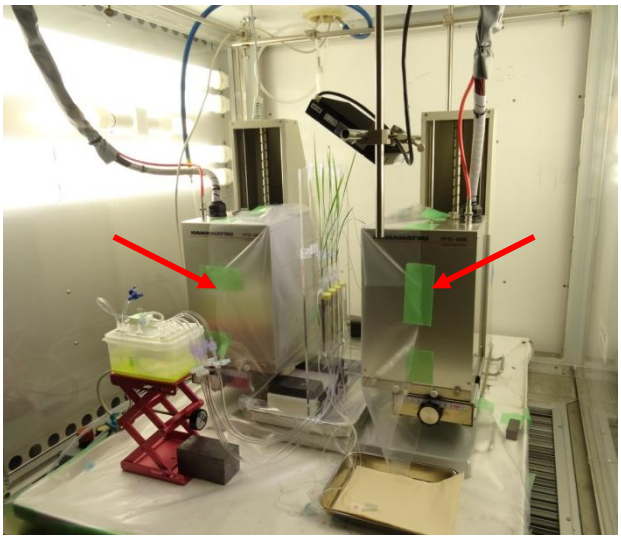
STATISTICAL ANALYSIS

Statistical analysis was carried out using SigmaPlot for Windows version 11.0 (Systat Software, Inc., Chicago, IL, USA). Quantitative values are presented as mean ± standard error of the mean (SE). Significance values were adjusted for multiple comparisons using the Bonferroni correction. Statistical significance was at $P < 0.05$. Student's t-test was used to assess the significance of the observed

differences between plants exposed or not to Cd in each Zn exposure condition analyzed. Statistical significance was at $P \leq 0.001$.

FIGURES AND TABLES

Figure 1. The setup of the PETIS imaging apparatus.



A panoramic view of the setup of the PETIS imaging apparatus; plants were set at the centre of two detector heads (indicated with red arrows) with LED lighting, reservoir tank and a bubbling pump (backside).

SECTION I

A zinc independent pathway for root-to-shoot cadmium translocation

RESULTS AND DISCUSSION

RESULTS

To better characterize the possible interactions between Cd and Zn translocation pathways we performed two set of experiments: in the first, rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM), in the absence or presence of a steady amount of Cd (0.1 μM), whilst, in the second, plants were exposed for 10 days to different Cd concentrations (from 0 to 1 μM) in the presence of a steady amount of Zn (1 μM). In all the experiments changes in metal concentrations did not produce either significant effects on the growth of both roots and shoots, or any apparent symptoms of stress (data not shown): at the end of the exposure period, root and shoot dry weights of a single plant were 0.128 ± 0.004 g and 0.588 ± 0.023 g, respectively.

Zn AND Cd PARTITIONING BETWEEN ROOT AND SHOOT

Results of the first experiment indicated that, in the absence of any Cd sources, Zn concentration in roots and shoots significantly increased as Zn availability in the external medium did. A similar trend was observed in the presence of a steady amount of Cd (0.1 μM); however, in this condition, Zn concentration in the shoots was lowered by the presence of Cd, whilst in the roots it was not significantly affected by the presence of the interfering metal (Fig. 1a,b). Finally, Cd accumulation in shoots and roots was significantly affected moving the external Zn concentration from 0.1 to 1 μM. Interestingly, a further increase in Zn availability in the medium - up to reach 10 μM - did not produced any additional decrease in Cd accumulation (Fig. 1c,d).

In the second experiment, the concentration of Zn in the shoots significantly decreased as Cd availability in the external medium increased, whilst Zn accumulation in the roots did not seem significantly affected by Cd availability (Fig. 1e,f). Under the same conditions a steady increase in Cd accumulation was observed in both shoots and roots moving the external Cd concentration from 0.01 to 1 μM (Fig. 1g,h).

ANALYSIS OF ROOT-TO-SHOOT TRANSLOCATION OF Zn AND Cd

Dynamics of root-to-shoot translocation of Zn and Cd were examined by measuring their concentrations in the xylem sap of rice plants exposed to the different combinations of the metals. In these experiments, translocation was estimated as the amount of Zn and Cd ions loaded and transported in the xylem sap for 45 min.

The amount of Zn ions transported in the xylem sap linearly increased as Zn concentration in the external medium did, both in the absence and in the presence of 0.1 μM Cd^{2+} (Fig. 2a). As expected the presence of a steady amount of Cd (0.1 μM) slightly decreased Zn translocation in all the conditions

analyzed (Fig. 2a). In the same experiment Cd translocation was significantly inhibited moving the external Zn concentration from 0.1 to 1 μM, and then resulted not affected following a further increase in Zn availability (Fig. 2b). On the other hand, enhancing Cd concentration in the external medium progressively reduced Zn translocation (Fig. 2c) and induced significant increases in the amount of Cd ions transported in the xylem sap (Fig. 2d). In the latter case, the translocation isotherm of Cd started to approach saturation at 0.1 μM.

In both the experimental setup, Zn and Cd translocation was linearly related to the total amount of Zn and Cd ions accumulated in the shoots over the 10-day period (Fig. 3).

EFFECT OF Zn AND Cd EXPOSURE ON NON-PROTEIN THIOL BIOSYNTHESIS

Since activation of thiol metabolism may potentially allow a greater proportion of Zn and Cd to be retained in roots through vacuolar sequestration, we measured the levels of non-protein thiols (NPTs) in roots of plants exposed to the different combination of the two metals. The NPT levels of the roots increased as the Zn concentration in the external medium did, either in the absence or in the presence of 0.1 μM Cd^{2+}. Interestingly, the levels of NPTs measured for each Zn exposure condition were significantly enhanced by the concomitant presence of Cd in the media (Fig. 4a). Finally, the NPT levels of the roots significantly increased as the external Cd concentration did (Fig.4b).

FRACTIONING OF Zn AND Cd IN RICE ROOTS

Fractioning of Zn and Cd retained by roots was carried out using a sequential extraction procedure with buffer and acid (Rauser and Meuwly, 1995; Nocito *et al.*, 2011). Table 1 and 2 summarize results obtained in representative experiments where the sum of Zn and/or Cd ions recovered in the different fractions accounts for at least 97% of the total Zn and/or Cd content of the roots. Following extraction, three main metal-ion fractions were obtained: i) buffer soluble (extracts 1-6); ii) acid soluble (extracts 7-9); iii) ash (non-soluble Zn and/or Cd). Extracts 1 to 6 were further resolved into two fractions, named anionic and cationic, by anion-exchange chromatography.

The procedure we used for metal ion fractioning allowed to discriminate Zn and Cd ions potentially mobile (cationic) from those retained in complexes with thiol-peptides or other soluble molecules negatively charged in the extraction buffer (anionic), or tightly adsorbed to cellular matrices or apoplast components (acid soluble and ash). So, the last three fractions should be considered not available for root-to-shoot translocation (Nocito *et al.*, 2011). An integrated analysis of data in which the total amount of each metal ions retained by roots is divided into two fractions – named mobile (cationic, i.e. potentially available for root-to-shoot translocation) and non-mobile (anionic + acid soluble + ash) – according to Nocito *et al.* (2011) is reported in Figure 5. Results indicated that both mobile and non-mobile fractions of Zn were linearly related to the Zn concentration in the external

medium, either in the absence or in the presence of Cd (Fig. 5a); however, the presence of a steady amount of Cd in the medium significantly enhanced the concentration of Zn ions in the mobile fractions because of the contraction in the amount of the metal in the non-mobile fractions. In these last conditions, increases in Zn external concentration decreased Cd content in the roots but did not produce any significant effect on the amount of Cd in the mobile fraction (Fig. 5b). On the other hand, increases in Cd external concentration significantly enhanced the amount of Zn and Cd ions measured in the mobile fractions (Fig. 5c,d).

Finally, the anionic buffer-soluble fractions were further resolved by gel filtration on a Sephadex G-50 column into peaks I, II and III (Fig. 6). Zn/Cd ions into peak I at the void volume of the column (V_0) were ascribed to non-specific adsorption of the metal ions to proteins. Peaks II and III – centred between V_0 and V_t of the column – were designated as the classical HMW thiol based Cd-binding complexes and LMW thiol based Zn- and/or Cd-binding complexes, respectively, since the amount of NPTs recovered in these fractions accounted for 22 (1 μM Zn^{2+}, 0 μM Cd^{2+}) to 89% (1 μM Zn^{2+}, 1 μM Cd^{2+}) of the total GSH equivalents measured in the roots; no thiols were found in peaks II and III of the anionic buffer-soluble fraction obtained from roots of plants grown under 0.1 μM Zn^{2+} in the absence of Cd^{2+} (data not shown). Neither Zn nor Cd ions were found at total volume (V_t) of the column (K_{av} = 1). Peaks II (HMW) were centred around K_{av} = 0.44 for Cd, whilst peaks III (LMW) were centred around K_{av} = 0.58 and K_{av} = 0.62, for Zn and Cd, respectively. Data analysis revealed that rice roots sequestered Zn and Cd differently. In all cases Zn appeared in LMW complexes. In the absence of any source of Cd LMW Zn-binding complexes significantly increased as the Zn external concentration did (Fig. 6a,b,c). Interestingly, the concomitant presence of Cd in the external medium significantly enhanced the amount of Zn ions found in LMW complexes. Moreover, the amount of Cd ions in LMW complexes remained constant in all the Zn conditions analyzed, differently from that found in HMW complexes, which instead decreased as Zn external concentration increased (Fig. 6e,f,g). On the other hand, LMW and HMW Cd-binding complexes appeared in a dynamic equilibrium depending on Cd external concentrations, as indicated by the ratio between the amount of Cd ions retained in each complex, which deeply changed moving toward the highest Cd external concentration, in the presence of a steady amount of Zn (Tab. 3, 4). Finally, in the same conditions, the amount of Zn found in LMW complexes significantly increased as Cd external concentration did (Fig. 6b,d,f,h).

KINETIC ANALYSIS OF ^{107}Cd SYSTEMIC MOVEMENT

The systemic movement of Cd in the whole plant was further analyzed using ^{107}Cd in short-term (24 h) PETIS experiments. Figures 7a shows the field of view of a typical experiment and the regions of interest (ROIs) used to estimate the dynamics of Cd in the plants. In particular, six ROIs (background, culture solution, distal roots, proximal roots, shoot base, and proximal shoot) were set for each plant.

The experiments were started by applying to the roots fresh marked (^{107}Cd; 0.55 MBq mL^{-1}) culture solutions containing 0.5 mM $CaCl_2$, 0.1 μM $CdCl_2$, and different concentrations of Zn^{2+} (0.1, 1, and 10 μM). ^{107}Cd absorption by roots was immediately observed after injection as clearly showed by the comparison of figure 7b with 7c. In all the conditions analyzed, the amount of Cd in the roots increased over the time reaching a common maximum plateau value at about 11 h for both plants exposed to 0.1 and 1 μM Zn^{2+}, and about 16 h for plants exposed to 10 μM Zn^{2+}. Concerning the shoots (Fig. 7d), ^{107}Cd signals appeared in the lower parts of the stems (shoot bases; Fig. 8) within 1 h from the injections and then linearly increased at least up to 10 h. Considering the initial slope of each curve (from 0 to 10 h) we estimated that the rate of Cd translocation was significantly higher in plants exposed to 0.1 μM Zn^{2+} (0.0126 ± 0.0003 nmol h^{-1}) with respect to those exposed to 1 or 10 μM Zn^{2+}, for which the estimated rate of Cd translocation was similar (0.0078 ± 0.0002 nmol h^{-1} or 0.0075 ± 0.0004 nmol h^{-1}, respectively). Similar results were obtained in a second independent analysis (Fig. 9).

DISCUSSION

It has been reported that Cd accumulation in plants can be inhibited by increasing the level of Zn in the soil. Such an observation could be mainly due to an effect of Zn on biological processes involved in Cd uptake and translocation, since it is unlikely to suppose this effect as the result of soil chemical processes (Basta and Tabatabai, 1992). In fact, the addition of Zn to the soil should increase the bioavailability of free Cd ions resulting in greater Cd uptake and accumulation in the plants. Several studies have shown that Zn and Cd ions may compete for the same transporter protein on a membrane. This means that increasing the concentration of Zn will decrease the movement of Cd through the transporter, and vice versa. Thus, the concept of competition necessarily implies the existence of a reciprocal interference between the two ions, which is intrinsically associated to their physical characteristics and to the selectivity of the transport system we consider. However, the presence of scarcely selective transport systems does not necessarily imply the existence of a "strong reciprocity" between the systemic fluxes of Zn and Cd in the whole plant, since the movement of the two ions across a plethora of biological membranes may involve several and often-different transport systems, each characterized by a specific selectivity. Finally, the level of complexity may be further increased considering that the expression level of each transporter may be often modulated by the nutritional status of the plant and that, once inside the cells, Zn and Cd ions are subjected to complex equilibria, which may differentially influence their relative mobility inside the plant. Such aspects have important technological implications in the fields of food safety, especially in cases where the strategies used for containing Cd accumulation in the crops be founded on Zn fertilization. In this view, we

performed a complete set of competition experiments with Zn and Cd in order to analyze their possible interactions and reciprocal effects at the translocation level using rice plants grown in a soil-free system.

The main results we obtained clearly indicate the lack of a fully reciprocity considering the effect of Cd on Zn accumulation, and vice versa, since the accumulation of Zn in the shoots was significantly inhibited by Cd increases in all the analyzed conditions, whereas those of Cd was only partially impaired by Zn increases (Fig. 1). In fact, Cd accumulation in the shoot was reduced by 16% moving the external Zn concentration of one order of magnitude (from 0.1 to 1 μM), then remained essentially unaffected by a further increase in Zn availability, from 1 to 10 μM, indicating Cd accumulation in this range as a Zn-independent process. However, from this data we cannot conclude that the effects of Zn on shoot Cd accumulation necessarily results from mechanisms involved in root-to-shoot Cd translocation, since Cd absorption by plants seemed affected by Zn concentration, as indicated by the total amount of Cd in the whole plant, whose value decreased as Zn concentration in the external medium increased (Fig. 1c,d). A similar consideration may be done for Zn accumulation in the shoot under different Cd concentrations (Fig. 1e,f). However, Zn and Cd translocation, evaluated at the end of the exposure period on the bases of metal concentrations in the xylem sap, resulted linearly related to total amount of Zn and Cd ions accumulated in the shoots over the 10-day period (Fig. 2, 3), suggesting the hypothesis that the differential effect produced by the two metals on Zn and Cd accumulation in the shoot was reasonably due to the existence of at least two translocation pathways with different metal selectivity. Moreover, it is also important to consider that the total amounts of Zn and Cd ions in the root tissues are often poor indicators of their actual availability to be loaded into the xylem and then translocated in a root-to-shoot direction, since, once inside the cells, Zn and Cd ions may be trapped into the root through selective binding sites or molecules with high affinity for the metals or through transfer across a membrane into an intracellular compartment (Clemens, 2006; Ueno *et al.*, 2010; Nocito *et al.*, 2011). Thus the concentration of Zn and Cd ions potentially mobile in the plants should result from different biochemical and physiological processes involved in metal chelation, compartmentalization and adsorption (Souza and Rauser, 2003; Nocito *et al.*, 2011; Olsen and Palmgren, 2014).

It has been shown that different photosynthetic organisms respond to excess of Zn and Cd ions by producing phytochelatins (PCs), a class of thiol compounds involved in metal chelation and vacuolar sequestration (Grill *et al.*, 1987, 1988; Zenk, 1996; Tennstedt *et al.*, 2009; García-García *et al.*, 2014; Song *et al.*, 2014), which may largely contribute to Zn and Cd root retention (Tennstedt *et al.*, 2009; Wong and Cobbett, 2009). In our conditions, rising Zn or Cd exposure progressively increased, with different efficiency, the level of NPTs in the roots (Fig. 4). Such a finding may be reasonably ascribable to the activation of PC biosynthesis, as revealed by the gel filtration analysis of the anionic fractions in which

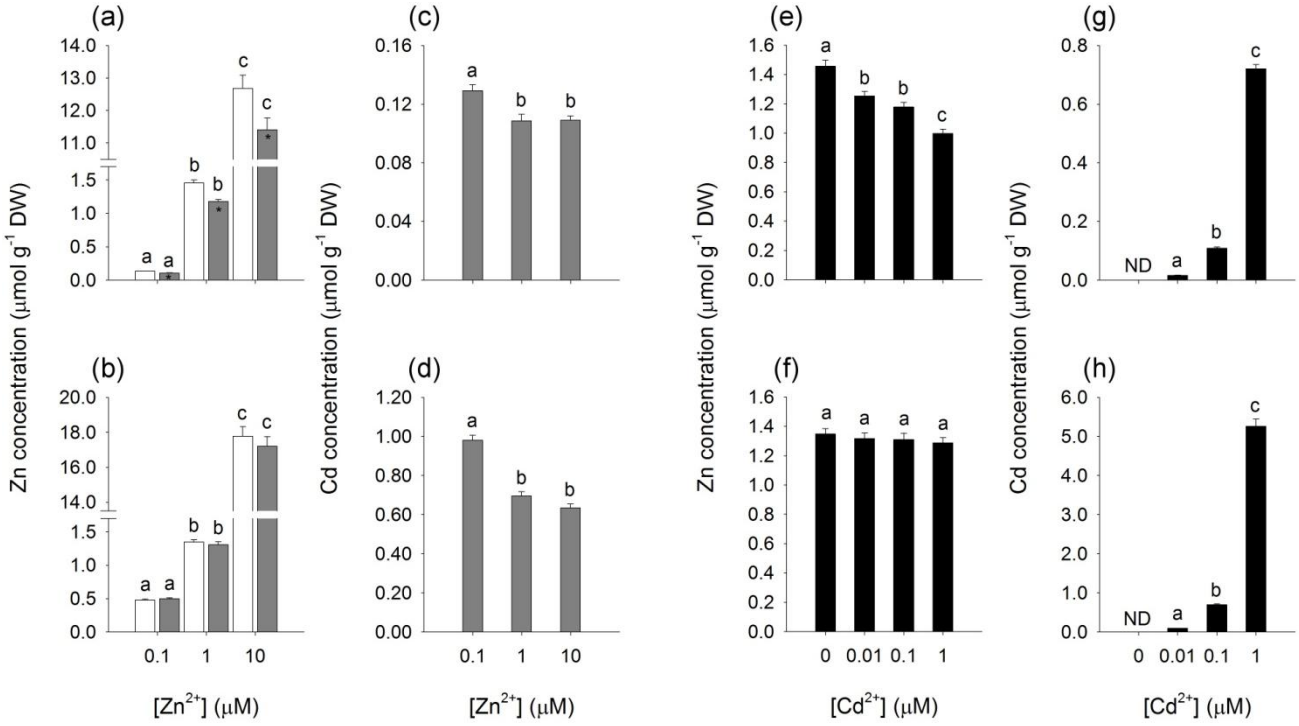
most of Zn and Cd ions resulted immobilized with thiol compounds in LMW and HMW complexes (Fig. 6; Tab. 3, 4) as previously reported in other papers (Rauser and Meuwly, 1995; Souza and Rauser, 2003; Nocito *et al.*, 2011). Moreover, fractioning of metals accumulated in the root also revealed that the three main fractions were in a dynamic equilibrium in which the increases in Zn or Cd external concentration, in the presence of a steady amount of Cd or Zn, respectively, resulted in changes in the amount of the two metal ions in each fraction (Tab. 1, 2).

Focusing the attention on the cationic fractions (Fig. 5; Tab. 1, 2) we can make some educated guesses about changes in the relative mobility of Zn and Cd into the root, since, as above mentioned, only the metal ions belonging to these fractions have all the requisites to be considered potentially available for root-to-shoot translocation (Nocito *et al.*, 2011). Increases in Zn external concentration, in the presence of a steady amount of Cd, did not produce significant changes in the amount of Cd ions in the mobile fractions but conversely increased the amount of free Zn ions in the same fractions. Such behaviors may be ascribable to competition phenomena between the two ions for both root absorption and negative charges on cellular matrices or apoplast components. In fact, the increases in Zn external concentration not only reduced the total amount of Cd in the root, but also displaced non-mobile Cd ions from cellular matrices (Fig. 5; Tab. 1, 2). On the other hand, the increases in Cd external concentration, in the presence of a steady amount of Zn, significantly enhanced the amount of both Zn and Cd ions in the mobile fractions (Fig. 5; Tab. 1, 2). Also in this case we can speculate that the gradual saturation of cellular matrices with Cd ions may have displaced Zn ions leading to a transient increase in the activity of the free Zn forms that, in turn, has been only partially counterbalanced by a week increase in the amount of Zn ions immobilized with thiol compounds in the LMW complexes (Fig. 6; Tab. 3, 4). Finally, by plotting the translocation data obtained in the two experimental setup as a function of the Zn/Cd or Cd/Zn concentration ratios in the mobile fractions we can easily evince that increases in Zn/Cd ratio did not produce fully reciprocal effects on Zn and Cd translocation, whilst increases in Cd/Zn ratio resulted in fully reciprocal effects (Fig. 10). Such a finding strongly confirms the hypothesis that Cd ions may use at least two distinct pathways to be translocated from roots to shoots. The first one – shared with Zn – is probably used for Zn translocation in physiological conditions, whilst the second one appears as a Zn-independent route that Cd may preferentially use when the first pathway is saturated with Zn. Moreover, the Zn-independent pathway we postulate seems to be also independent from Cd stress since the partial inhibitory effect exerted by Zn on Cd translocation was also observed in short-term PETIS experiments performed with unstressed rice plants (Fig. 7, 8, 9). In fact, time-course analysis of Cd systemic movement in the plants revealed that: i) the rate of Cd translocation was significantly reduced moving the external Zn concentration from 0.1 to 1 μM and then remained unaffected following a further increase in Zn availability (Fig. 7d, 9d); ii) the rates of Cd translocation measured in the presence of different Zn external concentrations significantly

differed just starting from the first hour of exposure (Fig. 7d, 9d), suggesting that the hypothetical Zn-independent pathway was constitutively expressed in rice plants. It is also noteworthy that rates of Cd translocation measured in the presence of 1 and 10 μM Zn^{2+} did not significantly differ even if the highest Zn concentration we tested strongly reduced the rate of Cd accumulation in the roots (Fig. 7, 9). Such a finding clearly confirms that the gradual saturation of the Zn-dependent pathway with Zn may force Cd ions to move through the second pathway, suggesting that the two translocation routes normally compete for free Cd ions into the root. Finally, our main conclusion seems to be further supported by the paper of Satoh-Nagasawa *et al.* (2012) which showed that rice mutants defective for OsHMA2 – the main transport system so far described in rice as involved in Zn^{2+}/Cd^{2+} translocation (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012, 2013; Takahashi *et al.*, 2012; Yamaji *et al.*, 2013) – had a residual capacity to translocate Cd to the shoots.

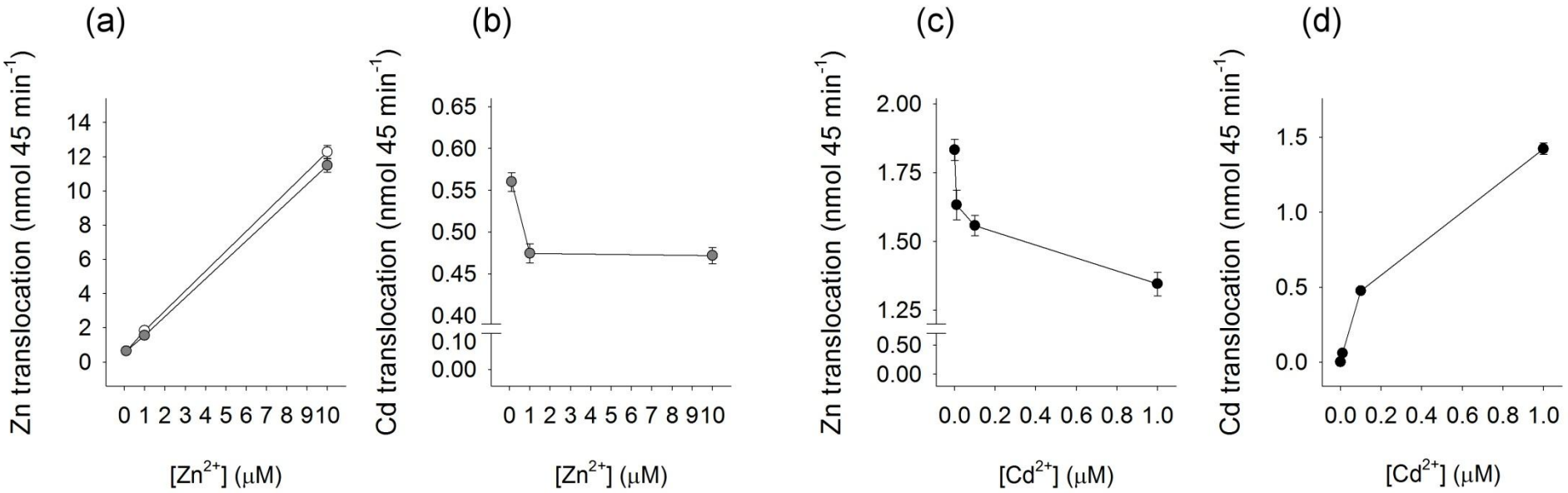
FIGURES AND TABLES

Figure 1. Zn and Cd accumulation in shoot and root**.**



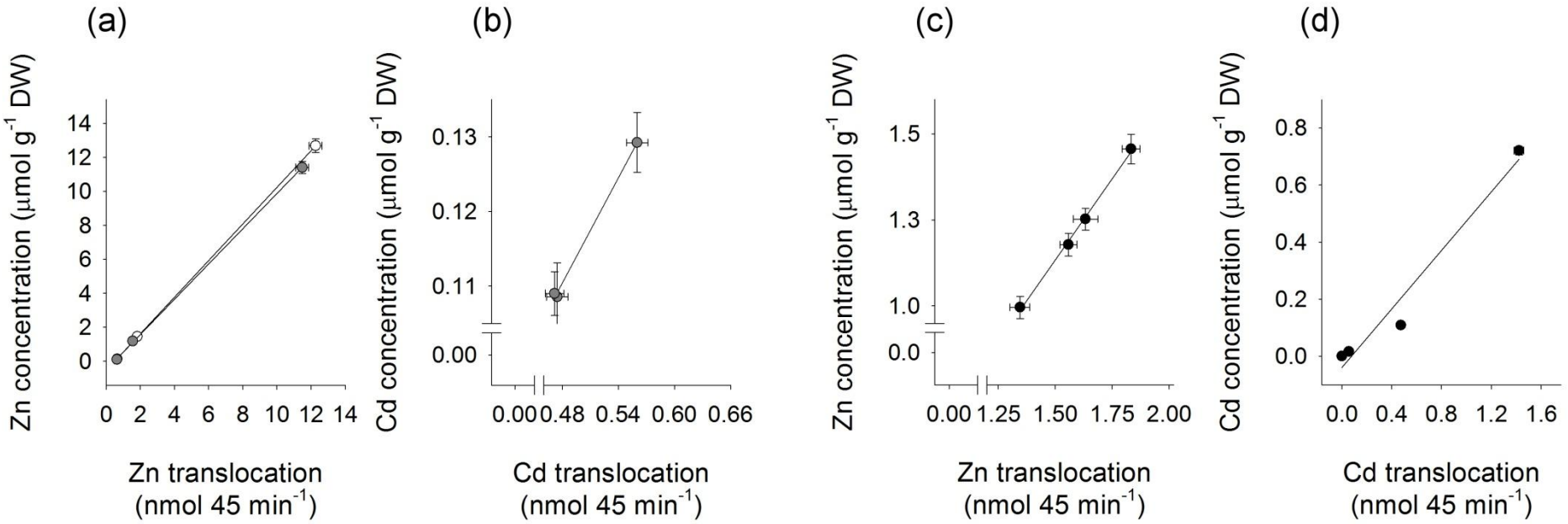
Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 µM), in the absence or presence of 0.1 µM Cd^{2+} (a-d), or to different Cd concentrations (from 0 to 1 µM) in the presence of 1 µM Zn^{2+} (e-h). (a,b) Zn concentration in shoot and root in the absence (white bars) and in the presence (grey bars) of a steady amount of Cd. (c,d) Cd concentration in shoot and root in the presence of a steady amount of Cd. (e,f) Zn concentration in shoot and root in the presence of a steady amount of Zn. (g,h) Cd concentration in shoot and root in the presence of a steady amount of Zn. Bars and error bars are means and SE of three experiments run in triplicate ($n = 9$). Different letters indicate significant differences between treatments ($P < 0.05$). Asterisks indicate significant differences between plants exposed or not to 0.1 µM Cd^{2+} ($P \leq 0.001$). ND, not detectable; DW, dry weight.

Figure 2. Zn and Cd translocation.



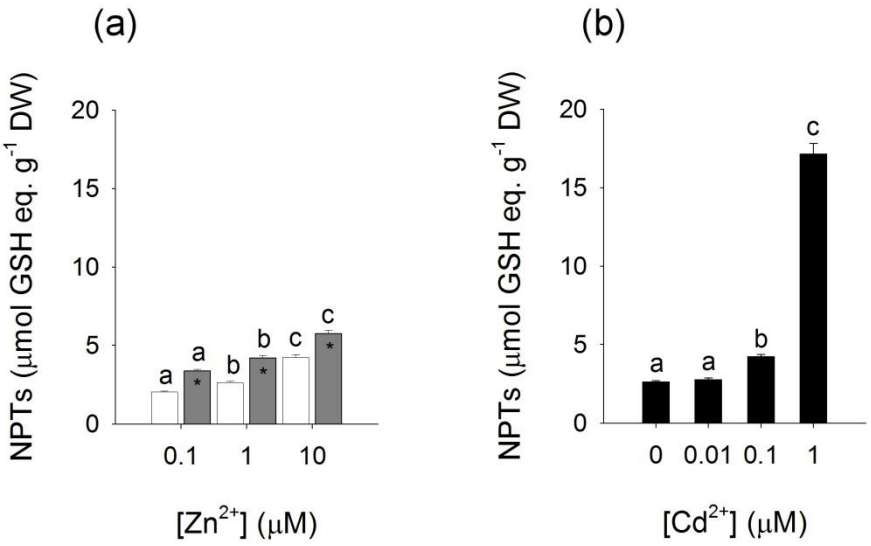
Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM), in the absence or presence of 0.1 μM Cd^{2+} (a,b), or to different Cd concentrations (from 0 to 1 μM) in the presence of 1 μM Zn^{2+} (c,d). At the end of the exposure period, shoots were separated from roots and the xylem sap exuded from the cut (root side) surface were collected over a 45 min period. (a) Zn ions loaded and transported in the xylem sap in the absence (white circles) and in the presence (grey circles) of a steady amount of Cd. (b) Cd ions loaded and transported in the xylem sap in the presence of a steady amount of Cd. (c,d) Zn and Cd ions loaded and transported in the xylem sap in the presence of a steady amount of Zn. Data are means and SE of three experiments run in triplicate (n = 9).

Figure 3. Relationship between Zn and Cd ions loaded in the xylem sap and Zn and Cd concentration in shoots.



Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM), in the absence or presence of 0.1 μM Cd^{2+} (a,b), or to different Cd concentrations (from 0 to 1 μM) in the presence of 1 μM Zn^{2+} (c,d). (a) Zn translocation vs Zn shoot concentration in the absence (white circles) and in the presence (grey circles) of a steady amount of Cd. (b) Cd translocation vs Cd shoot concentration in the presence of a steady amount of Cd. (c) Zn translocation vs Zn shoot concentration in the presence of a steady amount of Zn. (d) Cd translocation vs Cd shoot concentration in the presence of a steady amount of Zn. Data are means and SE of three experiments run in triplicate (n = 9). DW, dry weight.

Figure 4. Total non-protein thiols (NPTs).



Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 µM), in the absence (white bars) or presence (grey bars) of 0.1 µM Cd^{2+} (a), or to different Cd concentrations (from 0 to 1 µM) in the presence of 1 µM Zn^{2+} (b). NPT levels are expressed as GSH equivalents. Bars and error bars are means and SE of three experiments run in triplicate (n = 9). Different letters indicate significant differences between treatments ($P < 0.05$). Asterisks indicate significant differences between plants exposed or not to 0.1 µM Cd^{2+} ($P \leq 0.001$). DW, dry weight.

Table 1. Fractioning of Zn ions retained in root.

Metal concentration		Zn content				
Zn	Cd	Buffer soluble (1-6)		Acid soluble (7-9)	Ash	Total
		Anionic	Cationic			
μM		nmol g^{-1} DW				
0.1	0	42.05 ± 1.39 (a)	143.03 ± 3.86 (a)	240.95 ± 7.71 (a)	51.78 ± 1.91 (a)	477.81 ± 14.88 (a)
1	0	60.00 ± 1.56 (a)	420.00 ± 14.70 (a)	746.00 ± 22.38 (a)	121.00 ± 4.60 (a)	1347.00 ± 43.24 (b)
10	0	384.04 ± 13.44 (b)	3136.82 ± 128.61 (b)	13634.40 ± 545.38 (b)	618.49 ± 25.36 (b)	17773.74 ± 712.78 (c)
0.1	0.1	45.37 ± 1.72 (a)	206.02 ± 6.59 (a)	200.67 ± 7.22 (a)	46.79 ± 1.50 (a)	498.85 ± 17.04 (a)
1	0.1	110.00 ± 3.85 (b)	632.25 ± 22.13 (a)	475.01 ± 19.95 (a)	92.21 ± 3.13 (a)	1309.46 ± 49.06 (b)
10	0.1	534.96 ± 21.93 (c)	4541.08 ± 177.10 (b)	11546.99 ± 288.67 (b)	587.44 ± 25.26 (b)	17210.47 ± 512.97 (c)
1	0	60.00 ± 1.56 (a)	420.00 ± 14.70 (a)	746.00 ± 22.38 (a)	121.00 ± 4.60 (a)	1347.00 ± 43.24 (a)
1	0.01	95.59 ± 2.87 (b)	486.91 ± 17.04 (a)	620.59 ± 21.10 (b)	113.54 ± 3.29 (a)	1316.63 ± 44.30 (a)
1	0.1	110.00 ± 3.85 (b)	632.25 ± 22.13 (b)	475.01 ± 19.95 (c)	92.21 ± 3.13 (b)	1309.46 ± 49.06 (a)
1	1	145.12 ± 4.50 (c)	696.00 ± 24.36 (b)	364.00 ± 10.56 (d)	82.03 ± 3.12 (b)	1287.16 ± 42.53 (a)

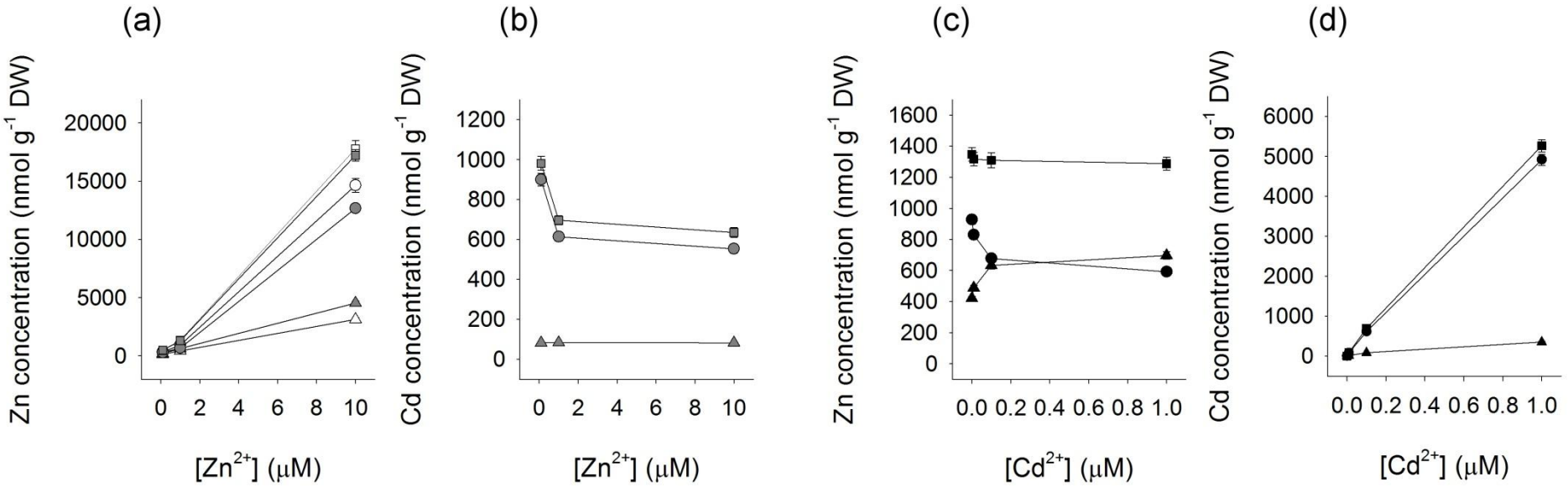
Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM), in the absence or presence of 0.1 μM Cd^{2+}, or to different Cd concentrations (from 0 to 1 μM) in the presence of 1 μM Zn^{2+}. Zn retained by root was 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). Different letters indicate significant differences between treatments ($P < 0.05$).

Table 2. Fractioning of Cd ions retained in root.

Metal concentration		Cd content				
Zn	Cd	Buffer soluble (1-6)		Acid soluble (7-9)	Ash	Total
		Anionic	Cationic			
μM		nmol g^{-1} DW				
0.1	0.1	140.10 ± 5.04 (a)	81.66 ± 2.61 (a)	608.83 ± 21.31 (a)	149.79 ± 5.69 (a)	980.36 ± 34.66 (a)
1	0.1	84.57 ± 3.55 (b)	83.00 ± 2.82 (a)	444.00 ± 14.21 (b)	84.00 ± 2.94 (b)	695.57 ± 23.52 (b)
10	0.1	72.76 ± 1.82 (b)	82.00 ± 3.53 (a)	397.07 ± 15.88 (b)	83.03 ± 3.40 (b)	634.86 ± 24.63 (b)
1	0	ND	ND	ND	ND	ND
1	0.01	5.85 ± 0.20 (a)	11.056 ± 0.32 (a)	62.79 ± 2.20 (a)	11.99 ± 0.36 (a)	91.69 ± 3.08 (a)
1	0.1	84.57 ± 3.55 (b)	83.00 ± 2.82 (b)	444.00 ± 14.21 (b)	84.00 ± 2.94 (b)	695.57 ± 23.52 (b)
1	1	624.33 ± 18.11 (c)	349.49 ± 13.28 (c)	3442.60 ± 92.95 (c)	845.28 ± 26.20 (c)	5261.71 ± 150.54 (c)

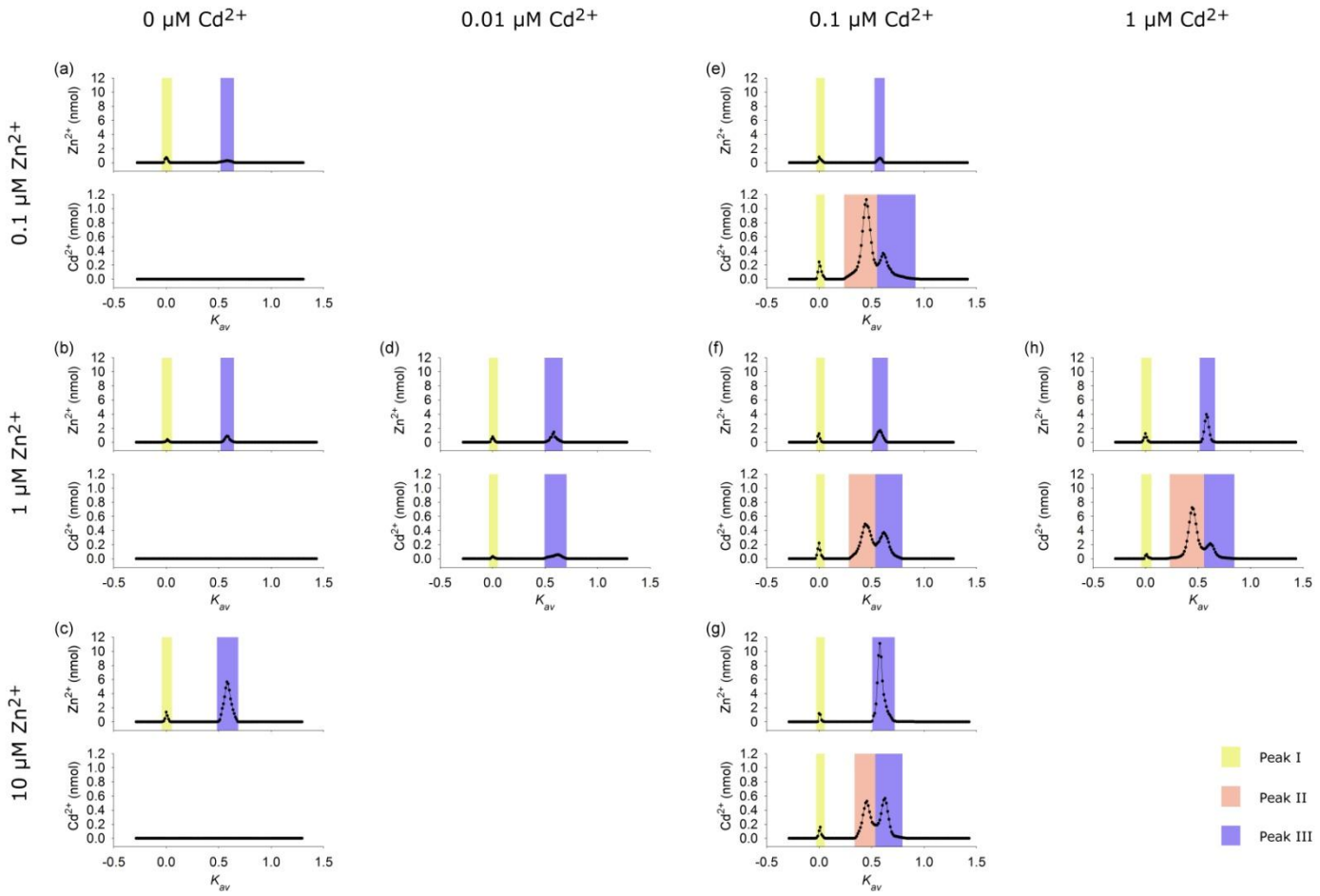
Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM) in the presence of 0.1 μM Cd^{2+}, or to different Cd concentrations (from 0 to 1 μM) in the presence of 1 μM Zn^{2+}. Cd retained by root was extracted with 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). Different letters indicate significant differences between treatments ($P < 0.05$).

Figure 5. Partitioning of Zn and Cd ions between potentially mobile and non-mobile fractions.



Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM), in the absence or presence of 0.1 μM Cd^{2+} (a,b), or to different Cd concentrations (from 0 to 1 μM) in the presence of 1 μM Zn^{2+} (c,d). (a) Total (square), mobile (triangle) and non-mobile (circle) Zn in the absence (white symbols) and in the presence (grey symbols) of a steady amount of Cd. (b) Total (square), mobile (triangle) and non-mobile (circle) Cd in the presence of a steady amount of Cd. (c) Total (square), mobile (triangle) and non-mobile (circle) Zn in the presence of a steady amount of Zn. (d) Total (square), mobile (triangle) and non-mobile (circle) Cd in the presence of a steady amount of Zn. Non-mobile (anionic + acid soluble + ash) metal ion fractions are calculated using data reported in Tab. 1 and 2. Data are means and SE of three experiments, each performed with eight plants (n = 3). DW, dry weight.

Figure 6. Zn- and Cd-binding complexes resolved by gel filtration chromatography.



Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 µM), in the absence (a,b,c) or presence of 0.1 µM Cd^{2+} (e,f,g), or to different Cd concentrations (from 0 to 1 µM) in the presence of 1 µM Zn^{2+} (b,d,f,h). The anionic fraction from buffer extracts 1-6 was chromatographed on a Sephadex G-50 column. Zn and Cd eluted were measured by ICP-MS. Void and total volume peaks are centred at K_{av} = 0 and K_{av} = 1, respectively. Peaks I, II and III are highlighted with different colors. Data are representative of one typical experiment repeated three times with similar results.

Table 3. Zn ions retained in the root in complexes with thiols.

Metal concentration		Zn		
Zn	Cd	HMW	LMW	Complexed with thiols
μM		nmol g^{-1} DW		
0.1	0	ND	21.74 ± 0.70 (a)	21.74 ± 0.70 (a)
1	0	ND	47.62 ± 1.76 (a)	47.62 ± 1.76 (a)
10	0	ND	343.45 ± 14.42 (b)	343.45 ± 14.42 (b)
0.1	0.1	ND	24.36 ± 0.83 (a)	24.36 ± 0.83 (a)
1	0.1	ND	84.75 ± 2.97 (b)	84.75 ± 2.97 (b)
10	0.1	ND	507.87 ± 19.61 (c)	507.87 ± 19.61 (c)
1	0	ND	47.62 ± 1.76 (a)	47.62 ± 1.76 (a)
1	0.01	ND	72.89 ± 2.70 (b)	72.89 ± 2.70 (b)
1	0.1	ND	84.75 ± 2.97 (b)	84.75 ± 2.97 (b)
1	1	ND	121.21 ± 4.97 (c)	121.21 ± 4.97 (c)

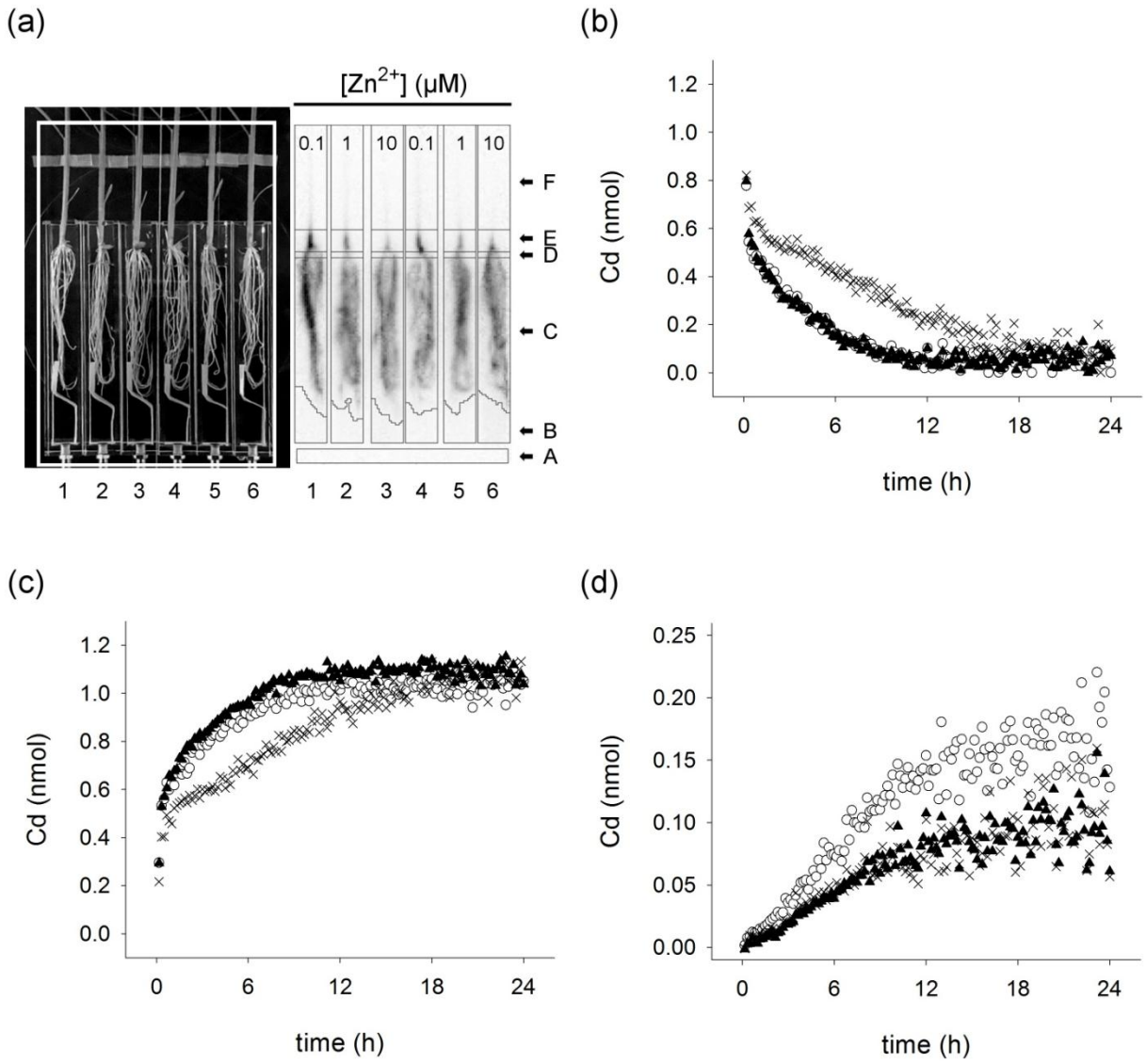
Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM), in the absence or presence of 0.1 μM Cd^{2+}, or to different Cd concentrations (from 0 to 1 μM) in the presence of 1 μM Zn^{2+}. The anionic fraction from buffer extracts 1-6 was chromatographed on a Sephadex G-50 column. Zn eluted was measured by ICP-MS. Data are means and SE of three experiments, each performed with eight plants (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$). LMW, low molecular weight Zn-binding complexes; HMW, high molecular weight Zn-binding complexes. ND, not detectable; DW, dry weight.

Table 4. Cd ions retained in the root in complexes with thiols.

Metal concentration		Cd		
Zn	Cd	HMW	LMW	Complexed with thiols
μM			nmol g^{-1} DW	
0.1	0.1	96.89 ± 3.00 (a)	34.18 ± 1.13 (a)	131.07 ± 4.13 (a)
1	0.1	45.78 ± 1.51 (b)	31.83 ± 1.18 (a)	77.61 ± 2.69 (b)
10	0.1	33.82 ± 1.29 (c)	33.19 ± 1.33 (a)	67.00 ± 2.61 (b)
1	0	ND	ND	ND
1	0.01	ND	5.00 ± 0.17 (a)	5.00 ± 0.17 (a)
1	0.1	45.78 ± 1.51 (a)	31.83 ± 1.18 (b)	77.61 ± 2.69 (b)
1	1	473.66 ± 17.05 (b)	134.35 ± 5.24 (c)	608.01 ± 22.29 (c)

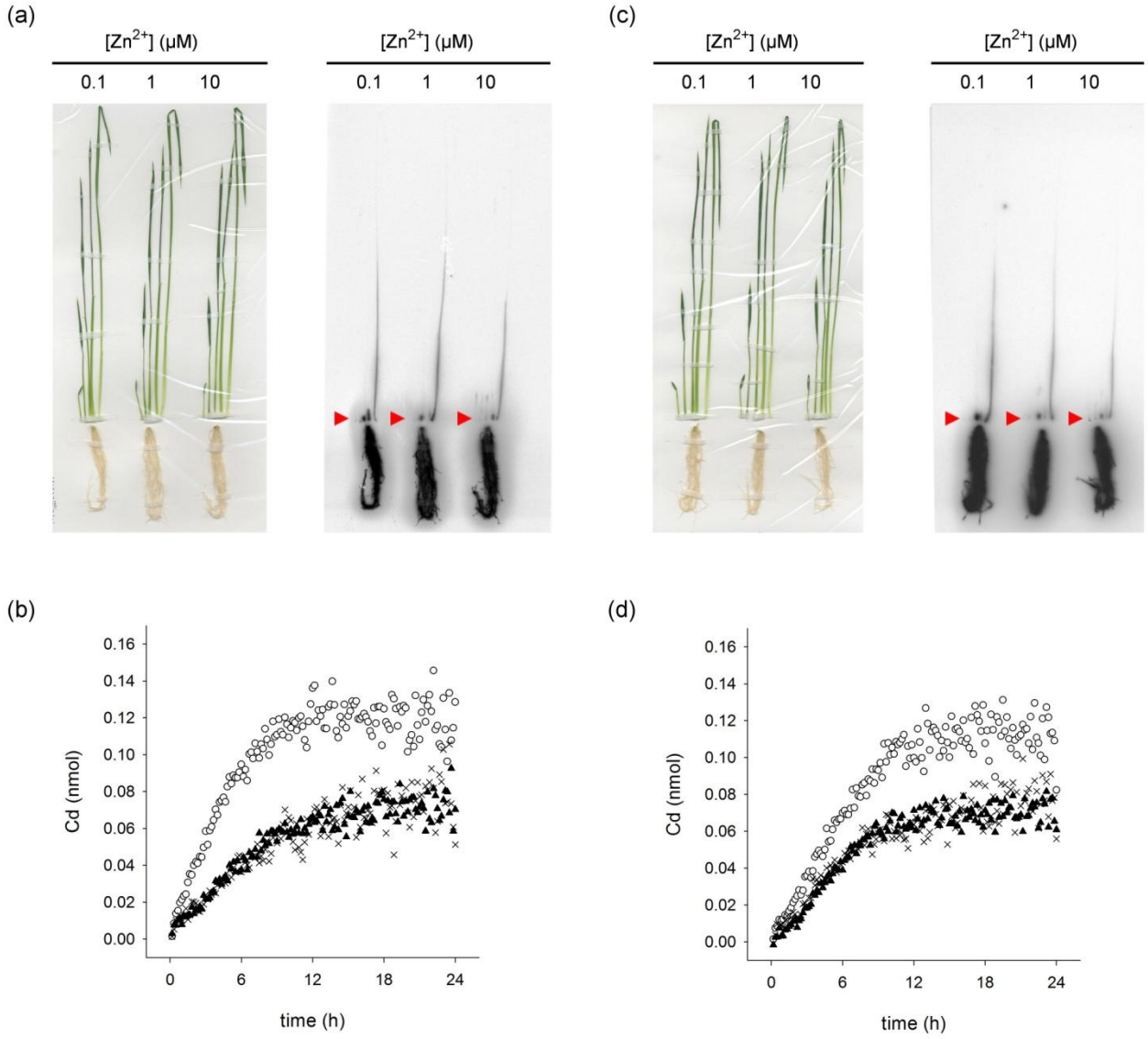
Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 μM) in the presence of 0.1 μM Cd^{2+}, or to different Cd concentrations (from 0 to 1 μM) in the presence of 1 μM Zn^{2+}. The anionic fraction from buffer extracts 1-6 was chromatographed on a Sephadex G-50 column. Cd eluted was measured by ICP-MS. Data are means and SE of three experiments, each performed with eight plants (n = 3). Different letters indicate significant differences between treatments (P < 0.05). LMW, low molecular weight Cd-binding complexes; HMW, high molecular weight Cd-binding complexes. ND, not detectable; DW, dry weight.

Figure 7. Time-course analysis of Cd systemic movement.



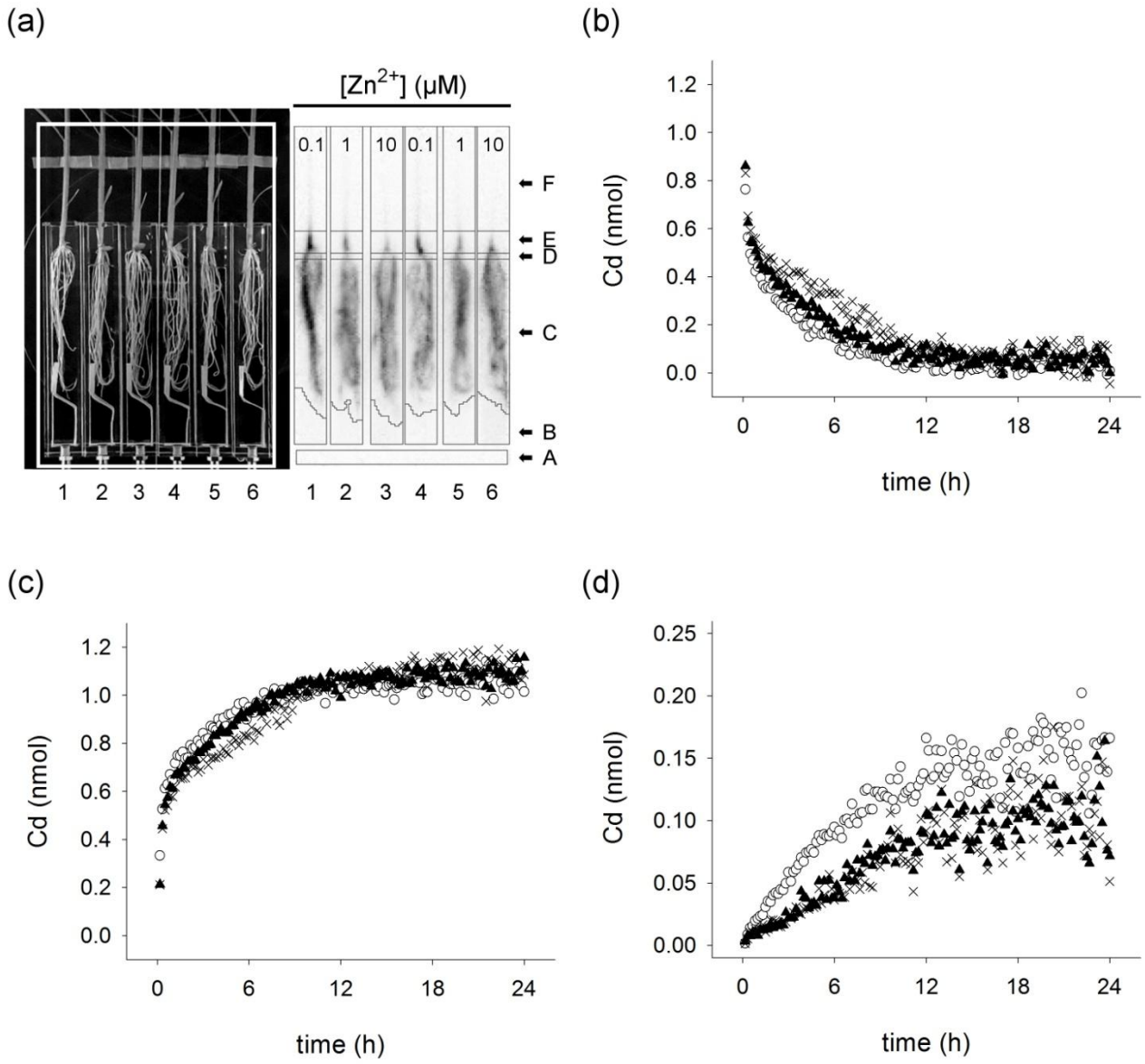
Rice plants were differentially exposed for a 24-h period to different Zn concentrations (from 0.1 to 10 μM), in the presence of 0.1 μM Cd^{2+} enriched with ^{107}Cd. (a) Image of rice plants used in a typical PETIS experiment. The field of view of PETIS is bordered by the white continuous line. ROIs used for time-course analysis are indicated with arrows in the adjacent panel. ROI-A, background; ROI-B, culture solution; ROI-C, distal root; ROI-D, proximal root; ROI-E, shoot base; ROI-F, proximal shoot. (b,c,d) Time-course analysis of Cd dynamics in the culture solution (b), whole root apparatus (c), and shoot base + proximal shoot (d). White circles, black triangles, and thin x refer to the experiments performed in the presence of 0.1 (No. 4), 1 (No. 5) and 10 μM Zn^{2+} (No. 6), respectively. A representative set of data from two independent experiments performed with two plants for each Zn exposure condition is given.

Figure 8. Autoradiography and time-course analysis of Cd accumulation in the shoot base.



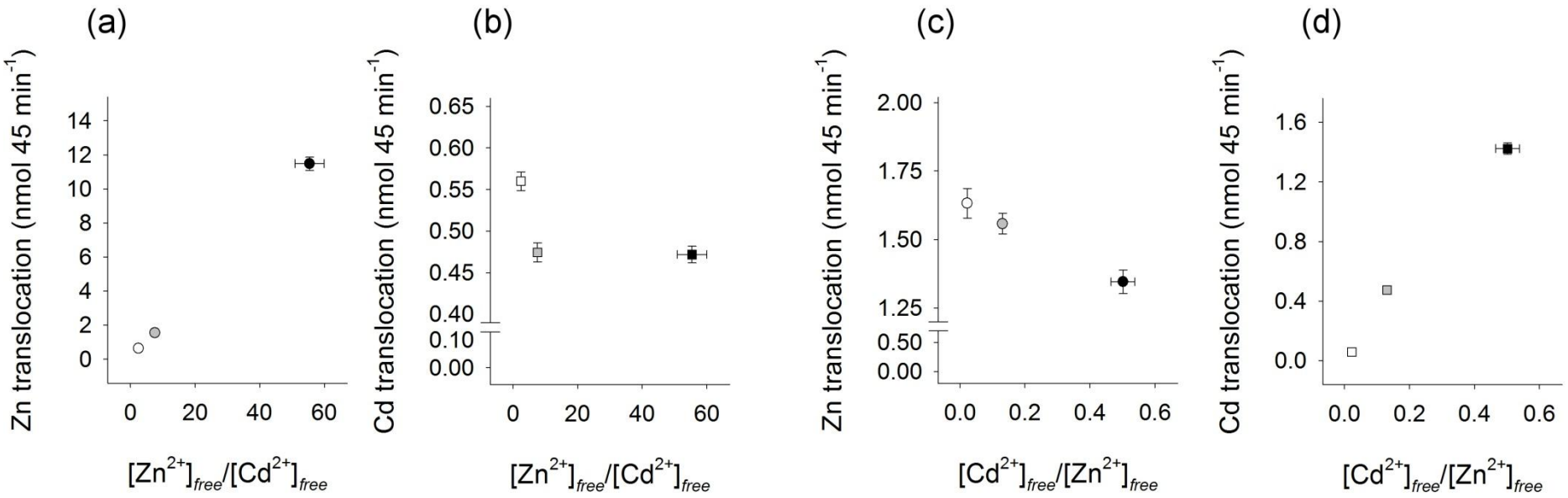
Rice plants were differentially exposed for a 24-h period to different Zn concentrations (from 0.1 to 10 µM), in the presence of 0.1 µM Cd^{2+} enriched with ^{107}Cd. (a,c) Optical observation of plants at the end of the PETIS experiments (left) and the corresponding autoradiography of ^{109}Cd after three days of exposure (right). Red triangles indicate the shoot bases. (b,d) Time-course analysis of Cd accumulation in the shoot bases. White circles, black triangles, and thin x refer to the experiments performed in the presence of 0.1, 1 and 10 µM Zn^{2+}, respectively. Two representative set of data from two independent experiments performed with two plants for each Zn exposure condition are given.

Figure 9. Another set of time-course analysis of Cd systemic movement.



Rice plants were differentially exposed for a 24-h period to different Zn concentrations (from 0.1 to 10 μM), in the presence of 0.1 μM Cd^{2+} enriched with ^{107}Cd. (a) Image of rice plants used in a typical PETIS experiment. The field of view of PETIS is bordered by the white continuous line. ROIs used for time-course analysis are indicated with arrows in the adjacent panel. ROI-A, background; ROI-B, culture solution; ROI-C, distal root; ROI-D, proximal root; ROI-E, shoot base; ROI-F, proximal shoot. (b,c,d) Time-course analysis of Cd dynamics in the culture solution (b), whole root apparatus (c), and shoot base + proximal shoot (d). White circles, black triangles, and thin x refer to the experiments performed in the presence of 0.1 (No. 1), 1 (No. 2) and 10 μM Zn^{2+} (No. 3), respectively. A representative set of data from two independent experiments performed with two plants for each Zn exposure condition is given.

Figure 10. Analysis of metal translocation as a function of Zn and Cd concentration in the mobile fractions.



Rice plants were hydroponically grown and differentially exposed for a 10-day period to increasing Zn external concentrations (from 0.1 to 10 µM), in the presence of 0.1 µM Cd^{2+} (a), or to different Cd concentrations (from 0.01 to 1 µM) in the presence of 1 µM Zn^{2+} (b). (a) Zn (circles) and Cd (square) translocation in plants exposed to 0.1 (white), 1 (grey) and 10 (black) µM Zn^{2+}, in the presence of 0.1 µM Cd^{2+}. (b) Zn (circles) and Cd (square) translocation in plants exposed to 0.01 (white), 0.1 (grey) and 1 (black) µM Cd^{2+}, in the presence of 1 µM Zn^{2+}. Data reported in figures derived from Fig. 2 and Tab. 1, 2.

SECTION II

Functional characterization of OsHMA genes in yeast

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Some members of the P_{1B}-type ATPase subfamily, which present several functions in the cell, ranging from metal delivery to cellular compartment to detoxification processes (Williams and Mills, 2005; Colangelo and Guerinot, 2006), have been identified as responsible for Zn and Cd xylem loading and thus particularly relevant in the determination of Zn and Cd accumulation in the shoots. In particular, AtHMA2 and AtHMA4 were found to be responsible for root-to-shoot Zn translocation in *A. thaliana* (Hussain *et al.*, 2004) and several reports also suggest their involvement in Cd transport (Mills *et al.*, 2003, 2005; Eren and Argüello, 2004; Verret *et al.*, 2005; Wong and Cobbett, 2009; Wong *et al.*, 2009). Moreover, they appeared predominantly expressed in the roots in correspondence of the vascular bundles and the encoded proteins localized on the plasma membrane, suggesting their function as efflux pumps extruding excess of metals into the apoplast (Mills *et al.*, 2003, 2005; Hussain *et al.*, 2004; Verret *et al.*, 2005). Acknowledging the importance of such transporters in the control of Zn and Cd translocation, the identification of the orthologs of AtHMA2 and AtHMA4 in rice would be of great concern in understanding and characterizing the root-to-shoot Cd translocation pathways, also in relation to possible competitions with Zn. Recently, HMA2 has been suggested as responsible for Zn and Cd xylem loading also in rice (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012), barley (Mills *et al.*, 2012) and *Triticum aestivum* (Tan *et al.*, 2013). However, the transport activity of OsHMA2 for Zn and Cd has been controversially described in a number of papers (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012; Yamaji *et al.*, 2013) in which the transporter was indirectly studied by in heterologous expression systems (*Saccharomyces cerevisiae*). A careful analysis of published studies reveals that both activity and function of OsHMA2 has not been unambiguously characterized, since three independent papers described this transporter as able to mediate Zn^{2+} and/or Cd^{2+} efflux for xylem loading (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012), whilst Yamaji *et al.* (2013) proposed OsHMA2 as an influx transporter for both Cd and Zn, involved in the preferential distribution of the two metal ions through the phloem to the developing tissues. Moreover, also the OsHMA2 tissue localization seems to be a controversial issue, since Takahashi *et al.* (2012) indicated the vascular bundle of the root as the main expression domain of the transporter, whilst Yamaji *et al.* (2013) localized OsHMA2 in the root pericycle and in the phloem regions of vascular bundles of the uppermost node connecting to panicle and flag leaf.

The results obtained in the first part of this work pointed out the existence of at least one additional Cd root-to-shoot translocation pathway that is Zn-independent and that finally contributes to Cd accumulation in the shoot. Thus, the specific aim of the second part of the work was to better characterize the transport activity and selectivity of OsHMA2 and try to find out the protein(s) responsible for the Zn-independent Cd translocation pathway.

Lee and coworkers (2007) provided a useful insight on the response to Cd exposure of some P_{1B}-type ATPases, *i.e.* OsHMA4 up to OsHMA9. In this study, *OsHMA5*, *OsHMA6* and *OsHMA9* were found to be upregulated by Cd both in shoots and roots, whereas *OsHMA4* showed an induction only in roots. The *in silico* analysis conducted on the rice P_{1B}-type ATPase subfamily aimed at searching for proteins functionally related to AtHMA2 returned that, apart from OsHMA2 and OsHMA3 that have already been partially characterized, OsHMA7 and OsHMA4 share the highest identity with AtHMA2 (21 and 20%, respectively). However, OsHMA7 did not seem involved in Cd movement since any response whatsoever was detected, even at high Cd concentrations (Lee *et al.*, 2007). The same analysis revealed that OsHMA4 falls in the same cluster as OsHMA5, OsHMA6 and OsHMA9 which, as mentioned earlier on, showed a clear induction upon Cd treatment. In addition, OsHMA4 is very close to OsHMA5 (58% of identity) (see the dendrogram adopted from Nocito *et al.*, 2011, and shown in the introduction as fig. 3). Interestingly, such transporter belongs to the same cluster of AtHMA5 and AtHMA7, which are classified as Cu/Ag ATPases (Hirayama *et al.*, 1999; Woeste and Kieber, 2000; Mandal *et al.*, 2004; Williams and Mills, 2005; Andrés-Colás *et al.*, 2006). According to these premises, OsHMA4 and OsHMA5 are likely to take part in the transport of Cd and/or Zn and could be good candidates to explain their xylem loading.

Thus, in order to identify the possible Zn-insensitive pathway involved in Cd translocation the transport activity of OsHMA2, OsHMA4 and OsHMA5 for Zn and Cd was better characterized. In particular, the full length genes were amplified by RT-PCR from total RNA isolated from rice roots and the relative cDNAs were heterologously expressed in wild type (INVSc1) and mutant (*zrt1zrt2*) strains of *S. cerevisiae*. In particular, we performed drop tests and growth analysis of the yeast strains expressing OsHMA2, OsHMA4 and OsHMA5 under the control of the *GAL1* or *GAL10* promoters.

The membrane topology prediction analysis of the three codified polypeptides performed by software MEMSAT3 confirmed OsHMA2 (Fig. 1), OsHMA4 (Fig. 2) and OsHMA5 (Fig. 3) as transporters belonging to the P_{1B}-type ATPase family, due to the presence of: (a) eight predicted transmembrane domains (TMs); (b) the CPC motif in TM6; (c) the signature sequences found in all P-type ATPases, *i.e.* DKTGT, GDGxNDxP and PxxK motifs, the first being particularly relevant for the ATPase activity since it contains the phosphorylatable aspartic residue.

OsHMA2

The amplified *OsHMA2* cDNA from the cultivar Roma encodes a polypeptide of 1067 amino acids, with a predicted mass of 116 kDa, is identical to *OsHMA2* cv. Volano (*OsHMA2v*, accession HQ646362) submitted to GenBank by Nocito and coworkers (2011). In particular, it shares 95% identity with the 1060 amino acid-long protein reported in PlantsT. The membrane topology prediction analysis (Fig. 1) also revealed signature sequences corresponding to $Zn^{2+}/Cd^{2+}/Pb^{2+}/Co^{2+}$ ATPases in

TM6 [CPC$(x)_4$SxP], TM7 [N$(x)_7$K] and TM8 [DxG] (Williams and Mills, 2005). Sequence analysis revealed OsHMA2 as a member of cluster 2 in the phylogenetic tree of the P_{1B}-ATPase subfamily; such a group is thought to include all $Zn^{2+}/Cd^{2+}/Pb^{2+}/Co^{2+}$ ATPases (Williams and Mills, 2005). Moreover, OsHMA2 has a relatively short N-terminal end (89 amino acids) with significant homology to the heavy metal-associated domain (Pfam: PF00403), containing the variant of the core consensus domain, GxCCxxE (^{17}**GICC**TS**E**^{23}), found in the *Arabidopsis* HMA2, HMA3 and HMA4 Zn^{2+}-ATPases (Eren *et al.*, 2007). Finally, OsHMA2 has a relatively long C-terminal end (373 amino acids) which contains numerous Cys-Cys repeat sequences and His residues that may be involved in heavy metal binding.

OsHMA4* AND *OsHMA5

The resulting amplicons codify a polypeptide of 978 and 1002 amino acids for *OsHMA4* and *OsHMA5*, respectively. They are characterized by a predicted mass of 105 and 108 kDa. Moreover, the aminoacidic sequence of OsHMA4 shares 99% identity with the protein predicted for OsHMA4 cv. Nipponbare, while those of OsHMA5 is identical to the protein predicted for OsHMA5 cv. Nipponbare. The membrane topology prediction analysis of the two proteins (Fig. 2 and 3) revealed signature sequences characteristic of the Cu^+/Ag^+ ATPase, *i.e.* CPC(x_6)P in TM6, N(x_6)YN(x_4)P in TM7 and P(x_6)M(x_2)SS in TM8 (Williams and Mills, 2005), in both OsHMA4 and OsHMA5. The amino acidic sequence also revealed the presence of short C-terminal ends, which are 26 and 16 amino acids long in OsHMA4 and OsHMA5, respectively. In this region, contrary to OsHMA2, neither Cys-Cys repeated sequences nor His stretches could be identified. By contrast, OsHMA4 and OsHMA5 presented relatively long N-terminal ends (275 and 376 amino acids, respectively), where two repetitions of the highly conserved GxxCxxC (OsHMA4: ^{45}**G**IS**C**AS**C**^{51} and ^{119}**GM**A**C**TS**C**^{125}; OsHMA5: ^{83}**G**MT**C**AA**C**^{89} and ^{161}**G**MT**C**TS**C**^{167}) metal binding sequence (Lutsenko *et al.*, 2003) were found. The length of the N-term of OsHMA4 and OsHMA5, as well as the presence and the positions of the GxxCxxC conserved motifs, resembles very much to the structure of AtHMA5, which is the Arabidopsis P-type ATPase with which both OsHMA4 and OsHMA5 share the highest identity with (59% and 71%, respectively). In addition, both proteins are also highly similar (49% identity) to AtHMA7. Both AtHMA5 and AtHMA7 present the same pattern in tandem of the conserved GxxCxxC motifs (AtHMA5: ^{59}**G**MT**C**SA**C**^{65}, ^{137}**GM**T**C**TS**C**^{143}; AtHMA7: ^{64}**G**MT**CA**A**C**^{70}, ^{141}**GM**T**C**AA**C**^{147}) of OsHMA4 and OsHMA5 (in bold the conserved amino acids).

FUCNTIONAL CHARACTERIZATION OF OsHMA2, OsHMA4 AND OsHMA5 IN YEAST

DROP TEST

To determine whether OsHMA2, OsHMA4 and OsHMA5 play a role in Cd transport, we performed classic functional assay (drop test) in yeast as shown in figure 4, in which two representative drop tests are reported. The yeast strains carrying OsHMA2, OsHMA4, OsHMA5 or empty vector were grown on SG media supplemented or not with different $CdCl_2$ concentrations (from 10 to 200 μM). In the drop test made using pYES2.1 vector (Fig. 4a), in the presence of Cd, both the yeast harboring the empty vector and those expressing the OsHMA proteins showed a slower growth with respect to the control plate without Cd^{2+}. Nevertheless, yeast strains expressing the three OsHMA proteins showed a stronger tolerance to Cd than the control, suggesting that OsHMA2 but also OsHMA4 and OsHMA5 are able to transport Cd, even though the *in silico* analysis indicated them as putative Cu/Ag ATPases (Williams and Mills, 2005) and recently Deng and coworkers (2013) characterized the OsHMA5 as a Cu transporter responsible for Cu xylem loading. Such a finding suggests that these proteins could function as detoxification systems in yeast by pumping excess of cytosolic Cd ions into the apoplast, as already reported for OsHMA2 by some authors (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012). However, other drop tests we performed, like the one showed in figure 4b, in which yeast strains carrying the chimeric OsHMA2-pESC-URA, OsHMA4-pESC-URA, OsHMA5-pESC-URA were compared to the yeast harboring the pESC-URA empty vector, did not show repeatable results. For instance, in this case, only OsHMA2 and OsHMA5 showed stronger tolerance to Cd than the yeast strain carrying the empty vector, whereas no evidence for enhanced tolerance to Cd was found for OsHMA4. Yamaji *et al.* (2013) reported that these inconsistencies may be attributed to many different factors, such as different expression vectors.

Moreover, to determine whether OsHMA4 and OsHMA5 play a role also in Zn transport, we performed drop tests using the yeast mutant strain ZHY3 (*zrt1zrt2*) (Zhao and Eide, 1996), sensitive to low Zn concentrations, using the pESC-TRP vector (Song *et al.*, 2010). The yeast strains carrying OsHMA2, OsHMA4, OsHMA5 or empty vector were grown on SG media supplemented or not with 0.2 mM $ZnCl_2$ and 0.2 mM EDTA (2,2',2",2'"-(Ethane-1,2-diyldinitrilo)tetraacetic acid) that chelates Zn creating a Zn-limiting condition. As reported in figure 5, this test did not reveal any significant difference between the empty vector and OsHMA4 or OsHMA5, suggesting that neither OsHMA4 nor OsHMA5 are able to transport Zn.

GROWTH ANALYSIS

In order to understand the reason why we did not obtain repeatable results with the drop tests, we analyzed the growth rates of the yeast strain carrying OsHMA2, OsHMA4, OsHMA5 or empty vector

(control) incubated in SD (no gene induction) or SG (gene induction) liquid media and the growth curves we obtained and the relative duplication times are reported in figure 6. The yeast strains carrying OsHMAs and empty vector did not significantly differ in their duplication times when grown in the presence of glucose (Fig. 5a). By contrast, the addition of galactose significantly enhanced the duplication time, which however resulted higher in the yeast expressing all the OsHMA proteins analyzed than in the control (Fig. 5b). Such a finding – reasonably due to an interfering effect of the OsHMA proteins induction on yeast growth – precluded the direct comparison of yeast growths in traditional functional assays performed with media containing excess of Zn or Cd.

GROWTH INHIBITION ANALYSIS

To solve this impasse, we grew the yeast strains carrying OsHMA2, OsHMA4, OsHMA5 or empty vector in liquid synthetic minimal media containing or not different excess of Zn^{2+} (from 1.5 to 16.5 mM) or Cd^{2+} (from 15 to 165 μM) in the presence of Gal (SG media). The preliminary tests we performed with the yeast harboring the empty vector (Fig. 7a,b) showed similar results, suggesting that this kind of test is quite repeatable. Moreover, in such a way, we were able to measure the inhibitory effect exerted by each metal concentration on the growth of each strain (control, OsHMA2, OsHMA4 and OsHMA5), in a comparison functional test independent from possible differences in the duplication time. Results indicated that both Zn and Cd significantly affected yeast growth, since the percentages of growth inhibition increased as the metal concentration in the medium did (Fig. 7c,d). Non-linear regression analysis of the growth inhibition curves allowed to calculate the metal concentrations required to inhibit the growth of each yeast strain by 50% (IC_{50}). The estimated IC_{50} values were significantly different between the yeast strains. In particular, for Zn the IC_{50} values of OsHMA2 and OsHMA5 were higher than those of the control; on the other hand, for Cd only the IC_{50} value of OsHMA2 was different from that of the control (Fig. 7). These findings are in agreement with the most part of the previous studies on the transport activity of OsHMA2 (Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012, 2013; Takahashi *et al.*, 2012), confirming that OsHMA2 removes Zn and Cd from the cytoplasm conferring a tolerance to excess Zn and Cd in yeast. A similar role was recently described for HMA2 in barley (Mills *et al.*, 2012) and *Triticum aestivum* (Tan *et al.*, 2013). In addition, AtHMA2 has been thoroughly studied and its role in Cd and Zn xylem loading is widely accepted (Eren and Argüello, 2004; Hussain *et al.*, 2004; Eren *et al.*, 2006, 2007; Wong and Cobbett, 2009; Wong *et al.*, 2009). For what concern OsHMA5, even if recently it has been characterized as a Cu^{2+} transporter, responsible for Cu xylem loading (Deng *et al.*, 2013), and the membrane topology prediction analysis (Fig. 3) also revealed signature sequences characteristic of the Cu^{+}/Ag^{+} ATPase (Williams and Mills, 2005), the growth inhibition test revealed the OsHMA5 is also able to transport Zn^{2+}, as shown by the growth inhibition curves and the IC_{50} values (Fig. 7c), disproving the result of the drop test for Zn

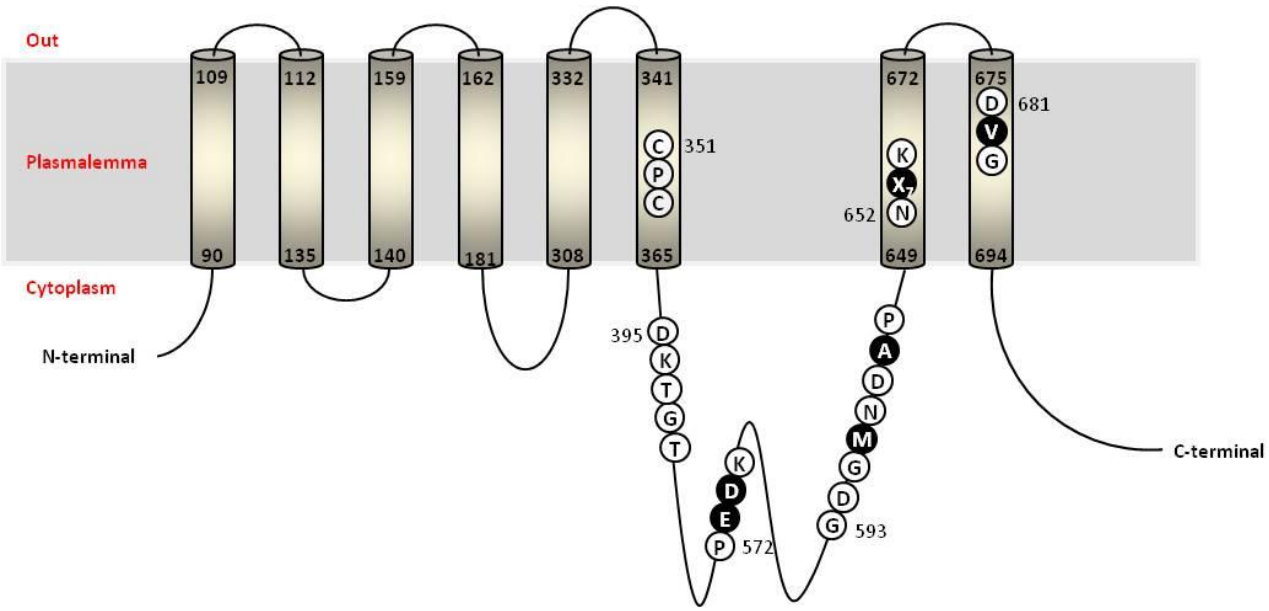
tolerance (Fig. 5). At the same time, the findings of the growth inhibition analysis refuted the results of both the drop tests for Cd tolerance indicating OsHMA5 as not able to mediate the transport of Cd^{2+} (Fig. 7d, 4). In the case of OsHMA4 the growth inhibition analysis excluded the hypothesis that OsHMA4 could mediate the transport of Zn^{2+} or Cd^{2+} (Fig. 7c,d), refuting the results we previously obtained in the drop tests which suggested OsHMA4 as able to mediate the Cd^{2+} transport (Fig. 4a). Unfortunately, the functional analysis of OsHMA4 and OsHMA5 in yeast we provided in this study excluded the involvement of these proteins in mediating Cd efflux from yeast cells leaving opened the question of which is (are) the protein(s) responsible for the Zn-independent root-to-shoot Cd translocation pathway(s).

The different growth analyses we performed clearly pointed out that great inconsistencies may be obtained using not only different yeast strains, experimental conditions, expression vector or medium components as reported by Yamaji and coworkers (2013), but also using different methods of analysis. Looking at the growth rate analysis (Fig. 6), the induction of the OsHMA proteins reasonably affect the yeast growth increasing the duplication times with respect to the control. Based on these findings, the drop test is not the correct method to study the transport activity of a protein because the direct comparison of yeast growths performed with media containing excess of Zn or Cd is precluded. In fact, results could be strongly affected by different duplication times of the yeast expressing the target proteins. In addition, the result is dependent from the inoculum, even though optical densities of cell suspensions of the different strains appear similar. Finally, the results, when different yeast colonies are compared, are strongly dependent from subjectivity and observer variability. To overcome these limits, we performed a growth inhibition analysis that enabled us to measure the inhibitory effect exerted by each metal concentration on the growth of each strain. This kind of functional test resulted independent from possible differences in the duplication time. A similar method was developed by Galgiani and Stevens (1976 and 1978) for studying yeast susceptibility to antimicrobial substances. Their evidences showed that this turbidimetric technique is reproducible, inoculums independent, rapid, free from subjectivity and observer variability. They also calculated the IC_{50} as the drug concentrations required to inhibit the yeast growth by 50%. Although the turbidimetric technique is known since a long time in clinical studies on yeast, concerning plant gene functional characterization in yeast the drop test has been largely used as functional assay so far (e.g. Gravot *et al.*, 2004; Southron *et al.*, 2004; Kobayashi *et al.*, 2008; Ueno *et al.*, 2010; Nocito *et al.*, 2011; Satoh-Nagasawa *et al.*, 2012; Takahashi *et al.*, 2012; Deng *et al.*, 2013; Yamaji *et al.*, 2013), even though the abovementioned limits. Other researchers only grew yeast in liquid culture and compared the growth rates of different transformants (Ramesh *et al.*, 2003; Gravot *et al.*, 2004; Nocito *et al.*, 2011), but only few related the yeast growth to different metal (Cd or Al) concentrations (Clemens *et al.*, 1999; Li *et al.*, 2014). All these

methods, however, still could depend on possible differences in the duplication time of the different transformants. Only in a recent paper on the characterization of Nramp aluminum transporter (NRAT1) the growth of the different yeast transformants was expressed as a 'relative growth', which is independent from the inoculums and the growth rate (Li *et al.*, 2014). Thus, probably, our study represents one of the first examples of growth inhibition analysis applied to plant gene functional characterization.

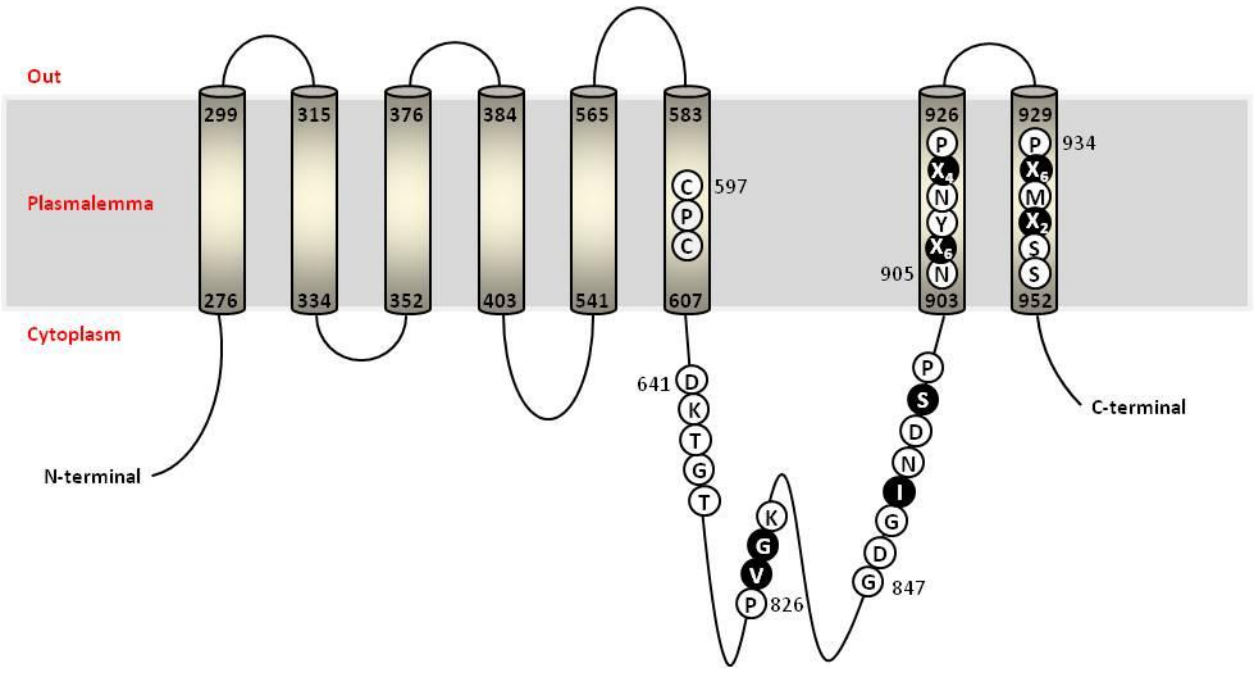
FIGURES AND TABLES

Figure 1. Membrane topology of OsHMA2.



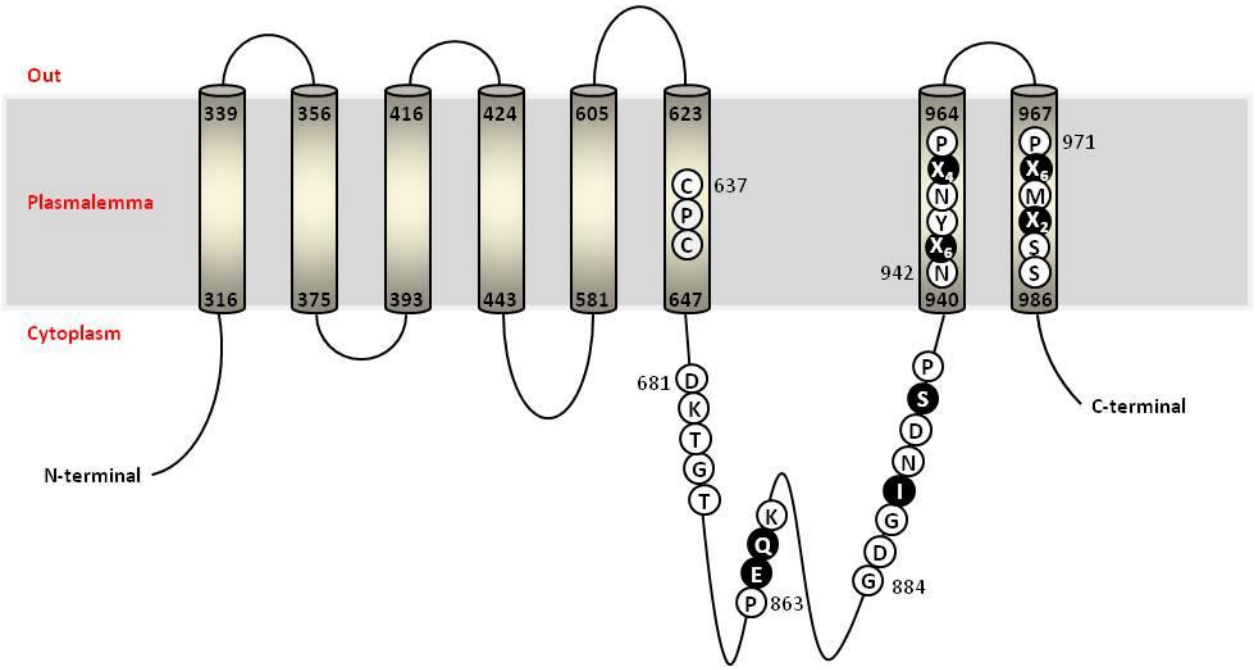
The OsHMA2 (cv. Roma) sequence has 100% identity to OsHMA2 cv. Volano submitted by Nocito and coworkers (2011). Eight TM helices are predicted in OsHMA2r using MEMSAT3. Numbers in bold indicate the position of TM segments within the OsHMA2 sequence. Signature sequences in TM6 [^{351}CPC^{353}], TM7 [^{652}N(x)$_7$K^{660}] and TM8 [^{681}DxG^{683}] are conserved in all $Zn^{2+}/Cd^{2+}/Pb^{2+}/Co^{2+}$ ATPases.

Figure 2. Membrane topology of OsHMA4.



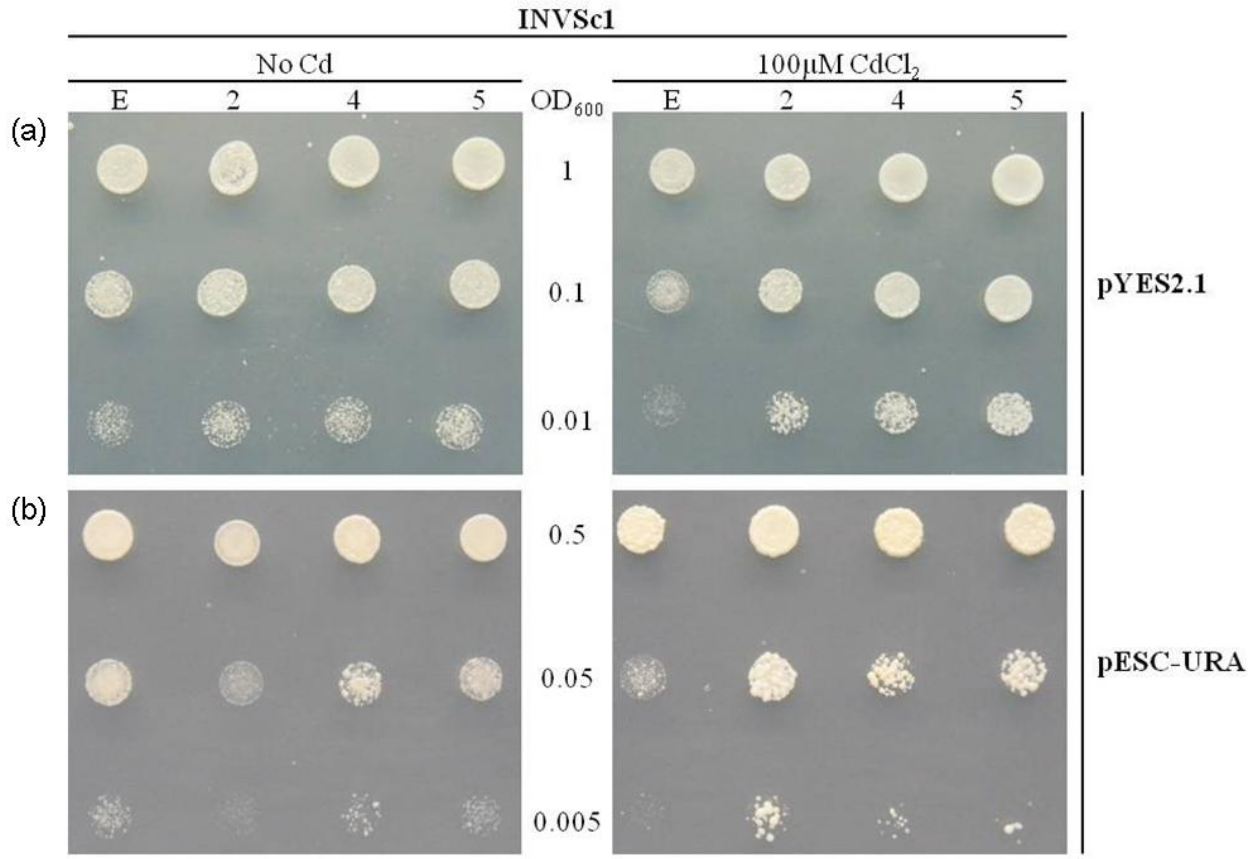
Eight TM helices are predicted in OsHMA4 using MEMSAT3. Numbers in bold indicate the position of TM segments within the OsHMA4 sequence. Signature sequences in TM6 [^{597}CPC^{599}], TM7 [^{905}N(x)$_6$YN(x)$_4$P^{918}] and TM8 [^{934}P(x)$_6$M(x)$_2$SS^{945}] are conserved in all Cu^+/Ag^+ ATPases.

Figure 3. Membrane topology of OsHMA5.



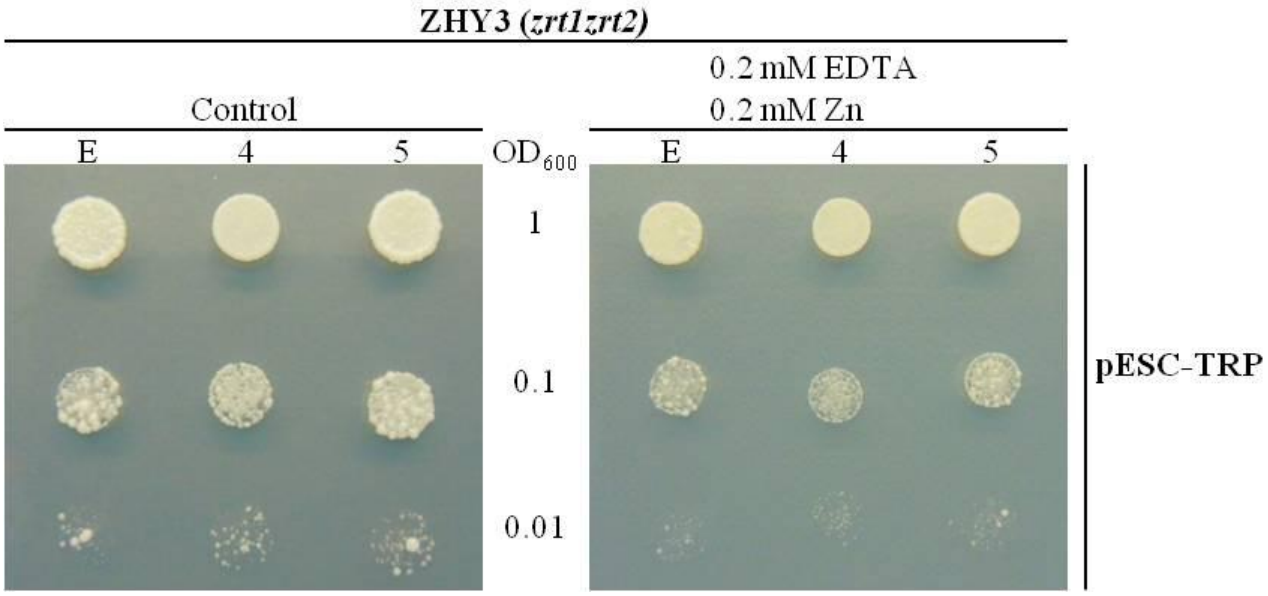
The OsHMA5 (cv. Roma) sequence has 100% identity to OsHMA5 cv. Nipponbare. Eight TM helices are predicted in OsHMA5 using MEMSAT3. Numbers in bold indicate the position of TM segments within the OsHMA5 sequence. Signature sequences in TM6 [^{637}CPC^{639}], TM7 [^{942}N(x)$_6$YN(x)$_4$P^{955}] and TM8 [^{971}P(x)$_6$M(x)$_2$SS^{982}] are conserved in all Cu^+/Ag^+ ATPases.

Figure 4. Drop test of yeast testing Cd tolerance.



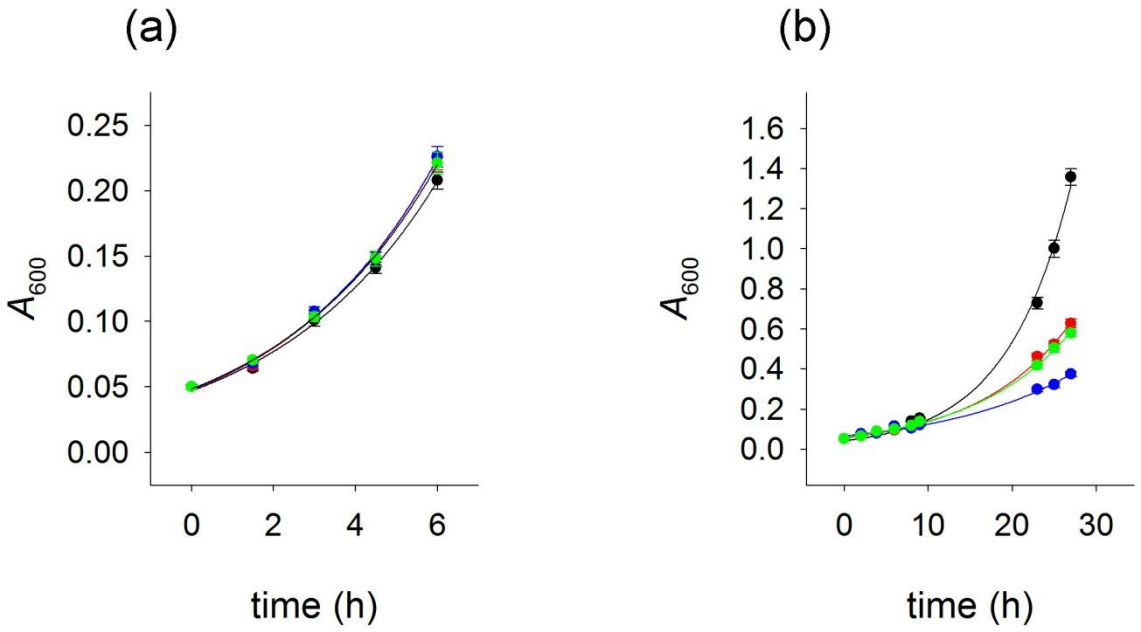
Cells of the wild type yeast strain INVSc1 expressing OsHMA2 (2), OsHMA4 (4) and OsHMA5 (5) under the control of the inducible *GAL1* or *GAL10* promoter or harboring the empty (E) pYES2.1 (a) or pESC-URA (b) vector were grown at 30 °C on SG media supplemented (picture taken after 5 days) or not (picture taken after 3 day) with 100 µM $CdCl_2$.

Figure 5. Drop test of yeast testing Zn tolerance.



Cells of the mutant yeast strain ZHY3 (*zrt1zrt2*) expressing OsHMA4 (4) and OsHMA5 (5) under the control of the inducible GAL1 or GAL10 promoter or harboring the empty (E) pESC-TRP vector were grown at 30 °C for 3 days on SG media with normal (control) or limiting levels of Zn (obtained adding 0.2 mM EDTA and 0.2 mM $ZnCl_2$).

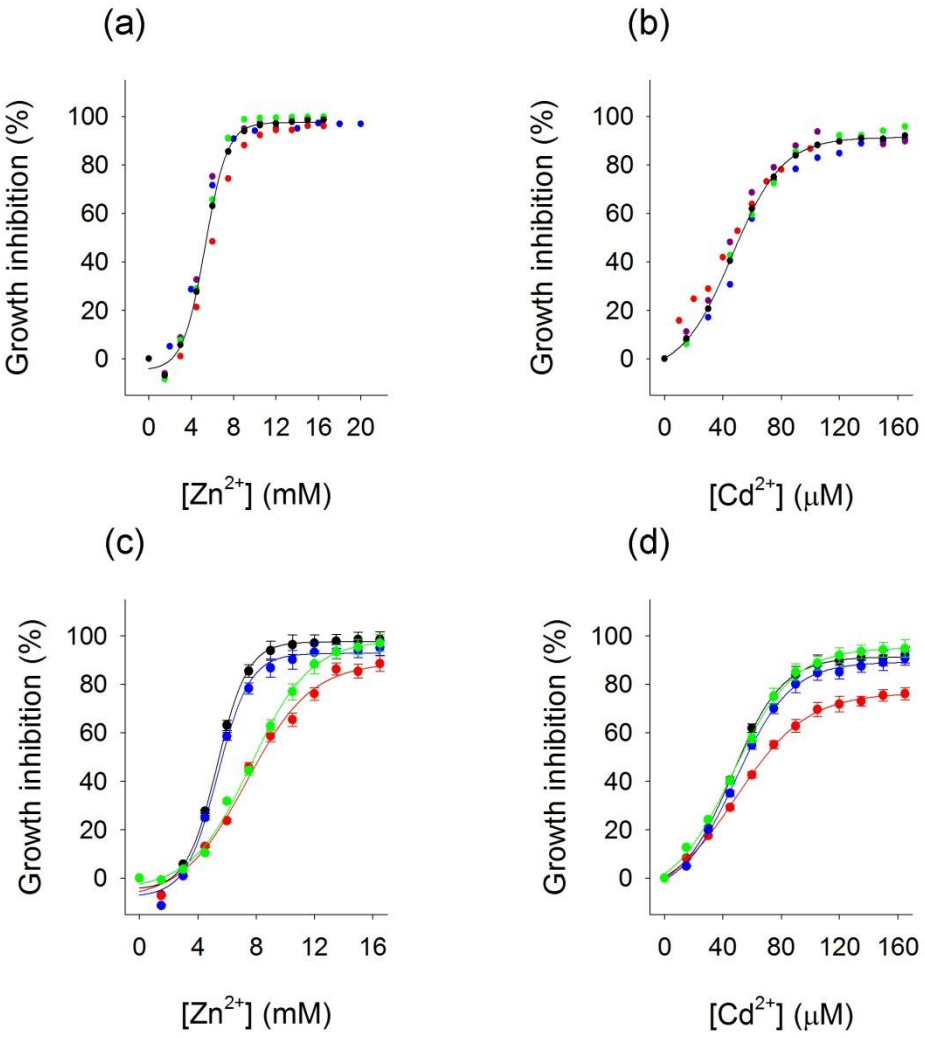
Figure 6. Kinetic analysis of yeast growth.



	Duplication time (h)	
	SD	SG
EMPTY	2.79 ± 0.09	5.37 ± 0.29
OsHMA2	2.68 ± 0.09 n.s.	7.70 ± 0.17 *
OsHMA4	2.69 ± 0.09 n.s.	10.66 ± 0.33 *
OsHMA5	2.75 ± 0.04 n.s.	8.15 ± 0.19 *

Growth of yeast cells of the wild type strain INVSc1 expressing OsHMA2 (red circles), OsHMA4 (blue circles) and OsHMA5 (green circles) or harboring the empty pESC-URA (control) vector (black circles) in liquid synthetic minimal media containing 2% (w:v) glucose (SD) (a) or 2% (w:v) galactose (SG) (b). The table reports the duplication times calculate for each strain. Data are means and SE of three experiments performed in triplicate (n = 3). Asterisks indicate significant differences between the control and yeast expressing one OsHMA protein ($P \leq 0.001$); n.s. indicates that there is not significant difference.

Figure 7. Yeast growth inhibition.



	IC_{50}	
	Zn^{2+} (mM)	Cd^{2+} (μM)
EMPTY	5.5 ± 0.2	52 ± 1
OsHMA2	8.3 ± 0.8 *	69 ± 4 *
OsHMA4	5.7 ± 0.3 n.s.	56 ± 3 n.s.
OsHMA5	7.9 ± 0.4 *	52 ± 4 n.s.

Growth inhibition curves of yeast cells of the wild type strain INVSc1 were incubated in SG media containing different concentrations of Zn (a,c) or Cd (b,d). (a,b) Preliminary tests with yeast harboring the empty pESC-URA (control) vector. The black circles are means of the means and the black curve is the result of the non linear regression analysis of that data. (c,d) Comparison functional tests of yeast cells expressing OsHMA2 (red circles), OsHMA4 (blue circles) and OsHMA5 (green circles) or harboring the empty vector (black circles). For each data point the percentage of growth inhibition was calculated with respect to the growth of the yeast cells in the absence of any excess of Zn and Cd. The table reports the duplication times calculate for each strain. Data are means and SE of three experiments performed in triplicate (n = 3). Asterisks indicate significant differences between the control and yeast expressing one OsHMA protein ($P \leq 0.001$); n.s. indicates that there is not significant difference.

CONCLUDING REMARKS

Several studies have shown that Cd, a non-essential and toxic metal, is taken up from soil and translocated in a root-to-shoot direction through transporters of essential elements such as Zn, suggesting that the two metal ions may compete for the same transporter protein on a membrane. This means that increasing the concentration of Zn will decrease the movement of Cd through the transporter, and vice versa. However, the movement of the two metal ions across several biological membranes may involve a wide range of transport systems, each characterized by a specific selectivity, not leading to a necessary "strong reciprocity" between the systemic fluxes of Zn and Cd in the whole plant. Finally, Zn and Cd ions are subjected to complex equilibria influencing their relative mobility inside the plant. Although divergent results have been obtained, they suggest that also Zn-independent pathways for Cd translocation in plants could be possible.

The proteins belonging to the HMA (Heavy-Metal ATPases) family have been partially characterized as the main actors of the process of translocation of trace elements (essential or non-essential) to all organs of the plant. In particular, OsHMA2 is the main transport system so far described in rice as involved in the xylem loading of Zn and Cd, even though both its activity and function has not been unambiguously characterized.

In such a contest took place the studies carried out in this PhD project. Indeed, the activity was focused at analyze possible interactions between Zn and Cd and reciprocal effects at the translocation level in rice plants, since these processes have been seen to be crucial in determining Cd accumulation in the shoots. The main results clearly indicate the lack of a fully reciprocity considering the effect of Cd on Zn accumulation, and vice versa, since the accumulation of Zn in the shoot was significantly inhibited by Cd increases in all the analyzed conditions, whereas those of Cd was only partially impaired by Zn increases. Such a finding suggests that Cd ions may use at least two distinct pathways to be translocated from the root to the shoot. The first one – shared with Zn – is probably used for Zn translocation in physiological conditions, whilst the second one appears as a Zn-independent route that Cd may preferentially use when the first pathway is saturated with Zn. Moreover, the Zn-independent pathway seems constitutively expressed in rice plants since the partial inhibitory effect exerted by Zn on Cd translocation was also observed in short-term PETIS experiments performed with unstressed plants. Since OsHMA2 appears to play an important role in Zn/Cd root-to-shoot translocation, in this work we also contributed to elucidate some aspects related to the OsHMA2 transport activity and selectivity by comparing the inhibitory effects exerted by Zn or Cd on the growth of yeast cells expressing, or not, OsHMA2. The results indicate that OsHMA2 enhances Zn and Cd tolerance in yeast, so we can reasonably conclude that OsHMA2 may pump excess of cytosolic Zn or Cd into the apoplast and thus has all the requisites to be considered the xylem loading system potentially involved in mediating the translocation of Cd through the Zn-dependent pathway. In addition, probably, this

study represents one of the first examples of growth inhibition analysis applied to plant gene functional characterization.

In conclusion our data provide several evidence to support the hypothesis that at least two competing pathways may be interested in mediating root-to-shoot Cd translocation in rice. The first one, prevailing at relatively low Zn concentrations, could involve OsHMA2 as Zn^{2+}/Cd^{2+} xylem loading system, while the second one appears to involve a Zn-independent system that still needs to be identified among the plethora of transporters involved in the metal homeostasis. The possible future identification of the transporter(s) responsible for the Zn-independent Cd translocation pathway(s) could allow the development of markers to select rice genotypes able to exclude Cd from the shoots. Furthermore, these activities could have important technological implications in the fields of food safety, especially in cases where the strategies used for containing Cd accumulation in the crops be founded on Zn fertilization.

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