UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI AGRARIA

Dipartimento di Produzione Vegetale

Doctoral Course in Plant Biology and Crop Production

XXIV Cycle

Doctoral thesis

CADMIUM EXCLUSION FROM RICE GRAINS: DEVELOPMENT OF MOLECULAR AND PHYSIOLOGICAL MARKERS

Scientific sector: AGR13

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Academic year 2010 – 2011

To Mirò and Big Man

“Yo aquí me despido, vuelvo
a mi casa, en mis sueños,
vuelvo a la Patagonia en donde
el viento golpea los establos
y salpica hielo el Océano.”

P. Neruda,
Oda a la paz

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Aims of the research

The well established tendency of rice (*Oryza sativa* L.) to accumulate Cd to levels often exceeding the international limits set up for the cereal grain trade highlights the need to apply sound strategies aimed at reducing the risk of grain Cd accumulation. However, the sole use of agronomic techniques, such as liming, application of organic matter or other soil amendments, has often been seen to fail, as most of the time the results obtained are just temporary. More promising, instead, looks the selection of rice cultivars which accumulate low Cd in the grains. Indeed, the natural variation in Cd distribution in plant tissues found across different varieties within rice species can be positively exploited to select those able to prevent Cd accumulation in the grains.
The research carried out in this PhD project took place in this context. Indeed, the general purpose was to deepen the knowledge of the physiological basis governing Cd distribution in rice, with particular concern on Cd root retention and Cd translocation, as they have been seen to be crucial in determining Cd accumulation. It is noteworthy that all the reported results were obtained by exposing rice plants to a broad range of relatively low Cd concentrations in order to simulate the real conditions in moderately contaminated soils, which is the most widespread situation.

Specifically, the aims of this study were: (I) to investigate the role of phytochelatins (PCs) in chelation and subcellular compartimentalization of Cd at the root level, thus in reducing the amount of Cd potentially mobile through the plant. This was done basing on physiological techniques aimed at isolating and quantifying Cd-PCs complexes, as well as on molecular analysis to better characterize the regulation of the enzyme phytochelatin synthase (PCS); (II) to identify genes encoding transporters putatively involved in Cd xylem loading, thus responsible for Cd translocation, 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 Arabidospsis were found to mediate the xylem loading of Cd; (III) to analyze the expression profile of such genes with respect to relatively low Cd concentrations, aiming at describing their response to Cd in the real conditions experienced by rice plants; (IV) to characterize the transporters encoded by the abovementioned genes by heterologous expression in *Saccharomyces cerevisiae*.

These activities provided useful information on the genes and on the physiological processes that determine the “Cd accumulation” trait in rice, thus constituting the basis to further develop molecular and/or physiological markers to early select rice genotypes able to exclude Cd from the grains.

Introduction

Section I

Cadmium in the environment: a potential risk for food safety

1. Trace elements

Trace elements can be defined as chemical elements that are needed in minute quantities for the proper growth and development of an organism (Bowen 1966). The main source of trace elements for humans are plants, particularly staple food crops as they make up a large proportion of dietary intake. Therefore, to enhance the beneficial effects of essential elements as well as limiting the adverse effects of non-essential elements on plants have direct consequences on human health. Concerning both essential and non-essential trace elements, there is a notable natural variation in the uptake, distribution and accumulation processes among crop species and in cultivars within the same species (Clarke et al. 1997a; Bell et al. 1997; Graham & Welch 1999; Huang & Graham 2001; Graham et al. 2007). This variation depends on several factors under genetic control that affect the abovementioned processes at different levels. Most of these factors have been widely investigated and are now well known. Others, instead, need to be clarified with the aim of improving crop quality, on one hand by increasing crop concentration of essential trace elements and, on the other, by reducing that of non-essential trace elements that may be harmful to human health, such as cadmium (Cd) (Graham & Welch 1999; Huang & Graham 2001; Gregorio 2002; Welch & Graham 2004; Graham et al. 2007).

1.1. Cadmium in soil

Cadmium (Cd) is a trace element that is present in the soil naturally and from anthropogenic sources. In the former case, natural mineral outcrops can been enriched in Cd through the weathering of Cd-rich parent material, whose real Cd content depends on the type of rock, mainly igneous, sedimentary or metamorphic. More rare is the case in which Cd presence in soil is due to geochemical occurrence. 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. As it is highly stable and resistant to heat, cold and light, Cd has been extensively used as protective plate on steel, in pigments synthesis, as stabilizer for plastic material and in dry cell batteries combined with nickel (Ni), just to mention some of its several applications. Thus, deriving from these activities are the sources of contamination that may originate highly Cd contaminated soils: atmospheric pollution from metallurgical industries, disposal of Cd containing wastes, burning of fossil fuels, deposition of aerosol particles from urban and industrial air pollution and, more specifically related to agricultural practices, application of sewage sludge and phosphate fertilizers which may contain Cd as an impurity, thus at low concentration but potentially dangerous due to their continual use. Notable is also the accidental supply of Cd due to the use of Cd containing irrigation waters (Alloway & Steinnes 1999).

Given a certain concentration of Cd, there are several factors affecting its availability, mostly depending on soil characteristics: generally Cd concentration increases with clay proportion (Mengel et al. 2001) as the soil sorptive capacity is higher compared to sandy soils, but Cd ions tend to remain adsorbed to soil particles. Cd availability is inversely related to soil pH (Mengel et al. 2001; Tudoreanu & Phillips 2004; Kirkham 2006), as an increasing acidity cause the dissolution of hydroxides and their co-precipitated metals, causing, in turn, the reduced Cd adsorption on colloids because of a decreased pH-dependent negative charge (Alloway & Steinnes 1999). The redox potential of the soil medium have also an important impact on Cd availability: in reductive conditions, in fact, Cd ions tends to precipitate in form of insoluble salt, such as CdS, thus not available for plant uptake. On the contrary, in oxidative conditions, Cd is mainly present in the free ionic form Cd^{2+} or as soluble salt in the soil solution and therefore likely to be taken by plants. Cd can also be present in soil bound to a broad range of organic compounds resulting in various organic complexes (Tudoreanu & Phillips 2004). Therefore, the real 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. However, compared to other metals including lead (Pb) and copper (Cu), Cd tends to be more mobile and thus more available to plants (Alloway & Steinnes 1999), potentially causing phtyotoxicity in case of elevated soil concentrations or, when it is taken up by roots and translocated to the edible portion of the plant, determining acute or chronic toxicity to humans even at low soil concentrations (Kawada & Suzuki 1998).

When introduced in soil as result of cycling through vegetation or external applications, either agriculture or industry activities, Cd tends to concentrate in the topsoil, the richest in organic matter to which Cd ions are adsorbed. On the long term, same as zinc (Zn) and Ni but different from Cu and Pb, it moves downwards, concentrating in the lowest horizons of the soil profile (Alloway & Steinnes 1999). Horizontally, Cd movement in the soil matrix is suggested to be dependent on the transpiration-driven mass flow of the soil solution, taking into account the low diffusion coefficient for Cd^{2+} in aqueous solutions (Sterckeman et al. 2004). This is also consistent with reports that Cd accumulation by plants grown in soil is directly related to the transpiration rate (Ingwersen & Streck 2005).

Apart from soil characteristics, Cd mobility, solubility and thus bioavailability are deeply affected by plants, especially in the rhizospheric soil, due to different root activities, such as: (a) proton efflux from root cells, especially considering non-graminaceous plants presenting the mechanism of iron uptake defined as “strategy I”, consisting in the reduction of Fe^{3+} to Fe^{2+} by proton extrusion

which, in turn, increases metal mobility; (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 aminoacids and metal chelating phytosiderophores. In particular, the efflux of such exudates which contain 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 is still to be investigated in details (Clemens 2006b; Prasad 1995).

1.2. Cadmium effects on plant metabolism

The effects of Cd on plant have been widely investigated and are now well known, even though most of Cd toxicity mechanisms are still unclear. It is commonly accepted that under long term exposure to Cd almost all physiological processes are affected, so that the recognition of primary toxicity symptoms is difficult (Prasad 1995). As proposed for other heavy metals, Cd cause oxidative stress, even though, contrarily to Cu, it does not seem to be directly involved in the production of the reactive oxygen species (ROS) (Sandman & Boger 1980). However, Cd inhibition of anti-oxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase is widely reported (Gallego et al. 1996). Other enzymes whose activity is inhibited by Cd are the chloroplast ribulose-1,5-bisphosphate carboxylase oxygenase, fructose-1, 6-biphosphatase, 3-phosphoglycerate kinase, NADP-gly-P-dehydrogenase, aldolase and plasma membrane H^+ATPase (Astolfi et al. 2005; Obata et al. 1996; Shoeran et al. 1990; Mattioni et al. 1997). Cd exposure cause also notable disruption in the metabolism of nutrients, as it reduces the uptake of nitrates and their transport from roots to shoots by inhibiting nitrate reductase (Hernandez at al. 1996). Even clearer is the inhibition of the Fe^{3+} reductase at root level which causes Fe deficiency in the whole plant, with deep consequences on photosynthesis (Alcantara et al. 1994). Cd directly affects photosynthesis by acting at different levels: it has been reported that Cd treatment reduces the total chlorophyll content as well as the chlorophyll a/b ratio in higher plants. Generally, carotenoids are less affected, so that Cd exposure results in a lower chlorophyll/carotenoid ratio (Krupa 1987). Moreover, Cd seriously inhibits the photosystem II, probably affecting the water splitting system at the level of the manganoproteins, and also the photosystem I, even though to a lesser extent (Siedlecka & Baszynsky 1993; Seidlecka & Krupa 1999). The disruption in photosynthesis process could also be due to the alteration of the content of phosphoatidylglycerol hexadecenoic fatty acid, widely accepted as a component responsible for the oligomerization of the chlorophyll protein complex. Also it has been observed

that the fine structure of chloroplasts results degenerated in Cd-treated plants due to disorganization in the lamellar pattern, similar to a senescence response (Krupa et al. 1993). Same wise, mitochondrial activity is affected in that the oxidative phosphorylation is inhibited by Cd, which appears to increase the passive permeability of the mitochondrial inner membrane to protons (Kesseler & Brand 1995); its action on membranes is also proved by an increase in lipid peroxidation (Somashekaraiah et al. 1992). Cd toxicity is also clear in the disturbance of the water status of the plant mainly due to an increase in the stomatal resistance (Barcelo & Poschenrieder 1990; Costa & Morel 1994): this is probably an indirect effect depending on the serious impairment in the movement of potassium (K), calcium (Ca) ions and abscissic acid to the guard cell (Barcelo et al. 1986; Barcelo & Poschenrieder 1990). Moreover, Cd generally decreases water stress tolerance of plants, causing cell turgor loss overall and degradation of the xylem cells, thus reducing water transport (Barcelo et al. 1988). Due to the interferences abovementioned, cell growth as well as whole plant growth is drastically inhibited by Cd: in bean plants exposed to Cd, for instance, leaf cell expansion growth and relative water content of primary leaves decreased by about 10%, probably because of an increase in the cross linking of pectins in the middle lamellae (Poschenrieder et al. 1989).

However, it is at the root level that Cd toxicity appears clear even at low concentrations, so that the quantification of the inhibitory effects of Cd ions on root elongation is one of the most commonly used method for monitoring plant responses to Cd (Godbold & Knetter 1991). First of all, in Cd treated plants an accelerated development of the endodermis and exodermis is observed (Seregin et al. 2004; Seregin & Kozhevnikova 2008). Also, elevated rhizosphere Cd concentrations often result in changes in the relative proportion and size of root tissues and cell types (Lux et al. 2010). Several studies have indicated that root diameter is increased by rhizosphere Cd concentrations that do not cause significant necrosis instead. Roots of willow, poplar, *Miscanthus sinensis* L. and maize grown in media containing Cd are shorter and thicker than plants grown in media lacking Cd, also presenting brownish apices (Lunačkovà & Lux 2010; Scebba et al. 2006; Maksimovič et al. 2007). The increase in root diameter in maize roots exposed to Cd has been explained as due to the increase of the size of parenchyma cells so that the enlargement of cortical tissues has a functional role by increasing resistance to radial flows of water and solutes (Maksimovič et al. 2007). Concerning the central part of the roots, just little information is available about changes induced by Cd in the development of cells and tissues. Vitória and coworkers (2003) report a proliferation in the cambial cells followed by a loss of organization of the cambial region in roots exposed to 0.5 mM Cd, which is a considerably high concentration, not reflecting the most common real conditions in agricultural soils. This topic would require more attention, especially considering the importance

of xylem loading in regulating Cd fluxes to the shoot (Papoyan et al. 2007; Ueno et al. 2008; Lu et al. 2009; Uraguchi et al. 2009; Verbruggen et al. 2009), tightly depending upon the organization of cells and tissues of this part of the root. Recently, an acceleration in the endodermal development and a lignification of cell walls of both inner cortical tissues and peripheral tissues of the vascular cylinder in maize roots has been interpreted as a way to restrict the radial apoplasmic movement of Cd, as well as Cd loading to the xylem, with the effect of protecting both unexposed root tissues and the shoot from Cd toxicity (Lux et al. 2010). This is also supported by the fact that the expression of genes involved in lignin biosynthesis is up-regulated in roots of *Arabidopsis thaliana* and *Noccaea caerulescens* upon Cd exposure (Herbette et al. 2006; van de Mortel et al. 2008). Taking into consideration the effects of Cd mentioned above, it is reasonable to suppose that the responses to local Cd exposure are initiated through Cd-induced oxidative stress, which has been seen to be implicated in the inhibition of root initiation and elongation in various plant species (Xiong et al. 2009).

1.3. Cadmium uptake by plant roots

Apart from aquatic plants, where Cd is also taken up by the shoot system (Ornes & Saiwan 1993), Cd enters plant organism through the roots. 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, therefore it can be described as a biphasic uptake kinetics. Estimates of the K_m value for the affinity to Cd in the uptake commonly fall between 20–1000 nM and it is well documented that Cd uptake and accumulation by plant roots is generally inhibited by Ca^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} or Mn^{2+} in the rhizosphere solution (Cataldo et al. 1983; Costa & Morel 1993, 1994; Lombi et al. 2001; Hart et al. 2002; Zhao et al. 2002; Berkelaar & Hale 2003; Han et al. 2006; Zhao et al. 2006). This is due to the fact that, as Cd does not have a biological function within the organism, no specific transporters have developed through evolution. Therefore, it is taken up by membrane permeases devoted to other cations transport but characterized by broad-range specificity, generating competition effects, particularly evident in the case of Ca^{2+} (Perfus-Barbeoch et al. 2002).

Indeed, there is evidence that Cd can enter root cells as free ion through ZIP (Zinc regulated transporter/Iron-regulated transporter-like Protein) transporters. Plants overexpressing *AtIRT1* have been seen to accumulate higher concentrations of Cd and Zn than wild types under Fe-deficient conditions (Connolly et al. 2002). This is also supported by transport studies in yeast (Eide et al. 1996; Korshunova et al. 1999). Same wise, TcZNT1/TcZIP4 can mediate Zn and Cd influx when expressed in yeast (Pence et al. 2000).

The Nramps (Natural resistance associated macrophage proteins) family has also been proved to take part in Cd transport: in yeasts, three Nramps have been identified (SMF 1-3) that mediate the uptake of Mn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, and Fe^{2+} (Supek et al. 1996; Liu et al. 1997; Chen et al. 1999). In Arabidopsis, AtNramps3 and 4 are able to support Fe and Mn uptake in mutant transport-deficient yeasts increasing also their Cd accumulation and sensitivity (Thomine et al. 2000).

Transporters of the CDF family also appear to mediate the cytoplasmic efflux of Cd as well as of other transition metal cations such as Zn^{2+},Co^{2+}, Ni^{2+} or Mn^{2+} and have been named MTP, standing for ‘‘metal tolerance protein’’. A plant CDF transporter gene was first characterized in Arabidopsis and designated ZAT (van der Zaal et al. 1999). A ZAT gene, *ZTP1*, has also been identified in the Zn hyperaccumulator *Thlaspi caerulescens* (Assunção et al. 2001). It is mainly expressed in the leaves and is probably also involved in Zn intracellular compartmentalization and tolerance (van der Zaal et al. 1999; Assunção et al. 2001). The same conclusion has been drawn by Persans and coworkers (2001) studying another hyperaccumulator, *Thlaspi goesingens*: they have characterized a CDF transporter (TgMTP1) that is thought to account for the accumulation of metal ions within the shoot vacuoles that confers tolerance to Cd, Co and Zn in yeast expression experiments.

The low affinity transporter LCT1 identified in wheat has been seen to increase Cd and Ca uptake when expressed in yeast (Clemens at al. 1998). Interestingly, increasing the external Ca concentration, Cd uptake results inhibited suggesting that LCT1 is actually a Ca transporter, accidentally mediating Cd movement across the plasma membrane. However, its subcellular localization *in planta* is still to be determined.

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

Functional analysis conducted in Arabidopsis demonstrated that AtDTX1, a transporter belonging to the MATE (Multidrug And Toxic compound Extrusion) family, serves as an efflux carrier for plant-derived alkaloids, antibiotics and other toxic compounds but, interestingly, it is also able of detoxifying Cd. Its localization on the plasma membrane supports the hypothesis that it could a role in Cd extrusion from the cell (Li et al. 2002).

In addition to the free ion form, Cd might enter root cells also as Cd-chelate through YSL (Yellow-Stripe 1-Like) proteins (Curie et al. 2009), which can mediate the uptake of different metals

complexed with plant-derived organic compound, such as phytosiderophores or non-proteic aminoacids (Curie et al. 2001). Apart from Cd, it is apparent that Fe and possibly Zn, Mn and Cu homeostasis are dependent on YSLs. However, further examination of the localization and substrate specificity of various YSLs and genetic analysis of mutants will help assigning functions to this recently identified plant-specific family of transporters (Colangelo & Guerinot 2006).

Once Cd enters the root system, plants respond to metal toxicity in a variety of different ways. Such responses include immobilization, chelation and compartmentalization of the metal ions, as well as the expression of more general stress response mechanisms such as ethylene and stress proteins (Sanità di Toppi & Gabbrielli 1999). Immobilization occurs when Cd ions are adsorbed to the negative charges present on the cell wall of root cells. 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 chelation of the metal by a ligand and, in some cases, the subsequent compartmentalization of the ligand-metal complex. Peptide ligands include the metallothioneins (MTs) which are a group of small gene-encoded Cys-rich molecules, which, in their reduced state, provide thiols for metal chelation. MTs have been found broadly distributed among animals, eukaryotic microorganisms, certain prokaryotes and plants (Rauser 1999). By contrast, the phytochelatins (PCs) are enzymatically synthesized Cys-rich peptides. After the structures of PCs had been elucidated and it was found that these peptides are widely distributed in the plant kingdom, it was proposed that PCs were the functional equivalent of MTs (Grill et al. 1987). There is now agreement that plants express both of these Cys containing metal-binding ligands and that the two play relatively independent functions in metal detoxification and/or homeostasis. PCs have first been reported in plant species and this has supported the notion that in animals MTs may well perform some of the functions normally contributed by PCs in plants. This is also why the use of term “phyto” as gradually replaced the name these compounds were previously known as, *i.e.* class III MTs (Cobbett 2000).

However, recently the enzyme catalyzing PC biosynthesis, commonly known as PC synthase (PCS), have been identified in a number of additional organisms. *Caenorhabditis elegans* has a single copy PCS gene that confers Cd resistance when heterologously expressed in yeast (Vatamaniuk et al. 2001). PCS genes or transcripts have also been identified in the parasitic nematodes *Brugia malayi* and *Parascaris univalens* (Vatamaniuk et al. 2002), in the trematode *Schistosoma mansoni* (Ray & Williams 2011) and in other metazoan organisms, including lower chordates (Clemens 2006a) and in several prokaryotic genomes (Clemens 2006a; Chaurasia et al.

2008; Tsuji et al. 2004; Harada et al. 2004). In contrast, there are no PCS genes encoded in the genomes of mammals, which instead use GSH and MTs to regulate metal homeostasis (Hamer 1986). PCs consist of only three amino acids: Glu, Cys and Gly with the Glu and Cys residues linked through a γ-carboxylamide bond. PCs form a family of structures with increasing repetitions of the γ-Glu-Cys dipeptide followed by a terminal Gly, thus presenting the general structure $(\gamma\text{-Glu-Cys})_n$-Gly, where *n* has been reported to be up to 11, being mostly within the range of 2 to 5. A number of structural variants, for example, $(\gamma\text{-Glu-Cys})_n$-β-Ala, $(\gamma\text{-Glu-Cys})_n$-Ser and $(\gamma\text{-Glu-Cys})_n$-Glu, have been identified in some plant species (Rauser 1995, 1999; Zenk 1996).

Interestingly, PCs are structurally related to glutathione (GSH; γ-Glu-Cys-Gly) and numerous physiological, biochemical and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC biosynthesis (Rauser 1995, 1999; Zenk 1996). Indeed, it has been observed that the induction of PC biosynthesis after exposure to metals corresponds to a temporary decrease in GSH level (Clemens 2006b; Nocito et al. 2007) and that, by treating plants with buthionine sulfoximine, an inhibitor of the GSH synthesis pathway, PC production is inhibited resulting in an increased Cd sensitivity (Blum et al. 2010).

The reaction leading to the biosynthesis of PCs from GSH involves the transpeptidation of the γ-Glu-Cys moiety of GSH onto initially a second GSH molecule to form PC_2 or, in later stages of the incubation, onto a PC molecule to produce an $n + 1$ oligomer. This γ-Glu-Cys dipeptididyl transpeptidase has been named PC synthase and it has been seen to be an oligomeric protein consisting in four M_r 25.000 different subunits (Grill et al. 1989). Interestingly, it has been observed *in vitro* that PCS is active only in the presence of metal ions. The best activator is Cd and the efficiency of other metals in inducing its activity is as follows:

Cd > Pb > Zn > Sb > Ag > Hg > As > Cu > Sn > Au > Bi (Zenk 1996)

These results have then been confirmed *in vivo* as PC biosynthesis has also been observed in plant cell cultures treated with metals. *In vitro* reactions, PC biosynthesis continues until the activating metal ions are chelated by the PCs formed (Loeffler et al. 1989). This provides an evidence for the fine tuning of PCs biosynthesis, as the product of the reaction chelates the activating metal, thereby reducing its availability and terminating the reaction. However, the control of PC biosynthetic pathway occurs at different levels. The first of these is likely to be the regulation of GSH biosynthesis. Studies of transgenic Indian mustard (*Brassica juncea*) plants, in which the expression of the enzymes of the GSH biosynthetic pathway was increased, show that PC biosynthesis and Cd tolerance can also be increased (Yong et al. 1999; Zhu et al. 1999): this could be interpreted as an

endogenous mechanism of control of PC synthesis. Moreover, exposure of Arabidopsis plants to Cd and Cu causes an increase in transcript levels of the two genes in the GSH biosynthetic pathway and of GSH reductase (Xiang & Oliver 1998).

However, the main point at which PC synthesis is regulated is the PC synthase activity. It has been proved that PC biosynthesis occurs within minutes of exposure to Cd and is independent of *de novo* protein synthesis, which is consistent with the observation of enzyme activation *in vitro*. Interestingly, the enzyme seems to be expressed independently of heavy metal exposure and this has been seen in different plant species: *S. cucubalis,* where it first was identified (Grill et al. 1989), tomato (Chen et al. 1997) and Arabidopsis (Howden et al. 1995a), all of them grown on medium where heavy metals were present only in traces. This means that PC synthase is somehow regulated by presence of heavy metals, thereby suggesting a post-translational control. First an allosteric control of the enzyme by heavy metals was hypothesized (Ha et al. 1999) but further studies clarified the mechanism of its activation (Vatamaniuk et al. 2000). It was proved that AtPCS1, and by implication other PC synthases, are indeed almost exclusively regulated by heavy metals at the post-translational level and catalyze a bi-substrate transpeptidation reaction in which both free GSH and its corresponding heavy metal thiolate are cosubstrates. Interestingly, it was also observed that both free GSH and its heavy metal thiolate are ordinarily required for maximal activity, but other compounds, for instance *S*-substituted GSH derivatives, can substitute for both, supposedly to overcome an obligatory requirement for heavy metals for activity (Vatamaniuk et al. 2000). The sufficiency of blocked thiol groups on at least one of the two substrate molecules required for core catalysis by PCS does not necessarily preclude the augmentation of activity by direct metal ion binding to the enzyme. Indeed, in presence of free metal ions and *S*-alkylglutathiones which act as substrates even with no metal ions available, the synthesis of *S*-alkyl-PC was enhanced. However, this effect is unlikely to be appreciable *in vivo* or *in vitro* when the dominant thiol peptide is unsubstituted GSH, as metal thiolate would quickly form causing the activation of the enzyme. According to these findings, PC synthesis from GSH in media containing heavy metal ions would take place following a scheme in which heavy metal thiolate, as, for instance Cd-GS_2 or Zn-GS_2, and free GSH interact via a substituted enzyme intermediate. Specifically, the kinetics of heavy metal-dependent PC_2 synthesis from GSH implicate the formation of an enzyme γ-Glu-Cys acyl intermediate with γ-Glu-Cys group coming from either free GSH or the heavy metal thiolate, which in turn plays the role of activated donor for transpeptidation of the second substrate (either Cd-GS_2 or GSH). Therefore, it seems that the minimum condition that must be satisfied for this reaction to proceed is that at least one of the thiol groups on one of the substrate molecules is blocked either

through heavy metal thiolate formation or *S*-alkylation (Vatamaniuk et al. 2000). An important physiological implication of this mechanism is that, contrary to the prevailing model (Zenk 1996; Loeffler et al. 1989), termination of the reactions catalyzed by PCS cannot be the directly caused by the chelation of heavy metals because GSH- and PC-metal complexes are the active substrate species. Instead, termination of PC reaction synthesis more likely results from exhaustion of the heavy metal pool such that free thiols (GSH and apo-PCs) compete with thiolates for the high affinity site of the synthase. Thus, it is probably the diminution of the substrate-active thiolate pool the determining factor ensuring that PC synthesis meets but does not exceed demand (Vatamaniuk et al. 2000).

PC synthase gene expression is, therefore, unlikely to play a significant role in regulating PC biosynthesis. As support to this, there are different studies indicating that PC synthase is constitutively expressed and levels of enzyme are generally unaffected by exposure of cell cultures or intact plants to Cd (Ha et al. 1999; Vatamaniuk et al. 1999). This could be interpreted to indicate a role for PCs in other than metal detoxification mechanisms, but, more generally, in the homeostasis of essential metal ion metabolism (Rauser 1995, 1999; Zenk 1996). *In vitro* experiments have shown in fact that PC-Cu and PC-Zn complexes can reactivate the apo-forms of the Cu-dependent enzyme diamino-oxidase and the Zn-dependent enzyme carbonic anhydrase, respectively (Thumann et al. 1991). Therefore, these experiments demonstrate that PC-metal complexes are capable of donating metal ions to metal-requiring enzymes. In addition, roles for PCs in Fe or sulfur metabolism have also been proposed (Zenk 1996; Sanita di Toppi & Gabbrielli 1999). Nevertheless, increased level of *TaPCS1* mRNA after exposure to Cd have been observed in wheat (Clemens at al. 1999). According to these apparently contrasting data, it is reasonable to suppose that, in some species PC synthase activity is regulated both at the transcriptional and post-translational level (Cobbett 2000).

It has been seen that both plants and yeast exposed to Cd accumulate low molecular weight (LMW) PC-Cd complexes, consisting in PCs and Cd, and high molecular weight (HMW) PC-Cd-S^{-2} complexes, containing additional acid labile sulfide (Murasugi et al. 1983; Speiser et al. 1992). Genetic and biochemical analysis suggest that the production of the sulfide moiety in the HMW PC-Cd-S^{2-} complex involves the purine biosynthetic pathway (Speiser et al. 1992). The HMW complex, a CdS crystallite coated by PC peptides (Dameron et al. 1989), has higher Cd-binding capacity than LMW PC-Cd and Cd ions are less susceptible to acid displacement (Reese & Winge 1988).

The role of these two types of complexes in heavy metals detoxification has been elucidated in studies conducted by Ortiz and coworkers (1992, 1995). They focused on a Cd-sensitive

Saccharomyces pombe mutant defective in production of the HMW PC-Cd-S^{-2} and identified the *hmt1* gene that has then been seen to share sequence identity with the family of ATP-binding cassette (ABC)-type transport proteins. This gene encodes a protein, HMT1, that is localized on the tonoplast (Ortiz et al. 1992). These findings added a key element in understanding Cd detoxification mechanisms in yeast and led to hypothesize a model that is still widely accepted (Fig. 1). According to it, the cellular uptake of Cd induces PC synthesis; the PCs produced chelate the free metal ions by forming the LMW complexes. These are then transported across the vacuolar membrane by HMT1, where additional sulfur (S) in form of sulfide is incorporated in the lumen of the vacuole to generate the HMW PC-Cd-S^{-2} complexes. In this model the LMW PC-Cd complex would function as a scavenger and carrier of cytoplasmic Cd, whereas the HMW PC-Cd-S^{-2} complexes would definitely function as storage of Cd, reducing its toxicity and thus increasing Cd tolerance of the organism (Ortiz et al. 1992, 1995). This role is consistent with the increased stability and metal-binding capacity of the HMW complex (Reese & Winge 1988). However, the fact that HMT1 transports only LMW PC-Cd complexes is not unequivocal, in that it could rather be sulfide or an enzyme involved in sulfide incorporation into the LMW PC-Cd complexes evolving in HMW PC-Cd-S^{-2}, which is the substrate of HMT1 (Ortiz et al. 1995). At present, no ortholog for HMT1 has been identified in plants. Recently two ABC transporters have been identified as possible PC transporters in Arabidopsis plants exposed to arsenic (As): AtABCC1 and AtABCC2 subcellular localization is on the vacuolar membrane and they have been proved to transport PC-As complexes and, with lower affinity, GS-As conjugates (Song et al. 2010). Interestingly, AtABCC2 has also been shown to transport chlorophyll catabolites which are potentially toxic because they can absorb light and produce ROS (Frelet-Barrand et al. 2008). Whether these transporters are specific for PC-As complexes is still unclear and their potential role in compartimentalizing other metal complexes would shed light on heavy metals detoxification mechanisms. These findings as well as the cytosolic localization of PC synthase recently demonstrated in Arabidopsis (Blum et al. 2010) are in agreement with the model proposed by Ortiz and coworkers (1992).

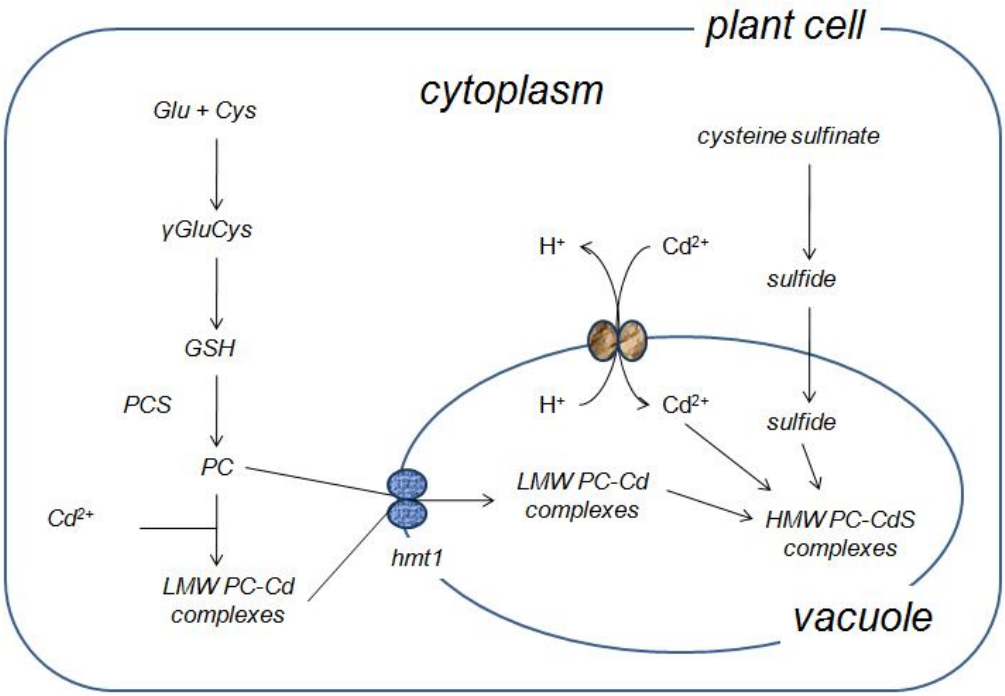

Fig. 1 : Cd detoxification mechanism based on phytochelatins (PCs) biosynthesis and compartimentalization in the vacuole according to Ortiz et al. 1992, 1995 (adapted from Cobbett 2000).

Another focal point that needs to be clarified is how LMW and HMW complexes are related to each other in terms of concentration within the cell and Cd distribution among them. In a population of dividing and aging cells, as yeasts are, the two complexes are part of a dynamic process. In *S. pombe* it has been observed that a nearly equal proportion of LMW and HMW complexes after 5 h of Cd exposure changed to a large, prevailing peak of HMW complex after 9.5 h of exposure (Murasugi et al. 1981). The same dynamic equilibrium from mostly a LMW PC-based complex to a dominant HMW complex component has been found in plants. Rauser (2003) observed changes in Cd distribution between LMW and HMW PC-based complexes in a time-course experiment in maize root. Same wise, it has been seen that not only time of exposure but also the severity of the stress affects the proportion of LMW and HMW: with increasing concentrations of Cd, in fact, the predominance of the HMW complex increases, as well as the proportion of root Cd chelated overall by HMW and LMW complexes (Rauser 2003). These data support once again the model from fission yeast that LMW Cd-PC complexes are synthesized first, transported into the vacuole and HMW complexes built up later by incorporating sulfide and additional Cd ions (Ortiz et al. 1995).
Regarding to this, it is important to note that, apart from being chelated and then transported into the vacuole in form of complexes, Cd can be also aspecifically transported across the tonoplast in form of free ion through antiporter mechanisms actuated by the so-called CAX Ca^{2+}/H^{+} transporters

(Salt & 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. 2010a, 2009a, 2009b).

The role of the vacuole as storage compartment is, therefore, essential in Cd detoxifying mechanisms as it allows the plant cell to maintain low concentration of the non-essential metal within the cytosol, thereby reducing its toxic effects.

1.4. Cadmium movement and translocation

Once Cd is within the cell not aspecifically adsorbed to cell wall and thus immobilized, nor chelated to organic compounds as PCs, nor compartimentalized in a subcellular compartment, it is, *de facto*, mobile throughout the plant.

It is possible for Cd^{2+} and Cd-chelates to reach the xylem either by radial symplasmic or extracellular, apoplasmic pathway. Although the relative contributions of these two pathways to the delivery of Cd to the xylem are unknown, it is likely that the proportion of the apoplasmic one will increase as the Cd concentration in the rhizosphere solution increases, as it has been proposed for both Zn and sodium (Na) (White et al. 2002; Plett & Møller 2010). This pathway is of particular relevance in regions of the root lacking the Casparian band (Berkelaar & Hale 2003; Lux et al. 2004) and it is in fact generally restricted to the extreme root tip and to regions in which lateral roots are being initiated (White 2001; Moore et al. 2002). As support to this, the root tip has been seen to be the most active region of the root for Cd influx (Piñeros et al. 1998); moreover, Cd accumulation by wheat cultivars is positively correlated with the number of root apices (Berkelaar & Hale 2000). A linear component to the concentration dependence of Cd uptake on Cd concentration in the nutrient solution is almost universally observed in short duration studies on hydroponically-grown plants. This fact can be interpreted as the result of an apoplasmic Cd flux to the xylem (Lux et al. 2010). By contrast, Xing and collaborators (2008) showed that, among accessions of *N. caerulescens* with different abilities to take up Cd and Zn, the amount of Cd taken up by roots and translocated to the shoot is inversely related to apoplasmic water flows. However, differences in the ability of accumulating Cd and Zn observed in *N. caerulescens* accessions can be also interpreted as evidence in transport selectivity. Furthermore, evidence supporting the occurrence of symplasmic delivery of Cd to the xylem is also the well proven competition between Cd and other cations for uptake by roots (Hart et al. 2002).

Once in proximity of the xylem, Cd is loaded from the symplasm into the xylem vessels by transporters which may aspecifically recognize Cd ions and/or Cd chelates. Putatively responsible for this mechanism are transporters belonging to the P_{1B}-type ATPase family; their biological function actually consists in Zn transport and xylem loading but there is evidence for their aspecific involvement in Cd transport. In particular, studies on *A. thaliana* double mutant led to identify two P_{1B}-type ATPases, AtHMA4 and AtHMA2, playing an essential role in Zn delivery to the shoot and also determining lower Cd concentration in the shoots when the genes encoding them were knocked-out (Hussain et al. 2004). This topic will be extensively treated in the following section of this introduction.

There is general agreement on the fact that the mass flux generated by the transpiration process is the driving force determining the movement of Cd along the xylem vessels up to the shoots (Senden at al. 1995). On the contrary, in which form Cd is translocated, distributed and, eventually, reallocated is still under debate. Initially, studies conducted on *B. juncea* making use of K-edge XAS technique, revealed different Cd species between roots and the xylem sap: in the case of root tissues, in fact, the EXAFS spectra fit the Cd-S interactions, with an interatomic distance of 2.53 Å. This most likely represents Cd-S coordination. On the contrary, the xylem sap fits either oxygen or nitrogen coordination at a distance slightly shorter than of 2.3 Å, which probably represents Cd coordinated with six ligands. According to these findings, whereas at root level Cd appears mostly bound to S-containing compounds, plausibly PCs, the transport of Cd via the xylem occurs in a PC-independent manner (Salt et al. 1995), through coordination to molecules still to be identified.
In apparent contrast with these results, Gong and coworkers (2003) demonstrated that Cd is translocated along the xylem bound to PCs which can undergo long-distance root-to-shoot transport: this has been found by expressing wheat *TaPCS1* cDNA in the *Arabidopsis* PC-deficient mutant *cad1-3*. In *cad1-3* plants PCs were not observed in roots, rosette leaves and stems but when *TaPCS1* was targeted expressed in the roots of *cad1-3* background, PCs were found both in rosette leaves and stems, although PCS protein and mRNA were detected only in roots; this proves that LMW-PC-Cd complexes can actually move throughout the plant. Also, as both ectopic and root-specific expression of *TaPCS1* did not significantly increase Cd content in root cells as it would be expected due to the enhanced capability of chelating and detoxifying Cd, Gong suggests that in addition to the known cellular protection function of PCs (Zenk 1996), these are involved in long-distance Cd transport with the aim of maintaining low Cd concentrations in roots. If that was true, PC-Cd detoxification model derived from unicellular organisms (Ortiz et al. 1992, 1995) would not be completely applicable to multicellular organisms as plants (Gong et al. 2003).

Experiments conducted on the Zn- and Cd-hyperaccumulator *Arabidospsis halleri* (Ueno et al. 2008) gave a better insight on the identification of Cd forms in the xylem sap. In this case, ^{113}Cd-NMR spectroscopy combined with a stable isotope (^{113}Cd) labeling technique was used and the chemical shifts of ^{113}Cd analyzed, as they change depending on the ligand the metal is coordinated to (Kostelnik & Bothner-By 1974). The NMR spectra clearly showed that Cd is predominantly transported in the free ionic form, both in plants exposed to 35 µM Cd and in computed speciation in the xylem saps from plants exposed to 1 µM Cd, which is indicative of more widespread conditions in contaminated agricultural soils. It was also seen that very little Cd was complexed with citrate, whereas citrate has been reported to play an important role in the xylem loading of metals such as Al and Fe (Ma & Hiradate 2000; Durrett et al. 2007). Other minor ligands identified where malate and histidine (Ueno et al. 2008).

Taking into account the apparently contrasting data reported so far, it is reasonable to suppose that the chemical forms of Cd being translocated in the xylem may vary among plant species. Moreover, because Cd is relatively mobile in terms of root to shoot transport compared to other metals and xylem vessels are not sensitive to metal toxicity, complexation with organic ligands may not be necessary for it to move in the hyperaccumulator *A. halleri* (Ueno et al. 2008). By contrast, this may be essential in plants that are not able to hyper-accumulate Cd and are more sensitive to it, *i.e. B. juncea*, where complexation of Cd to different ligands could be a way to reduce its toxicity.

As several studies conducted in different crops have suggested that Cd is translocated to fruit and grains mainly via the phloem (Popelka et al. 1996; Becher et al. 1997; Hart et al. 1998; Harris & Taylor 2001; Tanaka et al. 2003, 2007), it would be interesting to understand how this occurs in terms of Cd forms being transported.

At present, there is still a lack of knowledge on this topic, even though interesting results have recently come up. An elegant study conducted on Arabidopsis revealed the possibility that PCs undergo long-distances shoot-to-root transport (Chen et al. 2006). This research has been conducted on double loss-of-function mutant plants, defective for AtPCS1 (*cad1-3*; Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999) and AtPCS2 (Cazalè & Clemens 2001; Lee & Kang, 2005). As expected, the *cad1-3 atpcs2-1* double mutants showed no detectable PCs in all plant tissues. Interestingly, grafting of *cad1-3 atpcs2-1* double-mutant roots to wild-type shoots clearly showed transfer of PC_2, PC_3 and PC_4 from shoots to roots, demonstrating PCs movement downwards (Chen et al. 2006). Evidence that PCs can function in source-to-sink transport of metals and that phloem is the major vascular system for long-distance Cd transport was also found in wild type plants. A study conducted on *B. napus* highlights that thiols and Cd concentrations in xylem and phloem

vessels are so different to suggest distinct mechanisms of Cd transport in both vascular pathways (Mendoza-Cózatl et al. 2008). Cd appeared, in fact, more concentrated in phloem sap (20 μM) than in xylem sap (4 μM), as well as thiols (intended as Cys, γ-EC, PCs, GSH and GSH related peptides, whit PCs and GSH being more abundant) that were more than 50-fold more abundant in phloem sap than in xylem sap, with PCs almost exclusively present in the phloem. These findings suggest that phloem is indeed an important route for long-distance Cd transport and that Cd translocation via the xylem is not likely to be in the form of thiol-complexes, which is in agreement with the abovementioned conclusions drawn by Salt et al. (1995). This would mean that once Cd has been transported in the xylem, mostly in the free ionic form (Ueno et al. 2008), the phloem functions redistributing Cd to roots and younger leaves (sink-to-source transport) and this transport is highly likely to be as thiol–Cd complexes (Mendoza-Cózatl et al. 2008).

However, the mechanisms by which PCs undergo long-distance transport in plants remain largely unknown. Phloem transport of PCs would likely require several types of PC transporters for phloem loading and unloading, which still remain unidentified. In the Arabidopsis genome sequences of several potential peptide transporter families for PCs have been revealed but no evidence has been found for any of them. Same wise, the reason why PCs are transported from the shoots to the roots is still to be explained: it is possible to suppose that, beyond their detoxifying function, PCs may also serve as signaling molecules to communicate heavy-metal content between different tissue types. In other words, since Cd competes with the physiological transport of nutrients such as Ca, Fe, Mg, Mn, Cu, and Zn, as it is transported by the same transmembrane transporters (Clarkson & Lüttge 1989; Rivetta et al. 1997; Clemens et al. 1998, 2002; Grotz et al. 1998; Curie et al. 2000; Pence et al. 2000; Picard et al. 2000; Thomine et al. 2000, 2003; Connolly et al. 2002; Papoyan & Kochian 2004), PCs might serve as a signal to down-regulate nutrient transporters in roots to prevent further uptake of heavy metals (Chen et al. 2006).

2. Cadmium in rice

Rice is one of the most important staple foods in the world. Over 50 percent of the world population depends on rice for about 80 percent of its food requirements. At present, about 95 percent of the global output of rice is produced and consumed in developing countries. It is noteworthy that the improved production prospects planned for 2010 are behind the forecast for global rice consumption in 2011, which has been assessed to be in the order of 461 million tonnes, almost 3 percent above 2010. Much of the increase is likely to be in rice destined to food, with *per caput* consumption averaging close to 57 kilos in 2011, half a kilo more than in 2010. This increase

largely reflects rising demand in the fast growing economies, which are traditionally the major consumer of rice (FAO 2011).

Crop species and cultivars differ widely in their ability to absorb, accumulate and tolerate Cd. (Bingham et al. 1975; Kuboi et al. 1986; Li et al. 1995, 1997; Grant et al. 1999, 2000; Hocking & McLaughlin 2000; Clarke et al. 2002; Clemins et al. 2002; Miller et al. 2006). Among them, rice is the one presenting the highest risk of accumulating Cd in the aerial part of the plant, especially in the grains, so that along with other commercially grown crops destined for international trade such as sunflower, flax and durum wheat, it has been identified as Cd accumulator (Erdman & Moul 1982; Li et al. 1997; Clarke et al. 1997a,b; Hocking & McLaughlin 2000; Arao & Ishikawa 2006).

This is not only due to genetic reasons but also to the agronomic practices normally applied in its cultivation. As mentioned above, Cd availability in soil is tightly depending on soil characteristics, among which of particular relevance are pH and redox conditions. It has been shown that the enrichment of Cd in paddy rice grain occurs during soil oxidation, which accompanies pre-harvest drainage of the flooded paddy (Iimura 1981; Inahara et al. 2007). Pre-harvest drainage is typically initiated two weeks before plant maturity, which coincides with the grain-filling phase of rice growth. Therefore, it is during this time that Cd becomes more available to the plant with respect to Fe and Zn (Honma & Hirata 1977; Saito & Takahashi 1978). This may be due to higher Cd:Fe and Cd:Zn ratios in the soil solution, resulting in a lower competition from Fe and Zn with Cd for uptake at the root level (Smolders et al. 1997). The increased Cd:Fe and Cd:Zn ratios have been suggested to be due to higher concentrations of Cd in soil solution as a result of differential oxidation of sulfide minerals (Chaney et al. 1996). Alternatively, Cd:Fe and Cd:Zn ratios in soil solution may become higher by competitive sorption of Zn over Cd (Davranche & Bollinger 2000). Besides, Fe and Mn oxides and oxyhydroxides precipitate during soil oxidation, decreasing the Fe concentration in soil solution and providing available adsorbent surfaces. Different affinities of adsorbent soil constituents for each metal may result in higher Cd:Zn ratios in soil solution during oxidation (Atkinson et al. 2007; Charlatchka & Cambier 2000; Chuan et al. 1996). Recently, in temperature-controlled “reaction cell” simulating paddy soil conditions the effect of alternating anaerobic and aerobic conditions has been tested in relation to sulfur addition. In normal conditions, the ratios of Cd:Fe and Cd:Zn in solution increases during the aerobic phase while Cd concentrations is unaffected, meaning that the Fe and Zn concentrations decreases. However, in treatments with added sulfur in form of sulfate (SO_4), up to 34 % of sulfur precipitates as sulfide minerals during the anaerobic phase and the Cd:Fe and Cd:Zn ratios in solution during the subsequent aerobic phase is lower than without S addition. This depends on the fact that after supplying S, Cd solubility decreases whereas Fe and Zn are unaffected. Interestingly, when soil is

spiked with Zn, the Cd:Zn ratio decreases even in the aerobic phase, due to higher Zn concentrations resulting in competition effects (de Livera et al. 2011). Even though the real field conditions are likely to be a more complex system, these data show that higher concentrations of SO_4 than those normally applied as well as additional Zn application would likely limit the accumulation of Cd in paddy rice grain.

In order to take into consideration the likelihood of high Cd accumulation in rice grains as well as to meet the market needs, the Codex Alimentarius Commission of the FAO/WHO set the maximum allowable limits of Cd concentration as 0.4 mg kg^{-1} for polished rice, 0.2 mg kg^{-1} for wheat and 0.1 mg kg^{-1} for the other cereal grains (CODEX STAN 193–1995 2009). It is noteworthy that, even though the fixed limit for rice is the highest permitted, still problems due to rice stocks exceeding the maximum Cd concentration are of great concern in terms of food safety, also in soils characterized by low Cd contamination.
Concerning the geographical distribution of Cd contaminated soils and the resulting Cd content in rice, many surveys have reported that rice produced in Japan contained the highest cadmium levels (up to 65 ng/g) among samples collected from several different areas in the world, whereas the lowest observed came from Brazil (2 ng/g) (Rivai et al. 1990; Masironi et al. 1977). However, a considerable variation occurs even in more circumscribed geographical context and, with regard to this, interesting is the case of Java. The rice of West Java has been found to have double the Cd content of rice from Central and East Java (Suzuki et al. 1980), likely dependent on the soil characteristics which, as explained above, have a great impact on Cd availability: the soil type in West Java is *Ultisol*, as opposed to *Vertisol* in East Java. Interestingly, the soil type in Western Japan is also *Ultisol* and this may explain the higher cadmium levels (Rivai et al. 1990).

Focusing on Italy, rice is one of the most economically important cereal crops, in view of foreign exchange. The cultivated rice area has increased since the beginning of the European Community Policy, mainly owing to favourable market conditions for Italian paddy production. Rice cultivation is mostly located in the northern regions (Po Valley) and extends at present over about 240,000 ha (including some small areas cropped in Sardinia island and southern Italy) which represent only 1,4% of the total arable area (16,800,000 ha) (Russo & Callegarin 2007). From an analysis carried out referring to rice production in northern Italy in the two-year period 2006-2007, it has come up that some agricultural lots located in the provinces of Milan, Lodi, Pavia in Lombardia region, as well as in the provinces of Novara and Vercelli in Piemonte region, have yielded rice stocks exceeding the maximum Cd concentration allowed in the grains. In particular, the most critical area

has been found to be the southern province of Milan. Trying to find a connection between Cd concentration in rice grains and Cd content in soils, it has come up that there is no clear correlation whatsoever; this may depend on the fact that Cd in soil is actually subjected to complex dynamics depending on soil characteristics and vegetation effects, so that just a small fraction of Cd in soil is actually bioavailable thus being likely to be taken up by plants. As abovementioned, also agronomic practices can have a big impact in determining Cd availability in soil, making the risk of Cd accumulation in the edible part of the plant hardly predictable (Ente Nazionale Risi 2010).

2.1. Cadmium as a risk for human health

The accumulation of Cd in the edible part of plants may result in Cd-rich foodstuff, constituting a big issue in terms of food safety, as it is through consumption of Cd contaminated products that Cd enters the food chain likely having repercussions on human health.

The present levels of cadmium intake in most European countries are far below the limit recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) which has recently been called to evaluate the tolerable monthly intake (TMI) for cadmium of 25 µg/kg body weight (b.w.) set up in 2010, which corresponds to a weekly intake of 5.8 µg/kg b.w. (EFSA Panel on Contaminants in the Food Chain -CONTAM- 2011). The CONTAM Panel, basing on the current state of knowledge, has actually concluded that for Cd the current tolerable weekly intake (TWI) of 2.5 µg/kg b.w. established in 2009 should be maintained in order to ensure a high level of protection of consumers, including subgroups of the population such as children, vegetarians or people living in highly contaminated areas. However, it has been highlighted that taking non-dietary exposure into account, the total exposure of some subgroups of the population could exceed the CONTAM TWI limit. This means that adverse effects on human health due to Cd are unlikely to occur in an individual with current dietary exposure, but there is a need to reduce exposure to Cd overall at the population level (EFSA-CONTAM- 2011).

Nevertheless, it is important to note that as a result of numerous public health policies implemented over the past several decades, the Cd body burden of the general population appears to be rapidly declining (Friis et al. 1998), confirming that present levels of general population exposure to Cd are unlikely to have adverse health consequences. Existing standards such as the TMI are based upon biological models which associate Cd exposure and increased urinary excretion of low molecular weight proteins, such as creatinine, but it should be noted that the limits gradually set up over the last years have been estimated to occur in humans with a life-long daily intake much higher than the real one (EFSA 2009).

Cd overexposure has been seen to be harmful to human health in that it causes damages mainly to the skeletal system and the kidneys (Bhattacharyya 2009; Kobayashi et al. 2002). The most evident case is the situation occurred in the 1950s and 1960s in Japan where heavy Cd contamination of rice fields, along with nutritional deficiencies for Fe, Zn and other minerals, led to renal impairment and bone disease in exposed populations. In particular, Cd contamination from an upstream mine in the Jinzu River basin of the Toyama Prefecture led to the development of the so-called *Itai-itai* disease, representing the most severe stage of chronic Cd intoxication in many of the inhabitants of this region since the Second World War (Nogawa et al. 1975; Kido et al. 1991). Most *Itai-itai* disease patients were elderly postmenopausal women presenting renal and bone damage. The effect of Cd on human health has then been extensively studied in Cd-exposed experimental animals initially focusing on proteinuria, renal calcium stones and bone fractures, which are reported among persons exposed to high levels of Cd either in industry or via consumption of contaminated foodstuff. Urine cadmium concentrations among these populations are high, in the range of 20–30 µg Cd/g creatinine (Ezaki et al. 2003). More recently, extensive epidemiological studies have been conducted among populations in Belgium, Sweden, China, Japan and the United States to evaluate effects of much lower levels of Cd exposure via environmental routes (Alfvén et al. 2002; Honda et al. 2003; Kazantzis 2004; Järup & Alfvén 2005; Åkesson et al. 2006; Qian et al. 2006; Bernard 2008; Gallagher et al. 2008; Schutte et al. 2008; Engström et al. 2009; Järup & Åkesson 2009). These studies provide repeated demonstrations of longer Cd exposure correlating significantly with decreased bone mineral density (BMD) and increased fracture incidence at lower exposure levels than ever before evaluated. Moreover, recent studies with experimental animals have addressed the question whether very low concentrations of dietary cadmium can negatively impact the skeleton (Brzóska et al. 2005a, 2005b, 2005c, 2007, 2008; Brzóska & Moniuszko-Jakoniuk 2004a, 2004b, 2005a, 2005b). When applied to humans, the reports of Brzóska and collegues have extensive potential implications, in that rats reached somewhat less than their full peak bone mass as adults and experienced more extensive involution of the skeleton late in life when they had dietary cadmium exposures that resulted in end-of-life blood Cd concentrations of 0.79 ng Cd/ml. Many humans worldwide have blood Cd concentrations in this range. The current convergence of data in both humans and experimental animals, even though the question still needs to be fully addressed, highlights that lifetime skeletal development in a large number of humans is potentially being compromised by their lifetime, low-level exposures to Cd.

Same wise, as the main storage organs of Cd are the kidneys as the accumulation of Cd takes place especially in the proximal tubules, disruption in renal functions have also been studied (Bernard 1996; Wu et al. 2001; Buchet et al. 1990). At present, it is possible to detect mild alterations in

kidney functions at lower levels of exposure basing upon biomarkers developed over the last decades (Buchet et al. 1990). Studied markers, usually measured in urine, included kidney derived antigens or enzymes, plasma-derived proteins reflecting the permeability of the glomerular filter or the protein reabsorption capacity of proximal tubules (Bernard 1996; Wu et al. 2001). Among these approaches, the determination of molecular weight proteins (*i.e.* molecular weight <40 kDa) in urine is now recognized as the most useful tool for the early detection of Cd nephropathy. Because in healthy subjects the tubular reabsorption of these small proteins is almost complete (more than 99.9%), a small decrement in the tubular reabsorption capacity produces a marked increase of the urinary excretion of microproteins such as β2-m or RBP which, in most advanced cases of Cd nephropathy, can reach levels more than one thousand times above normal (Bernard 2004).

2.2. How to reduce Cd accumulation?

As abovementioned, the capability of plants to absorb and accumulate Cd depends both on how they are cultivated, *i.e.* the soil management practices (de Livera et al. 2011), and the species itself, whose characteristics may vary among different cultivars (Grant et al. 2008).

2.2.1. Agronomic practices

Concerning the soil management as possible intervention to reduce the likelihood of Cd accumulation by crops, a number of strategies are available to minimize Cd contamination. With this aim, liming is often suggested as a primary management tool. Beneficial effects of liming in reducing plant Cd concentrations have been clearly demonstrated in glasshouse experiments where Cd is added as metal salt in hydroponic conditions or in form of sewage sludge in soil trial (Street et al. 1978; Eriksson 1989). More recently, the same positive results have been confirmed in field experiments, where a decrease in rice plants Cd concentration was observed after lime application (Cattani et al. 2008). This is due to the slight increase in pH which stabilizes Cd adsorbed to soil particles and also to the competition effects deriving from the increase in Ca ions in the soil solution, limiting Cd uptake by the root system. Moreover, it is well documented that lime produces a rise in cation adsorption capacity of soil (Bolan et al. 2003), as well as a precipitation of Cd as $CdCO_3$ (Holm et al. 2003), reducing its bioavailability. The application of organic matter has been reported to have contrasting effect on Cd solubility and consequent uptake: on one hand, in fact, it increases the cation adsorption capacity, providing additional surface Cd ions can be adsorbed to and thus immobilized but, on the other hand, it is also possible that low molecular fractions, such as hydrophilic phases, have a strong affinity towards forming soluble Cd complexes. Cd-Dissolved

Organic Carbon (DOC) complexes, in fact, are more labile in soil and can soon release Cd weakly bound (Grant et al. 1999).

Additions of Zn to soil have been shown to reduce crop Cd concentrations significantly (Cataldo et al. 1983; Abdel-Sabour et al. 1988; McKenna et al. 1993; Moraghan 1993; Chaney et al. 1994; Oliver et al. 1994; Choudhary et al. 1995). This effect has been demonstrated in both common and durum wheat and is particularly well-expressed under conditions of Zn deficiency (Oliver et al. 1994; Choudhary et al. 1995). Another factor deeply affecting Cd availability in soil is the water regime. It has been shown that submersion is very useful to reduce Cd concentration in rice, compared with dry sowing. This is likely to be due to the redox potential stable around -400 mV under flooded conditions and fluctuating between +300 and -400 mV under dry conditions: the redox potential value in submersion cause the sulfate ions to reduce to sulfide ions that may form complexes with Cd ions, immobilizing them as insoluble salts (Cattani et al. 2008; Gimeno-Garcìa et al. 1996). The effect of alternating submersion and drainage, especially in correspondence of the grain-filling stage has already been mentioned; therefore, where water shortage is not an issue, flooded conditions are desirable in order to reduce Cd accumulation in rice grains.

Soil dressing techniques have also been taken into consideration but they are often hard to implement because of their high cost and difficulty in obtaining unpolluted soil. Same wise, electronic thermodynamic remediation and on-site soil washing/clean up techniques could be interesting in terms of efficiency but there are some factors to take into consideration, such as (1) selection of chemicals that have high effectiveness but also low environmental impact in that they could result in destruction of the physicochemical properties of soils and in secondary pollution of soil and groundwater, (2) development of an on-site washing and wastewater-treatment system, (3) ensuring favorable post-washing soil fertility and plant growth and (4) maintenance of the washing effect (Makino et al. 2008; Murakami et al. 2007; Mulligan et al. 2001). Taken together, these observations underline how interventions on soil are not always feasible nor cost-effective, thus do not solve the problem of Cd accumulation in plants grown especially on low contaminated soils.

2.2.2. Breeding and selection of low Cd crops

More promising is the opportunity to utilize plant breeding to select for genetically low-Cd concentration rice cultivars, taking advantage from the well documented broad variability in the Cd accumulation trait observed in Indica and Japonica cultivars, as well as in hybrids (Morishita et al. 1987; Arao & Ae 2001; Arao & Ishikawa 2006; Liu et al. 2007). This genotypic variation provides valuable information that may be used to analyze the physiological and genetic aspects of the low Cd accumulation phenotype which is essential to include in breeding strategies. Indeed, as things

standing now, in the absence of selection for the low-Cd trait, new cultivars may be randomly either higher or lower than traditional cultivars, determining no progress in Cd accumulation prevention.

In any cultivar selection process, after identifying the natural variation with respect of a particular trait, the following step is to find the inheritance and heritability of the genetic character.

Concerning low-Cd accumulation trait in rice, the first progresses in this direction have been made by Ishikawa and coworkers (2005): on the basis of a novel mapping population consisting of 39 chromosome segment substitution lines (CSSLs), Quantitative Trait Loci (QTL) for grain (brown rice) Cd concentration were detected on chromosomes 3, 6 and 8, the last being found to have a minor effect. By using a similar approach with Kasalath/Nipponbare backcross inbred lines, three additional different QTLs for Cd concentration, two on chromosome 4 and one on chromosome 11, have been also reported (Kashiwagi et al. 2009). A major QTL controlling shoot Cd concentration has also been detected on chromosome 11 in a population derived from two contrasting rice cultivars: Badari Dhan and Shwe War (Ueno et al. 2009a). More recently, a major QTL for Cd accumulation has been found on the short arm of chromosome 7 in a population derived from a low Cd-accumulating cultivar, Nipponbare, and a high Cd-accumulating cultivar, Anjana Dhan (Ueno et al. 2009b). This has been then identified as OsHMA3, a vacuolar transporter belonging to the P_{1B}-type ATPase family, functioning as a "firewall" to limit Cd translocation by sequestering it into the vacuole (Ueno et al. 2010a).

These findings constitute the preliminary step to include low-Cd trait in the selection breeding strategy for the release of rice varieties able to exclude Cd from the grains. However, it should be pointed out that the process is still long ahead, considering that low-Cd trait must be incorporated into a cultivar that has acceptable characteristics for yield, agronomic suitability, quality, disease resistance, herbicide tolerance and drought resistance, that are already listed in pre-existing selection programs (Grant et al. 2008). Also, it should be noted that even though selection can produce low-Cd cultivars, the Cd concentration of both low- and high-Cd cultivars will be influenced by both soil and management practices, as reported above (Grant et al. 1999). Therefore, it is through the combination of management practices that limit Cd accumulation with the use of low-Cd cultivars that the best results can be obtained in reducing Cd entrance into the food chain. Moreover, still the risk of long-term accumulation of phytoavailable Cd in agricultural soils that would increase the Cd concentration in both low- and high-Cd cultivars should be considered (Grant et al. 2008).

2.3. Phytoextraction: a potentially valuable remediation technique

The high genetic variability both in different species and in different cultivars within the same species can be also exploited for aims other than preventing Cd accumulation in the edible part of commercially grown crops. Indeed, since as mentioned earlier on on-site techniques are often not feasible because of high costs and low efficiency in low or moderately Cd contaminated soils, use of plants able to accumulate and thereby remove high quantities of Cd from soil is a valid alternative. With regard to this, phytoextraction has been proposed as a promising technique for decontaminating soil characterized by low level of Cd contamination. It basically consists in a cost-effective, environmentally friendly green technology that utilizes the capacity of hyperaccumulator plants to extract heavy metals from soil (Krämer 2005; McGrath et al. 2006; Pilon-Smits 2005). 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). Only *Alyssum*, a hyperaccumulator species used for Ni phytoremediation has been developed into a commercial technology (Chaney et al. 2007). Therefore, most of the hyperaccumulators tested so far cannot be unequivocally considered as commercially viable for phytoremediation (Robinson et al. 1998). However, among the Cd hyperaccumulators, *S. nigrum* L., *Populus* spp, *Salix'calodendron'* and *Arabis paniculata* (Wei et al. 2005; French et al. 2006; Maxted et al. 2007), have been found to be valuable candidates for field conditions due to their potentially high biomass, which, along with accumulation capacity and growth rate are the main determinants of phytoextraction process (Salt et al. 1998). Other plants commonly known to be able to accumulate high metal concentrations in the shoots, as *T. caerulescens* and *B. juncea*, might not be suitable for large-scale phytoextraction, the former for being easily infected by diseases whose development is favored by humid and warm weather conditions (McGrath et al. 2000), the latter for its slow growth and the difficulty of mechanical harvesting, which is an issue also for other hyperaccumulator plants (Ebbs et al. 1997; Ishikawa et al. 2006). The natural ability of plant to hyperaccumulate metal in their aerial portion can be enhanced by supplying a synthetic chelating agent to soil as, for instance, ehtylenediaminetetraacetc acid (EDTA) which has been found to significantly increase Pb accumulation (Huang & Cunningham 1996; Huang et al. 1997) but is also applicable to other metals, such as Cd (Blaylock et al. 1997). The use of chelating agents to enhance metal removal from soil by plants is also known as induced phytoextraction and its potential application is still under investigation, as the mechanisms of uptake, xylem loading and translocation of metal-chelate complexes are not fully understood (Salt et al. 1998). Furthermore, it is useful to underline that the success of phytoextraction processes also depends on the application of good agronomic practices and management as water regime, application of amendments and tillage, just to mention some

(Keller et al. 2003; McGrath et al. 2006). Moreover, to optimize the overall application of this technique it is important that the whole system is sustainable in terms of costs, suitability of the chosen plant with respect of the context and cultural practices required for plant growth and harvest. Indeed, factors as fertilization, water management and mechanical planting as well as harvesting, are well established for rice plants and, as already mentioned, there is a wide variability in the Cd accumulation trait in Indica and Japonica cultivars, as well as in hybrids (Morishita et al. 1987; Arao & Ae 2001; Arao & Ishikawa 2006; Liu et al. 2007). These features along with the considerably high biomass of rice plants and the possibility of double cropping and sequential harvesting make rice highly suitable for being implemented in phytoextraction techniques, especially if combined with sound water management regime, *i.e.* early drainage (Ibaraki et al. 2009).

In conclusion, understanding the molecular and genetic basis as well as the physiologic processes involved in Cd detoxification mechanisms would be an important aspect for developing plants able to prevent Cd translocation to the edible parts in order to ensure food safety, but also for exploring their potential use as agents in the phytoremediation of contaminated sites.

Section II

P_{1B}-type ATPase, a class of transporters with a major role in Cd movement throughout the plant

1. Biology, structure and mechanism of 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 *Arabidospsis thaliana* 46 transporters belonging to this class have been identified, pointing out their importance in vascular plant (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).

1.1. Substrate specificity

Type-1 ATPases. Type-1 ATPases are further divided into two subclasses: type-1A and type-1B. Type 1A is a small sub-class that contains bacterial ion pumps, with the *E. coli* Kdp K^+-pump being the best known and characterized. Whereas most P_{1A}-type ATPases translocate cations as H^+, Na^+, K^+,Ca^{2+} and Mg^{2+}, the substrates of P_{1B}-type ATPases are transition metal ions, as exhaustively discussed later on in this paper. Members of this class are, for instance, the bacterial metal-resistance proteins CopA (Rensing et al. 2000), ZntA (Okkeri & 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 (Rosen 2002; Nelson 1999). Close homologues of the P_{1B}-type ATPases that have been extensively studied in bacteria have been found in *S. cerevisiae* (Goffeau 1998), plants (Axelsen & Palmgren 1998) and animals (Lutsenko & Petris 2003).

Type-4 and -5 ATPases. The closest relatives of the type-1 enzymes are types 4 and 5. Type-4 ATPases have so far been found only in eukaryotes, in which they are involved in lipid transport and the maintenance of lipid-bilayer asymmetry. Sequence comparisons show that they present the main features of the ion-translocating P-type ATPases (Axelsen & Palmgren 1998), including the ion-binding site in the membrane. Type-5 ATPases, instead, have recently emerged in the

eukaryotic genome as a separate class and their substrate specificity as well as their biological roles are still unknown.

Type-2 and -3 ATPases. The most-investigated members of the P-type ATPase family are those that create and maintain the membrane potential in animal and plant cells, which results from the often significantly different ion concentrations on either side of the membrane. Almost everything that is known about P-type ATPases derives from the main representative transporters of these two classes. Type-2 ATPases are the most diverse. This class comprises the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (Lee 2002; Stokes & Green 2003), which has become the archetype of the P-type ATPase family, because its atomic structure is the only one so far to have been determined experimentally (Toyoshima et al. 2000; Toyoshima & Nomura 2002). Also, it includes the well-characterized Na^{+}/K^{+}-ATPase. Type-3 ATPases are H^{+}-pumps present almost exclusively in fungi and plants and are responsible for the maintenance of the proton potential across membranes (Jorgensen et al. 2003).

1.2. P_{1B}- type ATPases

P_{1B}-ATPases, also known as Heavy Metal Associated (HMA) ATPases, are a subgroup of P-ATPases that transport heavy metals (Cu^{+}, Cu^{2+}, Zn^{2+}, Co^{2+}) across biological membranes (Lutsenko & Kaplan 1995; Solioz & Vulpe 1996; Axelsen & Palmgren 1998; Rensing et al. 1999; Argüello 2003; Williams & 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.

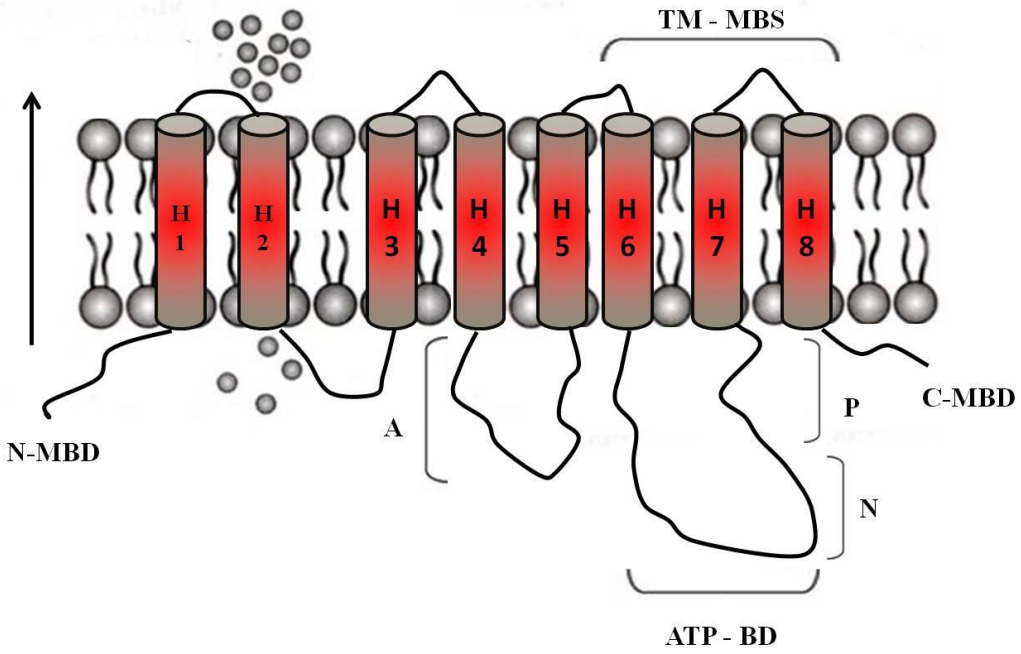

Fig. 2 : Schematic illustration of the topology and main domains present in P_{1B}-ATPases. Transmembrane segments, H1 to H8, are indicated. The relative locations of the cytoplasmic actuator (A), phosphorylation (P) and nucleotide (N) domains are shown, as well as the ATP binding domain (ATP-BD). The 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).

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 (Fig. 2). The described topology differentiate P_{1B}-ATPases from other subfamilies that contain ten (type-2 and -3) or twelve (type-4 and -5) TMs (Lutsenko & Kaplan 1995; Axelsen & Palmgren 1998). P_{1B}-ATPases also present a particular distribution of TMs with respect to the large cytoplasmic loop forming the ATP-BD. While P_{1B}-ATPases have two TMs on the C-terminal end of the ATP-BD, other P-ATPases have either four (type-1A) or six (type -2, -3, -4 and -5). However, in spite of the indicated differences, a common pattern is evident 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 (Argüello et al. 2007).

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 first available sequences indicate that P_{1B}-ATPases have a CPC sequence in the center of their sixth

transmembrane domain (H6). However, although the CPC sequences are the most frequently observed, a current analysis of the available genomes reveals the presence of 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.

Enzyme phosphorylation by ATP, subsequent turnover and transport, require metal binding to the TM-MBS. This is independent of metal binding to the N- and C-MBDs, since truncated ATPases lacking these domains or proteins carrying mutations yielding N-MBDs unable to bind metals are same wise phosphorylated and transport metals (Voskoboinik et al. 1999; Bal et al. 2001; Mitra & Sharma 2001; Fan & Rosen 2002; Mana-Capelli et al. 2003; Mandal & Argüello 2003).

Metals activate P_{1B}-ATPases with apparent affinities ($K_{1/2}$) in the 0.1–3 μM range (Okkeri & Haltia 1999; Voskoboinik et al. 1999; Sharma et al. 2000; Fan & Rosen 2002; Mandal et al. 2002; Tsivkovskii et al. 2002; Mana-Capelli et al. 2003; Eren & Argüello 2004). Most of these assays have been performed in the presence of various metal ligands such as DTT, Cys and ATP (Sharma et al. 2000; Fan & Rosen 2002; Mandal et al. 2002; Eren & Argüello 2004). Therefore, these $K_{1/2}$ values do not refer to free metal concentrations but to the total metal in the media. Considering the low dissociation constants for the soluble metal complexes (metal-thiolate, metal-chaperone, metal-ATP) (Martell & 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 domains 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).

Recently, more insight on the function of the A-domain has been given by the study of the isolated ATP-BD and A-domain of AfCopA, a Cu^+ transporter in *Archeoglobus fulgidus*. In spite of their small sequence homology (26%), the folding of AfCopA is highly similar to that of the A-domain

of SR Ca^{2+}-ATPase (Toyoshima et al. 2000; Toyoshima & Inesi 2004; Toyoshima et al. 2004). In particular, the presence of the highly conserved (S/T)GE(P/S) sequence on the side of the A-domain appears similar. 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 & Nomura 2002; Olesen et al. 2004; Toyoshima & 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 with a highly conserved CysXXCys 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. 2005a). Concerning Cu pumps, *in vivo*, N-MBDs receive Cu^{+} from the corresponding Cu-chaperones (Hamza et al. 1999; Larin et al. 1999; Huffman & O'Halloran 2000; Wernimont et al. 2000). The metal selectivity of this kind of pumps appears to be determined by the specific N-MBD-chaperone interaction via electrostatic and hydrophobic relations that align the metal binding sites and allow rapid ligand exchange (Wernimont et al. 2000; Arnesano et al. 2002). This metal exchange has not been established for Zn^{2+}-ATPases carrying CysXXCys containing N-MBDs, since no Zn-chaperone (or equivalent molecule) has been identified yet. Banci and co-workers (2002) have shown that besides the conserved CysXXCys sequences in N-MBD, metal coordination in bacterial Zn^{2+} N-MBD is accomplished with the participation of a carboxyl group from a Glu or, preferably, an Asp residue which is likely to be located prior to the conserved Cys residues (AspCysXXCys).

On the other hand, all eukaryote (plant) Zn^{2+}-ATPases possess a unique conserved CysCysXXGlu sequence in their N-term that appears to provide a novel metal coordination environment.

Previous studies indicate, in fact, that plant Zn^{2+}-ATPases N-MBDs coordinate both Zn^{2+} and Cd^{2+} with two conserved Cys and a Glu. Therefore, whereas the carboxyl group in bacterial Zn^{2+}-ATPases is provided by an Asp residue, in eukaryote proteins is usually given by a Glu residue. This is that the unique Zn^{2+} coordination via two thiols and a carboxyl group provides selective binding of the activating metals to these regulatory domains and it is possible to argue that, since the aminoacidic sequence is different, the specificity for the metal to be transported is actually dependent on the rearrangement of the residues (Eren et al. 2007). Homology modeling and

structural comparison by circular dichroism indicate, in fact, that the 75 amino acid long AtHMA2 N-term shares the βαββα folding present in most P_{1B}-type ATPase N-terminal metal binding domains (N-MBDs), including bacterial ones. This modeling also showed that the arrangement of metal binding sequences CysCysXXGlu within HMA2 N-MBD is similar to that of CysXXCys present in the homologous domains of other P_{1B}-type ATPases and Cu^{+}-chaperones. That is they might control enzyme turnover through similar intramolecular domain-domain interactions driven by metal binding. Concerning the functional role of the N-MBD, it has been observed in studies conducted in Arabidopsis on both ΔN-HMA2 and HMA2 carrying mutations in the metal coordinating residues Cys17, Cys18, and Glu21 that the structural modifications resulted in a 50% reduction of in the enzymes V_{max} without significantly altering the metal dependence for activity. Therefore, although the N-MBD is required for maximum enzyme turnover rate, it would not influence the metal binding to TMBDs and the resulting transport selectivity. In other words, it might not be essential for the transport of the metal but play 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. The putative C-MBD from *A. thaliana* HMA3 and its homologs have numerous Cys but no (or few) His residues. Alternatively, HMA2, HMA4 and homologous proteins have His- and Cys-rich C-MBD (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. In particular, the removal of the 244 amino acid C-term of AtHMA2 led to a 43% reduction of the enzyme turnover, having no significant effect on the Zn^{2+} $K_{½}$ for enzyme activation. This means that probably the C-term is required for the maximum turnover rate but it does not affect the interaction of metal with the transport sites, as already postulated for N-MBDs in Cu^{+}-ATPases (Mandal & Argüello 2003), Cu^{2+}-ATPases (Mana-Capelli et al. 2003) and Zn^{2+}-ATPase (Mitra & Sharma 2001). A part from the kinetics of the enzyme, circular dichroism analysis showed that the Zn binding led to a conformational changes in the C-MBD which probably is related somehow to the interaction of this domain with the cytoplasmic A, P and N domains in the ATP hydrolysis and energy transduction in the P-type ATPases (Toyoshima & Inesi 2004; Mana-Capelli et al. 2003). Moreover, the Zn binding sites seemed more likely to be constituted by the His residues more than the Cys residues even though the stoichiometry of the

binding is still to be fully clarified (Eren et al. 2006; Wong et al. 2009). The importance of His residues in metal coordination has also been observed in AtHMA4, which presents an eleven His stretch at its C-term. The deletion of this stretch clearly caused the transporter not to be functional, pointing out the importance of it in the transport of the metal and/or in the regulation of the enzyme. Therefore, it is possible to postulate a role in binding the metal which is then delivered to the active transport site, which is also supported by studies conducted on AtHMA4 (Mills et al. 2005) and TcHMA4 whose soluble C-term induced increased tolerance and accumulation of various metals, including Cd, in yeast (Papoyan & Kochian 2004; Bernard et al. 2004). Such a model of a "self-chaperone" has also been suggested for the His-rich domain of the Cu/Zn superoxide dismutase of *Haemophilus* (Battistoni et al. 2001). However, for an exhaustive understanding of the role of both C- and N- term still a biochemical characterization of these segments has to be carried out (Verret et al. 2005).

1.3. Catalytic mechanism

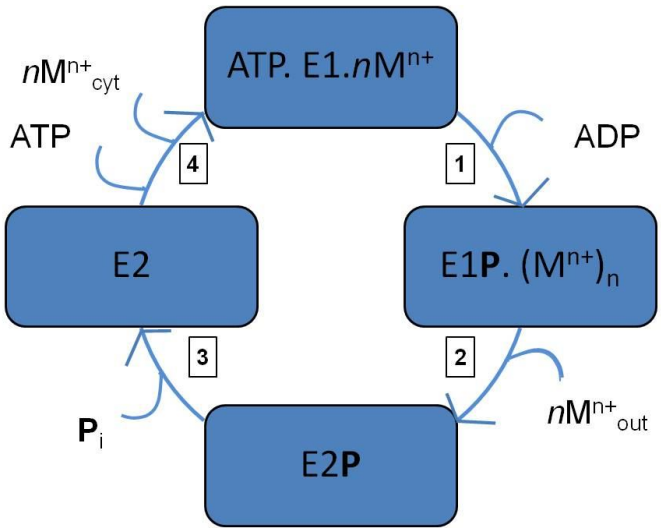

Fig. 3 : P_{1B}-ATPases catalytic cycle. E1, E2, E1P and E2P represent the basic conformations that the enzyme can assume. Mn^+ represents a metal transported by these enzymes. n indicates the uncertainty on the specific stoichiometry of transport. Mn^+_{cyt} and Mn^+_{out} represents the cytoplasmic or extracellular/luminal localization of the transported metal (adapted from Argüello et al. 2007).

P_{1B}-ATPases transport metals across membranes following the classical E1/E2 Albers-Post catalytical cycle (Fig. 3). This transport mechanism has been studied in detail especially in P_2-ATPases (Na^+/K^+-, Ca^{2+}-, and H^+/K^+-ATPases) (MacLennan et al. 1997; Kaplan 2002). Basically,

in the E1 state, the metal ion (M) binds to its high-affinity site in the TM-MBD, which is accessible from the cytoplasm. It is the binding of the metal itself that causes the P-domain to move into the E1 conformation. As a result of this conformational change, the key Asp residue within the P-domain can be phosphorylated by the Mg^{2+}-ATP, which is delivered to the phosphorylation site by the N-domain. In the E1-P state, the Asp is phosphorylated and it is then able to transfer the phosphoryl group back to an ADP molecule. In the rate-limiting E1-P to E2-P transition phase, the P-domain reorientates from its E1 to its E2 position, while the A-domain rotates to get in contact with the phosphorylation site with the apparent function of protecting the phosphoryl group against hydrolysis. At the same time, the ADP dissociates. The A-domain rotation is also involved in the shutting off of the cytoplasmic ion-access channel, which prevents the binding of additional metal ions. The P-domain movement then disrupts the high-affinity metal ion binding site so that the ion is released to the outside (extracellular/luminal side) through an exit channel. The ion-binding site is now available to bind a proton (H^+) from the outside for which it has a high affinity. The hydrolysis of the phosphorylated Asp results in the E2 state. Mg^{2+} and inorganic phosphate (P_i) dissociate so that 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 the main electrogenic P-type 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. The amount of fungal H^+-pump in the plasma membrane, for instance, is regulated at the level of gene expression (Portillo 2000). P-type ATPases of types -1B, -2B and -3 are regulated by domains that are situated within to the main chain of the enzyme. Well-documented examples include the plasma-membrane Ca^{2+}-ATPases in animals (Carafoli 1994) and, more recently, plants which have been found to have amino- or carboxy-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 & Rosen 2002; Mana-Capelli et al. 2003; Eren & 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 (E1-P state), for most P_{1B}-type ATPases the correct stoichiometry of the transport has not been established yet. Recently Mitra and coworkers 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 (Liu et al. 2005a). 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.

1.4. Distribution and physiological roles of P_{1B}-type ATPases

As no other P-ATPases subfamilies, P_{1B}-type ATPases are present in all life kingdoms. Due to their stability and diversity in terms of transported substrates, these proteins can be considered an important resource to explore and develop interesting and useful biotechnological applications (Argüello et al. 2007).

P_{1B}-type ATPases were first identified in bacteria like *Staphylococcus aureus* plasmid pI258 (Nucifora et al. 1989), *Rhizobium meliloti* (Kahn et al. 1989), *Escherichia coli* (Rensing et al. 2000), *Enterococcus hirae* (Odermatt et al. 1993) and *Synechococcus PCC 6803* (Tottey et al. 2001). In these organisms, their function is to 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. Cu^{+}-ATPases, Zn^{2+}-ATPases and a Co^{2+}-ATPase have been first identified and partially characterized in bacteria. As mentioned above, functional and biochemical assays showed that these ATPases can also transport non-physiological substrates. For instance, Cu^{+}-ATPases also transport Ag^{+} (Fan & Rosen 2002; Rensing et al. 2000; Solioz & Odermatt 1995). Similarly, Zn^{2+}- ATPases can transport Cd^{2+} and Pb^{2+} (Rensing et al. 1997; Sharma et al. 2000; Tsai & Linet 1993). Same wise, also archeal P_{1B}-type ATPases present a notable variety of substrate specificities (Argüello 2003; Baker-Austin et al. 2005; Mana-Capelli et al. 2003; Mandal et al. 2002). However, archeal P_{1B}-type ATPases, when compared to bacterial P_{1B}-type ATPases have characterizing features that make them deeply different, due to the extremophilic conditions in which their hosting organisms have gradually adapted to. For instance, increased Cu^{+}-ATPase transcript levels have been observed in *Ferroplasma acidarmanus*, an organism that tolerates Cu concentration up to 20 g/l (Baker-Austin et al. 2005). Another 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 (Mana-Capelli et al. 2003; Mandal et al. 2002). 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 & Cox 1994; Bull et al. 1993; Lutsenko et al. 2003; Vulpe et al. 1993).

MNKP and WNDP mutant proteins manifest distinct phenotypes due to their differential expression patterns in human tissues. MNKP is expressed in almost all the cells except the hepatic cells. Mutations in MNKP lead to poor Cu uptake from the intestine resulting in severe neurological disorders and connective tissue abnormalities. WNDP is instead mainly expressed in hepatocytes and mutations in this ATPase result in high Cu levels in the liver, blood and brain causing neurological disorders and cirrhosis. In the cell, both proteins are localized in a trans-Golgi compartment and undergo Cu-dependent trafficking (Hung et al. 1997; Petris et al. 1996). As in the case of SR Ca^{2+}-ATPase, studies on these proteins have considerably contributed to the understanding of P_{1B}-type ATPase functions.

Plants significantly differ from other organisms both in number and selectivity of their P_{1B}-type ATPases. So far, several studies have been conducted in the model plant Arabidopsis that have led to a classification of these transporters. More recently, the focus on P_{1B}-type ATPases has also been extended to rice (*Oryza sativa*).

From evolution studies and phylogenetic analysis it emerged that higher plants evolved with a relatively high number of P_{1B}-type 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}-type 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 & Mills 2005). Preliminary phylogenetic analysis enabled to identify the relationships between P_{1B}-type ATPase of different plant species, as shown in the dendrogram reported in Fig. 4.

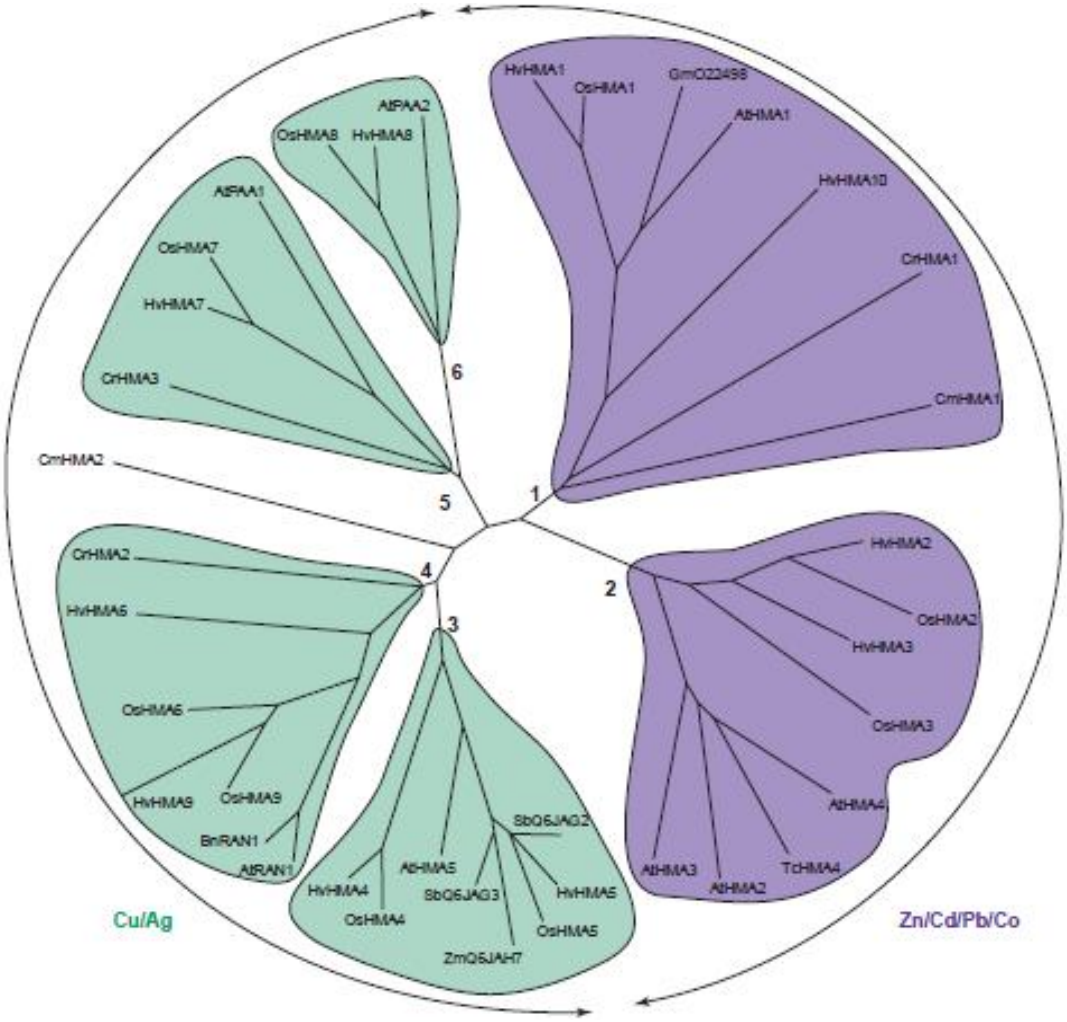

Fig. 4 : Dendrogram showing families of P_{1B}-ATPases in Arabidopsis, rice, barley and the algae *Chlamydomonas reinhardtii* and *Cyanidioschyzon merolae*. Six clusters (1–6) can be identified (highlighted in green or purple) that contain Arabidopsis, rice and barley sequences, suggesting that the angiosperm ancestor of these species had at least six P_{1B}-ATPases (Baxter et al. 2003). Sequences from maize, soybean, sorghum and *T. caerulescens* are included. Based on genetic and functional studies of the Arabidopsis P_{1B}-ATPases, pumps in cluster 2 (and possibly cluster 1) are predicted to be capable of transporting one or more of the group Zn, Cd, Pb Co (highlighted in purple), whereas pumps in clusters 3–6 (highlighted in green) could transport Cu and potentially Ag. The dendrogram was constructed using Clustal W 1.82 (Chenna et al. 2003). Accession numbers for Arabidopsis are: AtHMA4, O64474; AtHMA2, Q9SZW4; AtHMA3, Q9SZW5; AtHMA1, Q5JZZ1; AtHMA5, Q9SH30; AtPAA1, Q9SZC9; AtRAN1, Q9S7J8; AtPAA2, Q7YO51. Accession numbers for Oryza sativa are OsHMA1, PlantsT 64489; OsHMA2, PlantsT 64494; OsHMA3, PlantsT 64499; OsHMA4, amended version of PlantsT 64504; OsHMA5, PlantsT 64509; OsHMA6, BAD25508.1; OsHMA7, PlantsT 64519; OsHMA8, PlantsT 64524; OsHMA9, supplementary data from (Baxter et al. 2003). Accession number for *Thlaspi caerulescens* TcHMA4, Q70LF4. BnRAN1, Q941L1. Accession numbers for *Glycine max*, *Zea mays* and *Sorghum bicolor* are as shown on the dendrogram. The algal sequences used were obtained from the JGI (Joint Genome Initiative), C. reinhardtii database, or the C. merolae genome project. Accession numbers for *C. reinhardtii*: CrHMA1, C_10220; CrHMA2, C_650007; CrHMA3, C_260147; CmHMA1, CMS330C; CmHMA2, CMP215C. Preliminary analysis for barley has identified partial P_{1B}-ATPase sequences in the Plant Genome DataBase . Barley P_{1B}-ATPases have been named HvHMA1-HvHMA10, according to their nearest rice relatives; the additional sequence (a possible second HMA1 homologue) is referred to as HvHMA10 (adapted from Williams & Mills 2005).

So far, eight genes have been found in dicot Arabidopsis (*AtHMA1* to *AtHMA5*, *AtHMA6/PAA1*, *AtHMA7/RAN1* and *AtHMA8/PAA2*), nine in the monocot Oryza sativa (*OsHMA1–OsHMA9*) and ten in *Hordeum vulgare*, another monocot (*HvHMA1–HvHMA10*). The green alga, *Chlamydomonas reinhardtii*, and the red alga, *Cyanidioschizon merolae*, are also included in the dendrogam which shows that they contain fewer P_{1B}-ATPases in their genomes than do higher plants. It is interesting to note that six clusters can be clearly identified, containing Arabidopsis, rice and barley sequences. This mean 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}-type ATPases within the same clusters 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 draw some conclusions on the metal specificity of these pumps which have been divided into two big groups, either transporting Cu/Ag (clusters 3 - 6), or alternatively Zn/Cd/Co/Pb (clusters 1-2) (Williams & Mills 2005).

1.4.1. P_{1B}-type ATPases in Arabidopsis

1.4.1.1. AtHMA6 and AtHMA8

AtHMA6/PAA1 (the first plant P_{1B}-type ATPase to be identified) and AtHMA8/PAA2 are closely related to each other and their characterization has paved the way to study the function and the physiological role of P_{1B}-type ATPases in plants. They are both involved in copper delivery to Cu-requiring proteins in the chloroplast. In particular, AtHMA6/PAA1 transports Cu across the plastid envelope for incorporation in the stromal enzyme Cu/Zn superoxide dismutase (AtCSD2). It is present in both roots and shoots, so it is reasonable to suppose it functions both in green end non-green plastids. AtHMA8/PAA2, instead, is responsible for the transport of Cu into the thylakoid lumen to supply plastocyanin, a Cu-requiring protein involved in the electron transport chain. It was found only in shoots, which suggests a role exclusively in the thylacoid membrane of photosynthetically active chloroplasts. Studies conducted on double mutants *paa1 paa2* provided evidence of a seedling-lethal phenotype: the most straightforward explanation for this is a very severe inhibition of Cu delivery to plastocyanin, which is the only protein that can accept electrons from the cytochrome b6f complex in vitro (Molina Heredia et al. 2003).

AtHMA6/PAA1 and AtHMA8/PAA2 can be considered functional homologues of, respectively, CtaA and PacS, two P_{1B}-type ATPases in *Synechocystis*, a cyanobacterium that shares ancestry with chloroplasts. Similarly as in Arabidopsis, CtaA and PacS are involved in the transport of Cu to plastocyanin: in particular, CtaA takes Cu into the cell and PacS transports it across the thyloacoid

membrane. From CtaA to PacS Cu is trafficked by Atx1, a metallochaperone. Due to the homology these transporters present with AtHMA6 and AtHMA8, it is reasonable to raise the question whether in Arabidospis there is an interaction between the Cu transporting P_{1B}-type ATPase and metallochaperones. So far, one chloroplast metallochaperone has been identified, AtCCS, putatively involved in the supply of Cu to AtCSD2, but there is no evidence yet that it also interacts with chloroplast P_{1B}-type ATPases. A study conducted on AtHMA5, which belongs to the Cu/Ag, group revealed an interaction between the transporter and an Atx1-like Cu chaperone in yeast two-hybrids experiments. However, this result has not been confirmed yet *in planta* even though it is reasonable to suppose that the mechanism of interaction would be similar, since metal uptake and trafficking systems are conserved in plants and other organisms (Abdel-Ghany et al. 2005; Shikanai et al. 2003).

1.4.1.2. AtHMA1

Closely related to AtHMA6/PAA1 in terms of functions is AtHMA1, identified by means of proteomic approach specifically targeted to the Arabidopsis chloroplast envelope. Transient expression in Arabidopsis leaves and cultured cells and Western blot analyses performed on subplastidial fractions provided evidence for chloroplast envelope localization. From sequence analysis, HMA1 lacks the N-terminal HMA regulatory domain usually found in P_{1B}-ATPases. Instead, it has a long His stretch reminding the N-terminal domain of the P_{1B}-ATPase Bxa1 from *Oscillatoria brevis* that was described as transporting both copper and zinc (Tong et al. 2002). It is interesting to note that OsHMA1 in rice shares the same structural properties (Baxter et al. 2003) and is similarly predicted to be chloroplast located by Predotar (Small et al. 2004), even though there is no evidence yet. Moreover the green alga *Chlamydomonas reinhardtii* displays two putative organelle-targeted P_{1B}-ATPases, CrHMA1 and CrHMA3, that are respectively orthologues of HMA1 and PAA1/PAA2 (Hanikenne et al. 2005), suggesting that the presence of two subgroups of copper transporting P_{1B}-ATPases is necessary even in this phylogenetically distant photosynthetic eukaryote. Heterologous expression of AtHMA1 in yeast revealed its involvement in Cu homeostasis, whereas the deletion of the N-term His domain partially affected the metal transport, resulting in a higher sensitivity to high Cu concentration. Experiments conducted on *hma1* Arabidopsis mutant showed a lower total chloroplast superoxide dismutase activity and a photosensitivity phenotype under adverse light conditions (high light). According to the results gained so far, it is possible to argue whether AtHMA6/PAA1 and AtHMA1 have partially overlapping functions as they are both involved in importing Cu in the chloroplast envelope to incorporate in the stromal enzyme superoxide dismutase. P_{1B}-ATPases are generally specific to

either monovalent or divalent cations. Since Cu can be found both as a monovalent and divalent ion, the apparent redundancy between AtHMA1 and AtHMA6/PAA1 at the chloroplast envelope could hide their specific role in the transport of divalent and monovalent Cu ions. In order to ascertain this hypothesis, further biochemical work is needed (Seigneurin-Berny et al. 2006)

1.4.1.3. AtHMA7

AtHMA7/RAN1 is the first P_{1B}-type to be functionally characterized in plants and it is also involved in Cu transport. In particular, AtHMA7/RAN1 plays an important role in the ethylene signaling pathway. The hypothesis that this pathway was somehow dependent on metal transport dates back to a study conducted by Burg and Burg in 1967 when it was arisen the question whether ethylene receptor(s) may be liganded to a transition metal, given the positive correlation between the effectiveness of ethylene and related agonists (such as CO) and their capacity to interact with metals. Further evidence of this came also from spectroscopic and X-ray diffraction analysis and, more recently, from *in vitro* association studies of copper ions with the ethylene-binding domain of ETR1 purified from yeast. The function of AtHMA7/RAN1 was elucidated by a genetic approach, first identifying a novel mutant *ran1* (responsive-to-antagonist1) from a genetic screen for mutations that alter the sensitivity to ethylene. Seedlings isolated were those that showed the ethylene-induced triple response phenotypes after treatment with the receptor-antagonist *trans*-cycloctene (TCO). As TCO is a highly competitive inhibitor of ethylene binding to its receptors, all the mutants identified should have altered or relaxed ligand specificity. The involvement of AtHMA7/RAN1 in Cu transport is proved by the fact that addition of copper ions to *ran1* plants was sufficient to revert the TCO-dependent ethylene dependent phenotype of the mutants. Moreover, the cloning of AtHMA7/RAN1 revealed that this gene encodes a protein with high similarity to Cu-transporting P_{1B}-type ATPases, including the human Menkes/Wilson disease proteins, respectively ATP7A and ATP7B, and *S. cerevisiae* CCC2. With regard to this, expression of RAN1 in yeast complemented the defect of a *ccc2* deletion mutation indicating that RAN1 possesses Cu-transporting activity. Therefore, by analogy with CCC2, AtHMA7/RAN1 is proposed to transport Cu, which is delivered by a cytoplasmic Atx1-like metallochaperone, into a post-Golgi compartment. Then, Cu is incorporated into the membrane-targeted ethylene receptor apoprotein ATR1, allowing it to coordinate ethylene. However, the sub-cellular localization of AtHMA7/RAN1 is still to be confirmed, as any possible interaction with metallochaperones.

More recent studies showed that AtHMA7/RAN1 has also other functions: the Arabidopsis double mutant *ran1-3* showed a rosette-lethal phenotype which might be caused by the disruption of Cu delivery mechanisms to an unidentified Cu-containing enzyme, likely involved in cell expansion.

Moreover, AtHMA7/RAN1, as well as the Cu chaperone AtCCH1, has been observed to be up-regulated during senescence so it is likely to be implicated in Cu mobilization from the leaves during this process (Hirayama et al. 1999; Woeste & Kieber 2000).

1.4.1.4. AtHMA5

Analysis of the membrane topology of AtHMA5 show that, apart from the main signatures of P-type ATPases, it conserves the specific transmembrane metal-binding residues that have been described as probably being implicated in Cu coordination during transport in CopA (Mandal et al. 2004). In HMA5 these residues correspond to Cys617 and Cys619 (at the sixth transmembrane domain), Tyr930 and Asn931 (at the seventh transmembrane domain) and Met959 and Ser963 (at the eighth transmembrane domain). *HMA5* results mostly expressed in roots and flowers, although a very low level of expression is also found in above-ground plant tissues. Interestingly, the addition of $CuSO_4$ at different concentrations to the growth medium results in a dose-independent induction of *HMA5* which is not observed in case of exposure to other metals assayed, as Fe, Ag and Zn, suggesting *HMA5* mRNA levels in Arabidopsis are specifically regulated by Cu (Andrés-Colás et al. 2006). Given that biochemical and genetic studies in bacteria, yeast and mammals have established that delivery of Cu to regulatory amino-terminal metal-binding domains of P_{1B}-type ATPases occurs through specific interactions between the metal-binding domains of the ATPase and a cytosolic ATX1-like Cu chaperone (Hamza et al. 1999; Huffman & O'Halloran 2000; Larin et al. 1999; Strausak et al. 2003; Walker et al. 2002; Wernimont et al. 2004), the question whether AtHMA5 could interact with a metallochaperone can be raised. Andrés-Colás and coworkers (2006) demonstrated by two-hybrid experiments that HMA5 interacts with Arabidopsis ATX1-like Cu chaperone *in vivo* and suggest a potential regulatory role for the C-terminal domain present in plants. It is interesting to note that that expression of RAN1 follows a similar pattern to HMA5, both being genes induced by Cu and mostly expressed in roots and pollen (Hirayama et al. 1999; Woeste & Kieber 2000; Andrés-Colás et al. 2006). In addition, analysis of the RAN1 and HMA5 promoters reveals the presence of putative *cis*-regulatory elements responsible for their similar expression patterns. However, a microarray study of Arabidopsis gene expression in different root cell types has shown that whereas RAN1 is abundant in all root tissues, HMA5 is mostly expressed in the pericycle cells (Birnbaum et al. 2003). Moreover, while addition of Cu to the medium induces toxicity in *hma5* plants, it alleviates the ethylene-related phenotypes of *ran1* mutants (Hirayama et al. 1999; Woeste & Kieber 2000). Therefore, a more detailed analysis of both HMA5 and RAN1 would be necessary to assess their specific roles acknowledging that they are both involved in Cu homeostasis but probably having divergent roles.

1.4.1.5. AtHMA4 and AtHMA2

AtHMA4 is the first eukaryotic member of the Zn/Cd/Co/Pb cluster to be cloned (Mills et al. 2003) and heterologous expression both in *E. coli* and yeast give some support to this. The expression of *AtHMA4*, in fact, rescues the sensitivity to Zn of the *E.coli zntA* mutant which is defective in a P-type ATPase responsible for the efflux of Zn, Cd and Pb (Beard at el. 1997; Rensing et al. 1997, 1998), in that it almost completely restores growth at high Zn concentrations. By contrast, no differences exist when expressing *AtHMA4* in the *copA* mutant which has a disruption in a Cu-translocating efflux pump (Rensing et al. 2000), suggesting a role of AtHMA4 specifically in Zn efflux from the cytoplasm (Mills et al. 2003). A function of AtHMA4 in Zn homeostasis is also confirmed by its expression in the *zrc1cot* yeast double mutant background which is hypersensitive to Zn as it lacks two vacuolar membrane transporters. *AtHMA4* expression restores the ability of *zrc1cot1* to grow at Zn concentrations up to 300 µM, as the wild type strain (Mills at el. 2005). Same wise, the putative involvement of AtHMA4 in Cd transport has been proved by means of heterologous expression: AtHMA4 is, in fact, also able to confer resistance to yeast when exposed to Cd concentrations toxic to wild type yeast strains (Mills at al. 2003; Courbot at al. 2007), as well as to *ycf1* background which lacks a vacuolar ABC transporter of glutathione-S conjugates and is, therefore, hypersensitive to Cd (Mills et al. 2005). Interestingly, analysis conducted with radiolabeled Cd (^{109}Cd) and Zn (^{65}Zn) reveal a small but significant difference in the rate of uptake of Cd and Zn being lower in AtHMA4 transformants than the vector controls, which could be interpreted as AtHMA4 acting as a metal-efflux pump determining a reduction in metal accumulation (Mills et al. 2005).

This would mean that AtHMA4, as well as contributing to Zn homeostasis, serves an important role in metal detoxification, functioning as en efflux pump when the external concentration of metal cations is high. The expression pattern of AtHMA4 both by RT-PCR and GUS activity also confirms this hypothesis: AtHMA4 is present in all tissues, predominantly in roots, stems and flowers and, in particular, at root level, it is expressed in the stellar cells, the tissues surrounding the vascular vessels (Verret et al. 2005; Hussain et al. 2004). Moreover, the investigation on the localization of AtHMA4 protein at the subcellular level *in planta* by EGFP fusion transient expression in mesophyll protoplasts clearly suggests that AtHMA4 is localized at the plasma membrane (Verret et al. 2005), as it is also observed for its orthologous in the hyperaccumulator *Arabidospis halleri*, AhHMA4, transiently expressed in tobacco plants (Courbot at al. 2007). The analysis of metal content in leaves of *Athma4* mutant reveal lower concentrations of Zn and Cd in comparison to wild-type plants. By contrast, both Zn and Cd concentrations in the roots of the recessive mutants are higher. Interestingly, when overexpressed *AtHMA4* leads to greater Zn

concentrations in the leaves and to an increased tolerance to Zn, Cd and Co overall, suggesting an improved capability of translocating metals up to the shoots (Verret et al. 2005). Taken together, the results obtained so far indicate that AtHMA4 could help in removing toxic concentrations of metals from the roots and delivering them to the shoots where they might have a less damaging effect as leaves cells are more highly vacuolated than roots ones and, therefore, can sequester toxic ions in subcellular compartments. The role of HMA4 in metal detoxification processes has also been recently confirmed by QTL analysis on the hyperaccumulator *A. halleri* and its metal-sensitive non-accumulator relative *A. lyrata* backcross progeny which made possible the identification of three major QTLs responsible for Zn and Cd hypertolerance, among which the one accounting for the greater variance colocalizes with HMA4 (Courbot at al. 2007; Willems et al. 2007). The role of AhHMA4 in Zn hyperaccumulation and Cd tolerance is also demonstrated through RNAi-mediated silencing, where *A. halleri* plants with a lower expression of *AhHMA4*, behaved similarly to wild-type *A. thaliana* plants with respect to their Zn and Cd tolerance and Zn accumulation abilities. On the other hand, *A. thaliana* overexpressing *AhHMA4* showed increased Zn translocation to the shoot, as well as increased transcript abundance of Zn deficiency genes in analogy with wild-type *A. halleri*. Compared to the syntenic segment in *A. thaliana*, in *A. halleri* there is a complex triplication of a region that includes *HMA4* and the two downstream genes. Within coding sequences, the three *AhHMA4* copies are on average 99% identical to each other, but share only 88% sequence identity with *AtHMA*4, suggesting that the triplication may have occurred recently. Sequence divergence from *A. thaliana* is more pronounced at the 5'-flanking regions of *AhHMA4* genes, supporting the hypothesis that possible *cis*-regulatory changes along with gene copy number expansion and high expression level of HMA4 are responsible for the enhanced Zn efflux from the root symplasm into the xylem vessels necessary for shoot Zn hyperaccumulation (Hanikenne et al. 2008).

From phylogenetic analysis conducted in Arabidopsis, HMA4 encoded on chromosome 4 and is closely related to HMA2 and HMA3 encoded by tandem genes on chromosome 2. *HMA4* and *HMA2/3* probably result from an ancestral duplication of an extensive region, which has subsequently undergone additions or, more probably, deletions. The relationship existing between *HMA2/3/4* genes is also reinforced by the analysis of intron positions. Within the coding sequence of each gene there are eight introns and each intron in series occurs in an identical position with respect to the corresponding amino acid sequence. Interestingly, the polypeptides HMA4 and HMA2 encoded by genes on different chromosomes are more similar to each other than to HMA3, whose encoding gene exists in tandem with the one encoding HMA2 (Cobbett et al. 2003).

As abovementioned, most, but not all, P_{1B}-type ATPases contain one or more copies of a particular metal-binding domain (MBD) motif. The MBD motif has a core signature sequence, CxxC (which in most cases appears within a conserved GMxCxxC), in addition to a number of additional well-conserved positions. A variant of the MBD motif at the N-term appears to occur in HMA2, HMA3 and HMA4. This sequence, in fact, lacks the canonical CxxC (or GMxCxxC) and is replaced instead with a CC (or GICC). In addition to this variant at the N-term, HMA2 and HMA4 proteins differ from all other described type P_{1B}-type ATPases in that both have extended C-terminal regions. HMA4 and HMA2 extend approximately 500 and 250 amino acids, respectively, C-term to the eighth transmembrane domain and contain twelve and six CysCys dipeptide sequences, respectively, in addition to a number of HisCys or CysHis sequences. By contrast, HMA3 has only a small C-terminal extension that contains two CysCys dipeptide sequences. Furthermore, HMA4 has eleven consecutive His residues close to the C-term, which are repeated to a lesser extent in HMA2. Within the 500 amino acid C-terminal extension of HMA4 there is evidence of internal homologies suggesting the extension has evolved through a series of duplications of a shorter ancestral sequence. The hypothesis that HMA4 and HMA2 derive from the duplication of an ancestral gene that has subsequently diverged is also confirmed by the fact that their C-terminal extensions exhibit some conserved segments in the sequences, even though they are notably different in length (Cobbett et al. 2003). If that was true, it would be reasonable to argue whether HMA4 and HMA2 have some overlapping functions and, therefore, are somehow redundant. Hussain and coworkers (2004) observed that whereas *hma2* and *hma4* mutants do not differ from the wild type, *hma2 hma4* double mutant shows a deficiency phenotype characterized by uneven chlorosis in leaves of reduced size, rosetting of leaves on a stem because of shortened internodes and infertility, previously reported to be Zn deficiency symptoms (Marschner 1995). This phenotype can be compensated for by the application of additional exogenous Zn. Moreover, Zn content in the shoots of the double mutant is approximately twofold less compared to the wild type and below the threshold of 20 ppm, believed to be the minimum requirement for normal growth (Marschner 1995), which may explain the stunted and chlorotic phenotype. By contrast, Zn accumulation in the roots is almost twofold higher in the double mutant compared to the wild type, indicating that HMA4 and HMA2 are not involved in Zn uptake at root level. Interestingly, the involvement of HMA4 and HMA2 in transport of Cd ions is also proved *in planta* on a PC deficient background (*cad1-3* mutant) which lacks detectable PCs and shows an approximately 40-fold increase in Cd sensitivity (Howden et al. 1995b; Ha et al. 1999). *hma2-1 cad1-3* and *hma4-1 cad1-3*, obtained crossing *hma4* and *hma2* single mutant with the *cad1-3* mutant, exhibited approximately twofold increased sensitivity to Cd compared with the *cad1-3* mutant alone (Hussain at al. 2004).

Even though the fact that Zn deficient phenotype of the double mutant can be rescued by adding Zn in excess can be interpreted as sufficient Zn can still be translocated throughout the plant by other transporters, there is evidence to conclude that HMA4 and HMA2 play a role in the loading of Zn to the xylem. Moreover, as the expression of both is also observed in phloem tissue, they are probably involved also in the remobilization of Zn from the shoot to the root. Same as for HMA4 (Verret et al. 2005), the localization of HMA2 on the plasma membrane (Hussain et al. 2004) is consistent with transporting Zn in or out of the cell and supports a role in the traslocation of Zn within the plant, as well as a detoxification function in presence of Cd. The functional characterization of HMA2 confirm that it is a Zn^{2+}-dependent ATPase, as expected by its signature sequence in the transmembrane region. Interestingly, HMA2 is also highly activated by Cd, as well as by other divalent cations to a lesser extent. The kinetic parameters describing the metal interaction with HMA2 show that both Zn and Cd are transported with high affinity (Zn^{2+} $K_{½}$= 0.11 ± 0.03 µM and Cd^{2+} $K_{½}$= 0.031 ± 0.007 µM). Concerning the expression pattern, a slightly higher expression in roots is observed but the detection of *HMA2* mRNA in all tissues indicates that it is actually ubiquitous. However, there is no evidence of up-regulation of *HMA2* expression after exposure to various metals, differently from *HMA4* which appears to be up-regulated in presence of Zn^{2+} and Mn^{2+} and down-regulated in presence of Cd^{2+} (Mills at al. 2003; Gravot et al. 2004). According to these findings, same as HMA4, HMA2 is a Zn^{2+} transporting ATPase that drives the efflux of the metal into the extracellular compartment. The enzyme is characterized by high affinity as well as broad specificity, thus also controlling the transport of non-physiological heavy metals as Cd^{2+} (Eren & Argüello 2004).

1.4.1.6. AtHMA3

Even though HMA4, HMA2 and HMA3 present high similarities in their sequences (Cobbett et al. 2003), HMA3 is slightly different in that it has the two first introns remarkably shorter compared to the two other transporters (around 200 bp). Conceptual translation of the cDNA predict a polypeptide of 760 amino acids with significant amino acid similarity with many P-ATPases involved in the Cd/Zn transport, including CadA from *Listeria monocytogenes*. The N-terminal part of AtHMA3 exhibits only one degenerated HMA signature 29-31 residues as defined by PROSITE (PS01047), with a replacement of the canonical motif GMxCxxC by GICCxxx. As abovementioned, this modification is an unusual feature shared with AtHMA2 and AtHMA4. Nevertheless, AtHMA3 does not present the long AtHMA4 C-terminal extension, as AtHMA2 and AtHMA4 do instead. Preliminary studies conducted in yeast showed that AtHMA3 expression complements the Cd- and Pb-hypersensitive mutant *ycf1*, suggesting a role of this protein in the

detoxification of Cd and Pb. Whether this is due to chelation or transport processes is clarified by using the AtHMA3-D397A mutant, in which the Asp-397 of the phosphorylation consensus domain is replaced by an Ala: this is not able to complement the *ycf1* mutant strain, thus invalidating any chelation hypothesis. Interestingly, ICP experiments showed that the *ycf1* complementation is not correlated to an intracellular Cd decrease, suggesting that Cd is not expulsed from the cells but rather sequestered in a subcellular compartment, which is also supported by observations with the confocal microscope that localized the AtHMA3: :GFP fusion protein on the tonoplast (Gravot et al. 2004). The subcellular localization on the vacuolar membrane is also confirmed *in planta* (*A. thaliana*, ecotype Ws) (Morel et al. 2009). Moreover, the *in vivo* visualization of the intracellular Cd patterning by using fluorescent probe shows a strong fluorescence after incubation in presence of Cd inside the vacuole of overexpressing AtHMA3 protoplasts, providing further evidence of AtHMA3 functioning as transporter pumping toxic metal (Cd) and excess of physiological metal (Zn) within the vacuole, which is also demonstrated by an increased tolerance to Co, Cd, Zn, and Pb in the AtHMA3 overexpressing lines (Morel et al. 2009). Different transcriptomic studies conducted *in planta* have shown that AtHMA3 mRNA is present at a very weak level in all plant parts and that its expression is only slightly modulated by Zn or Cd treatments, if affected at all (Becher et al. 2004; Gravot et al. 2004; Talke et al. 2006). In particular, AtHMA3 results to be highly expressed in the vascular bundles and root apex as well as in correspondence of the guard cells and the hydathodes: the expression of AtHMA3 in the root tip is essential to maintain the homeostasis of both physiological and toxic metals in the part of the plant that first face the presence of heavy metals in the soil. The expression of AtHMA3 in the guard cells and the hydathodes instead can be explained taking into account that also many other metal transporters that can promote a cytoplasmic Cd influx are similarly expressed in guard cells (Leonhardt et al. 2004). The ZIP4 homolog in the hyperaccumulator species *T. caerulescens*, for instance, is specifically expressed in guard cells (Küpper et al. 2007) and transports Cd and Zn (Pence et al. 2000). Additionally, it has been shown that Cd can penetrate the guard cells through hyperpolarized Ca^{2+} channels (Perfus-Barbeoch et al. 2002). Therefore, a large activity of AtHMA3 could participate in cytoplasmic detoxification of Cd in guard cells, these cells being located at the end of the transpiration stream, where heavy metals coming from the soil or from aerosols can accumulate after evaporation of the apoplastic solute. AtHMA3 results constitutively expressed as its expression level does not changed after Cd and Zn exposure, differently from the its ortholog in the tolerant hyperaccumulator species *A. halleri* which is upregulated upon Zn exposure (Becher et al. 2004; Gravot et al. 2004; Talke et al. 2006). It is interesting to note that AtHMA3 is nonfunctional in the Arabidopsis ecotype Col-0 (Hussain et al. 2004). In this accession, in fact, the cDNA presents a base pair deletion inducing a

stop codon, producing a truncated protein that lacks the essential large cytosolic loop and the seventh and eighth transmembrane helices. This may explain why various proteomic analyses performed on the tonoplast in the Col-0 background failed to detect AtHMA3 (Carter et al. 2004; Shimaoka et al. 2004; Jaquinod et al. 2007). Among 15 accessions, spanning the world, only three present this mutation. The ecotypes sharing the mutation were all found to be less tolerant of Cd, with some variability, than those exhibiting the full length AtHMA3 cDNA. However, a segregation analysis would be necessary to precisely determine the weight of AtHMA3 among other factors in plant tolerance to heavy metals.

Tab. 1 : Summary of distribution and metal specificity of *A. thaliana* P_{1B}-ATPases.

	Tissue expression	**Cellular localization**	**Metal specificity**
HMA1	Roots, shoots	Chloroplast envelope	Cu^{+}
HMA2	Vasculature of roots and shoots	Plasma membrane	Zn^{2+}, Cd^{2+}
HMA3	Roots and leaves	Vacuole	Cd^{2+}, Pb^{2+}
HMA4	Vasculature of roots and shoots	Plasma membrane	Zn^{2+}, Cd^{2+}, Pb^{2+}
HMA5	Roots, flowers	?	Cu^{+}
HMA6	Roots, shoots	Chloroplast envelope	Cu^{+}
HMA7	?	Post Golgi compartment	Cu^{+}
HMA8	Shoots	Thylakoid membrane	Cu^{+}

1.4.2. P_{1B}-type ATPases in *Oryza sativa*

The completion of two shotgun sequences of the rice (*Oryza sativa*) genome (Goff et al. 2002; Yu et al. 2002) provided the first opportunity to compare the full complement of P-type ATPases from a monocot with that of a dicot, Arabidopsis. Baxter (2003) and coworkers in their studies identified 43 members of the P-type ATPase family in rice. This is three fewer than found in Arabidopsis. The rice genome is approximately 3.5 times the size of Arabidopsis (430 Mb compared with 120 Mb; Goff et al. 2002; Yu et al. 2002). This size difference has been attributed to (a) a 1.25- to 2.5-fold increase in the number of genes (32,000–55,000 in rice versus 26,000 in Arabidopsis), (b) an increase in intergenic distances, and (c) an increase in intron size and number. In the case of P-type ATPase genes, the overall increase is certainly due to a 2-fold increase in the average size of introns (356 to 164 bp). It is interesting to note that in the context of a possible 2.5-fold increase in the number of rice genes, the number of P-type ATPases did not show a parallel expansion. This same observation has been noted for 15 other gene families (Banuelos et al. 2002; Goff et al. 2002; Baumberger et al. 2003; Jasinski et al. 2003), raising a question about the function of the additional 6,000 to 29,000 rice-specific genes. At present, the expectation is that much of the increased

number of genes is due to “over-predictions,” because many of the novel rice genes are not supported by expressed sequence tag (EST) data and do not show similarity to known proteins in any organisms, including corn (*Zea mays*; Yu et al. 2002).

There are nine P_{1B}-ATPases in rice, whereas in Arabidopsis they are eight. The similarity in the number of this type of transporters suggests that the expansion occurred with respect to P_{1B}-ATPases in prokaryotes was important for the evolution of angiosperms (Baxter et al. 2003). Moreover, the number of introns in these two species presents the same average (13), having highly similar positions within potential orthologs. Therefore, it is possible to speculate that the approximate number and organization of P-type ATPases appear to have been maintained during the evolution of rice and Arabidopsis (Baxter et al. 2003).

Despite the economical and agricultural importance of rice, very little is known about the function of P_{1B}-ATPases in this species, even though the focus is gradually moving on it and various studies have recently come up.

The first study conducted on *O. sativa* dates back to 2007 (Lee et al. 2007) and identifies OsHMA9 as a metal efflux protein. From preliminary analysis, *OsHMA9* results upregulated by Cu, Zn and Cd treatments, as well as *OsHMA5* and *OsHMA6*. The determination of the expression pattern reveals that the *OsHMA9* gene is mainly expressed in vascular tissues, including the xylem and phloem, an observation consistent with a role in the loading and unloading of heavy metals in those tissues. In particular, in the leaves, *OsHMA9* is weakly expressed in the mesophyll tissues, a major storage site for heavy metal ions, indicating internal detoxification (Ma et al. 2004). As OsHMA9 is also found in the developing spikelets at the anthers level, it is possible to suppose that it probably plays a role in metal delivery to rice anthers, as it has been proposed in Arabidopsis (Hussain et al. 2004). The involvement of OsHMA9 in metal transport and detoxification processes is also proved by the observations of *oshma9* knockout plants on medium containing elevated levels of Cu, Zn, Pb, and Cd. In these conditions, in fact, growth is inhibited compared with the wild type, as manifested by their reduced height, fresh weight and chlorophyll content. Moreover, the *oshma9* mutant accumulate more Cu, Zn, Pb, and Cd than do the wild type in the shoots. In apparent contrast with these results, the expression of *OsHMA9* in *E.coli* partially rescues the sensitivity to high Cu in the the *copA* mutant, which is defective in its endogenous Cu efflux pump but it does not complement the *zntA* mutant, leading to the conclusion that OsHMA9 is a Cu- rather than a Zn-transporter. As the subcellular localization of OsHMA9 is on the plasmamembrane, it is reasonable to suppose that it is heavy-metal efflux protein but further works are needed to verify its specificity and, in particular, as it is in the subclass phylogenetically related to AtHMA7/RAN1, which

functions in the delivery of Cu ions to the ethylene receptor (Hirayama et al. 1999) and there is evidence that it also transports Cu, it would be interesting to explore the possible role of OsHMA9 in ethylene-signaling pathways (Lee et al. 2007).

OsHMA3 has been recently and extensively studied combining map-based cloning techniques with molecular biology and classical physiology approaches. Following previous studies that detected on the short arm of chromosome 7 a major QTL for Cd accumulation accounting for 85.6% of the variance observed in a population derived from a low Cd-accumulating cultivar, Nipponbare, and a high Cd-accumulating cultivar, Anjana Dhan (Ueno et al. 2009a), Ueno and coworkers identified OsHMA3 as a key element functioning as a "firewall" to limit Cd translocation from roots to shoots. The analysis of sequences show very high similarity between the two cultivars showing different behaviours in Cd accumulation. By contrast, both of them result very different when compared with *A. thaliana* and *A. halleri*. Interestingly, OsHMA3 from Anjana Dhan (OsHMA3a) present a 53-amino acid deletion in the C-terminal region in addition to several amino acid changes compared to OsHMA3 from Nipponbare (OsHMA3n). However, the expression of chimera proteins in yeast obtained by fusing the N-term from OsHMA3a with the C-term of OsHMA3n or fusing the N-term from OsHMA3n with the C-term of OsHMA3a indicate that not the 53 residues missing in the C-term of OsHMA3a but the amino acid substitutions in the N-terminal region in OsHMA3n make the difference in Cd sensitivity. As evidence of this, turning the His at the position of 80 in OsHMA3a to Arg as it is in OsHMA3n by site-directed mutagenesis in yeast led to the enhanced sensitivity to Cd, contrarily to what observed when substituting Val at the position of 638 to Ala, which does not alter the Cd sensitivity. These results indicate that the amino acid at the position of 80 might be critical for the function of OsHMA3n. The amino acid substitution found in OsHMA3a probably cause topology change as predicated by the SOSUI program; according to this prediction, in fact, OsHMA3n has eight predicted transmembrane domains, whereas mutation of amino acid at position 80 from Arg to His results in an additional transmembrane domain between TM1 and 2, likely affecting the functionality of the transporter. Therefore, considering also that the analysis of the expression pattern and of the subcellular localization reveal that *OsHMA3* is constitutively expressed in roots and localized on the tonoplast, it is reasonable to suppose that OsHMA3n from a low Cd-accumulating cultivar (Nipponbare) functions as a firewall by sequestrating Cd into the vacuoles in the roots, keeping Cd away from the above-ground tissues. In contrast, probably because of the mutation of amino acid at the position of 80, OsHMA3 in a high Cd-accumulating cultivar (Anjana Dhan) has lost its function to sequester Cd into the vacuoles, resulting in high translocation of the toxic metal from the roots to the shoots (Ueno et al. 2010a).

These results have been confirmed soon thereafter comparing Nipponbare cultivar to Jarjan, an *indica* cultivar from Bhutan, which, similarly to Anjana Dhan, show very high Cd accumulation in the shoots, being the highest among 146 accessions from a rice core collection (Ueno et al. 2010b). In a progeny obtained by crossing Nipponbare and Jarjan, the phenotype characterized by high Cd accumulation in the shoot displays a segregation pattern of 1:3, indicating that this phenotype in Jarjan is controlled by a single recessive gene, later identified as OsHMA3. The expression pattern as well as the subcellular localization in Jarjan are the same as Anjana Dhan, *i.e. OsHMA3* being constitutively expressed in roots and the protein localized on the tonoplast. Furthermore, interestingly Anjana Dhan and Jarjan have the same sequence, presenting the 53 residues deletion in the C-term and the amino acid changes in the positions 80 and 638, the former probably causing the loss of function of OsHMA3 (Ueno et al. 2010a, 2010b).

More recently, linkage analysis of progeny from a cross between Cho-Ko-Koku (high Cd translocation) and Akita 63 (low Cd translocation) confirmed the qCdT7 locus as responsible for difference in the trait "Cd accumulation in the shoots" and indentified in that segment the gene encoding OsHMA3. In particular, sequence analysis show that OsHMA3 of Akita 63 (AB559522) encoded a 1004-aa protein, whereas that of Cho-Ko-Koku (AB559521) encoded a protein of 951 aa as a result of deletions in exon 7. Exon 7 of the Akita 63 OsHMA3 contain a repeat sequence consisting of five direct repeats. By contrast, exon 7 of Cho-Ko-Koku has a 153-bp (51-aa) deletion caused by variation in the number of tandem repeats. This tandem repeat may have resulted through either duplication or deletion during rice evolution. In addition to this 51-aa deletion, 2-aa deletion and 33-aa substitutions were also found comparing Akita 63 and Cho-Ko-Koku OsHMA3. These differences may cause OsHMA3 from Cho-Ko-Koku to be non-functional as proven by heterologous expression in *ycf1*, since sensitivity to Cd can be rescued by Akita 63 OsHMA3 but not by Cho-Ko-Koku OsHMA3. Interestingly, experiments *in planta* expressing OsHMA3 from Nipponbare in Cho-Ko-Koku result in a drastic decrease in the rate of Cd translocation up to the shoots. Even though it could be argued that the deletions and mutations in the C-term region may underlie the differences in the function of OsHMA3 between Akita 63 and Cho-Ko-Koku, it should be considered that AtHMA3, the ortholog of OsHMA3 in *A. thaliana*, has only a short C-term (Gravot et al. 2004). In addition, the C-terminal region has been reported to be inessential for HMA2 function (Wong et al. 2009). Therefore, it is possible that structural mutations in the C-term of OsHMA3 in Cho-Ko-Koku are not responsible for the loss of function of the protein, as already noted in the cultivar Ajana Dhan (Ueno et al. 2010a). By contrast, the amino acid changes in the transmembrane domains of OsHMA3 predicted by different transmembrane prediction programs are more likely to cause the protein to bind to the membrane with an inverted orientation, causing

the protein to assume a conformational change responsible for the loss function of OsHMA3 in Cho-Ko-Koku (Miyadate et al. 2011). Therefore, according to the results obtained so far, it is possible to hypothesize that Cd taken up by root cells is sequestered into the vacuole being mediated by OsHMA3 to reduce the concentration of this toxic metal in the cytoplasm. The sequestration of a large portion of Cd means that a limited amount is loaded into the xylem from the root cells and, consequently, translocated to the shoots from the roots, which would explain the correlation between a functional HMA3 and low accumulation of Cd in the shoots. However, it should be noted that still the form of Cd transported by OsHMA3 remains to be determined (Miyadate et al. 2011).

As abovementioned, HMA2 and HMA4 in Arabidopsis seem to play an essential role in controlling root-to-shoot Zn translocation acting as metal pumps involved in the xylem loading (Hussain et al. 2004) and there is evidence that they also take part in Cd transport (Mills et al. 2003, 2005; Eren & Argüello 2004; Verret et al. 2005). However, at present no studies have been conducted on the orthologs of these genes in rice, even acknowledging their possible role in determining Cd translocation and thus accumulation in shoots and grains. Therefore, further research should be carried out to: (a) explore their potential in ensuring food safety by preventing metal translocation or (b) in enhancing remediation of contaminated soil through phytoextraction techniques by boosting their expression and transport activity.

Chapter 1:

Cadmium retention in rice root is influenced by cadmium availability, chelation and translocation

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This manuscript was published in *Plant, cell & environment* (2011), **34** (6), 994-1008

ABSTRACT

Analysis of rice plants exposed to a broad range of relatively low and environmentally realistic Cd concentrations showed that the root capacity to retain Cd-ions rose from 49% to 79%, corresponding to increases in the external Cd^{2+} concentration in the 0.01-1 μM range. Fractioning of Cd-ions retained by roots revealed that different events along the metal sequestration pathway (i.e. chelation by thiols, vacuolar compartmentalization, adsorption) contributed to Cd immobilization in the roots**.** However, large amounts of Cd-ions (around 24% of the total amount) predictable as potentially mobile were still found in all conditions, whilst the amount of Cd-ions loaded in the xylem seemed to have already reached saturation at 0.1 μM Cd^{2+}, suggesting that Cd translocation may also play an indirect role in determining Cd root retention, especially at the highest external concentrations. *In silico* search and preliminary analyses in yeast suggest OsHMA2 as a good candidate for the control of Cd xylem loading in rice. Taken as a whole, data indicate Cd chelation, compartmentalization, adsorption and translocation processes as components of a complex "firewall system" which acts in limiting Cd translocation from the root to the shoot and which reaches different equilibrium positions depending on Cd external concentration.

Key-words: cadmium, cadmium root retention, cadmium-binding complexes, translocation, P_{1B}-ATPase transporters, *Oryza sativa* L.

1. Introduction

Cadmium is a toxic heavy metal naturally present in soils or anthropogenically released in natural and agricultural environments. Important sources of soil contamination are short- or long-range atmospheric depositions from mining activities, phosphate fertilizers and manures, municipal sewage wastes, urban composts and industrial sludges (Alloway & Steinnes 1999; McLaughlin, Parker & Clarke 1999).

The presence of Cd in soils is an increasing concern with respect to human food-chain accumulation as well as to crop production. Although this metal is not essential for plant nutrition, it can be easily taken up by roots and accumulated in vegetative and reproductive plant organs. In this way Cd-rich soils potentially result in Cd-rich foods, although natural variation occurs in both the uptake and the distribution of Cd in crop species (Guo, Schulz & Marschner 1995; Grant et al. 1998; Cakmak et al. 2000), and in cultivars within species (Clarke et al. 2002; Dunbar, McLaughlin & Reid 2003; Grant et al. 2008; Uraguchi et al. 2009).

Among cereals, rice (*Oryza sativa* L.) is a staple crop that tends to accumulate Cd to levels sometimes exceeding the proposed international limits for the cereal grain trade (Arao & Ae 2003; Ishikawa et al. 2005; Liu et al. 2005b; Grant et al. 2008). Recently, the Codex Alimentarius Commission of the FAO/WHO fixed the official maximum allowable limits of Cd concentration as 0.4 mg kg^{-1} for polished rice, 0.2 mg kg^{-1} for wheat, and 0.1 mg kg^{-1} for the other cereal grains (CODEX STAN 193–1995 2009). However, in spite of the more permissive limit for polished rice, the problem of Cd concentration in rice grains still remains of great concern, even in situations where trace quantities of the metal are found in soils. It is well established that long-term consumption of rice grown in paddy soils contaminated by Cd can cause a high prevalence of renal proximal tubular dysfunction (Kobayashi 1978; Cai et al. 1990; Kobayashi et al. 2002). Moreover, subsistence rice diets have been shown to increase the soil Cd risk, since the low total and bioavailable levels of Fe, Zn and Ca in grains may substantially increase net Cd absorption by humans (Reeves & Chaney 2002; Chaney et al. 2004). In these contexts, it is desirable to develop both agronomic management practices and breeding strategies to reduce Cd accumulation in edible parts of plants, not only to comply with the restrictive standards for the cereal grain trade but also to ensure the safe consumption of plant foodstuffs.

Despite several efforts aimed at developing agronomic techniques to reduce Cd concentration in paddy soils (Murakami, Ae & Ishikawa 2007; Makino et al. 2008), the introduction of rice cultivars which accumulate low levels of Cd in their grains seems to be the most promising approach, in terms of effectiveness and economic value, to minimize the dietary intake of Cd (Grant et al. 2008).

As already noted, there are well-documented differences across plant species in the partitioning of Cd between tissues. Several investigations report on the capability of many plant species to retain much of the Cd taken up within the roots (Jarvis, Jones & Hopper 1976; Wagner 1993; Lozano-Rodríguez et al. 1997; Puig & Peñarrubia 2009; Verbruggen, Hermans & Schat 2009), suggesting the existence of conserved mechanisms limiting Cd allocation through the whole plant and preventing excessive Cd accumulation into the seeds. Thus, the knowledge of the physiological bases governing root retention of Cd in plants is thought to be critical to reduce Cd accumulation in the edible portions by defining pollution-safe cultivars and/or by improving agronomic practices to be used in moderately contaminated soils (Grant et al. 2008).

Several mechanisms may be involved in determining the root capacity to retain Cd-ions. Transport of Cd across the plasma membrane is only the first step in metal uptake and accumulation into the roots. Once inside root cells, Cd-ions have to be trapped by a "firewall system" through selective binding sites with high affinity for the metal, or through transfer across a second membrane into an intracellular compartment (Clemens 2006b). The efficiency of these processes may contribute to the natural variation in Cd distribution between roots and shoots observed in crop species, since only Cd-ions escaping these trapping pathways may be potentially available to be translocated via the xylem in a root-to-shoot direction.

One of the principal classes of heavy metal chelators known in plant cells is the phytochelatins (PCs), 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 & Goldsbrough 2002). PCs are non-translationally synthesized from glutathione (GSH) in a transpeptidation reaction catalyzed by the enzyme PC synthase (PCS; Grill et al. 1989; Rea, Vatamaniuk & Rigden 2004). PCs are able to form thiolate bonds with Cd-ions in complexes that accumulate in the vacuoles (Clemens 2006b). Meticulous studies on maize seedlings showed that most of the total Cd retained by roots is bound in high- and low-molecular weight complexes containing PCs and related thiol-compounds, revealing these peptides as crucial for Cd root retention in cereals (Rauser & Meuwly 1995; Rauser 2003).

Recently, Ueno et al. (2010a) described a new component (*OsHMA3*) of the network affecting Cd partitioning in rice, which has been isolated by using a mapping population derived from a cross between a high (Anjana Dhan) and a low (Nipponbare) Cd-accumulating cultivar. Such a gene encodes a member of the P_{1B}-ATPase transporter sub-family (Axelsen & Palmgreen 2001; Williams & Mills 2005), mainly expressed in the tonoplast of root cells, which functions as a "firewall" to limit translocation of Cd-ions from the root to the shoot. The authors also described this transporter as constitutively expressed in the roots and highly selective for Cd sequestration into the root vacuoles. Interestingly, a single amino acid mutation in OsHMA3 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 Cd translocation from root to shoot (Ueno et al. 2010a).

Translocation of Cd-ions to the shoots requires, at the root level, both radial symplastic passage and active loading into the xylem (Colangelo & Guerinot 2006). It is generally accepted that Cd-ions compete with Zn-ions for accumulation in the shoots, since they probably use the same transport systems to be loaded into the xylem. Good candidates for such an activity are members of the P_{1B}-ATPase sub-family belonging to the cluster 2 (Axelsen & Palmgreen 2001; Baxter et al. 2003; Williams & Mills 2005). In particular, AtHMA2 and AtHMA4 seem to play essential roles in controlling 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 & Argüello 2004; Verret et al. 2005). Interestingly, the *Athma2-Athma4* Arabidopsis double mutant shows a near-complete abolition of root-to-shoot Cd translocation (Wong & Cobbett 2009), whereas decreasing HMA4 transcript levels by RNA interference in Zn hyperaccumulator *Arabidopsis halleri* resulted in enhanced Cd root retention capacity (Hanikenne et al. 2008). Concerning rice, a positive and strong correlation between Zn and Cd concentrations in the shoots was recently observed in 69 rice varieties; authors also found that root-to-shoot Cd translocation via the xylem was the major and common physiological process determining Cd accumulation in shoots and grains of rice plants (Uraguchi et al. 2009).

Considering the importance of Cd root retention in limiting the amount of the metal that may enter the food chain, here we present a physiological analysis aimed at better characterizing this trait in rice plants exposed to a broad range of relatively low Cd concentrations, with the aim of simulating the actual Cd availabilities experienced by roots in moderately contaminated soils (Sauvé et al. 2000). With this aim we focused our attention on the main physiological and biochemical components of the root "firewall system" thought to be related to Cd root retention, to provide information about the relative relevance of each of them in determining the trait "Cd root retention capacity" in rice. Finally, we also present for the first time a preliminary functional characterization of *OsHMA2*, a gene encoding a putative Zn^{2+}/Cd^{2+} ATPase which could be involved in root-to-shoot Cd translocation in rice.

2. Results

2.1. Cd partitioning between root and shoot

Rice plants were exposed for a 10 d period to different Cd concentrations (from 0.01 to 1 µM) in the growing medium. Cd exposure did not produce either significant changes in growth of both roots and shoots with respect to the control, or any apparent symptoms of stress (data not shown): at the end of the treatment period, root and shoot FWs of a single plant were 0.5 ± 0.025 g and 0.8 ± 0.038 g, respectively.

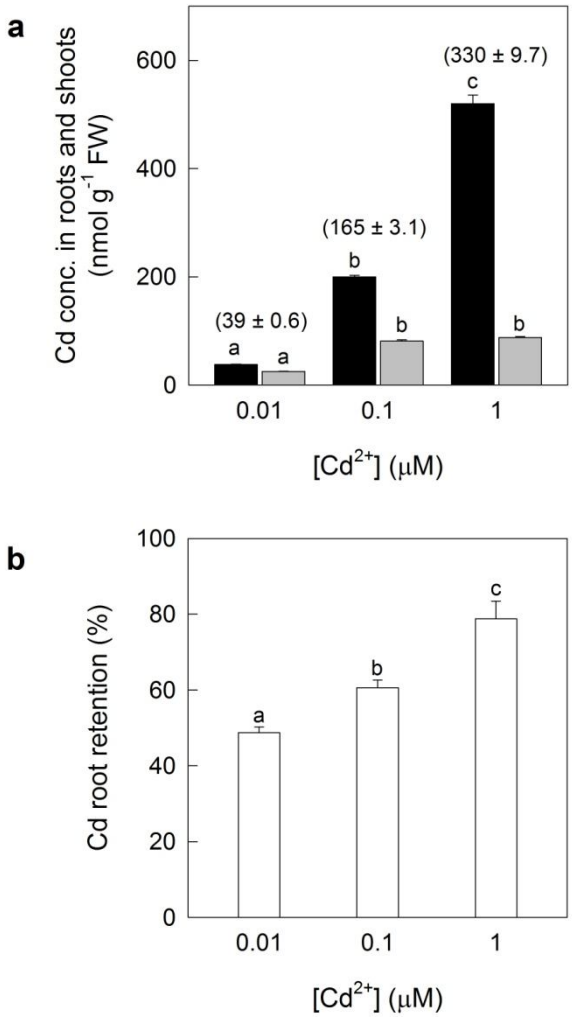


Fig. 1 : Cd accumulation and partition in rice plants. Plants were exposed to different Cd concentrations (0.01, 0.1 and 1 µM) for 10 d. (a) Cd partition between roots (black bars) and shoots (grey bars). Data reported in brackets indicate the total Cd content in a whole plant expressed as nmol ± SE. (b) Percentage of total Cd retained by roots. Bars and error bars are means and SE of three experiments run in triplicate (n = 9). Different letters indicate significant differences between Cd treatments ($P < 0.05$). FW, fresh weight.

The total amount of Cd absorbed by plants was enhanced by increasing the external metal concentration (Fig. 1a). Considering roots and shoots separately, two different trends of Cd accumulation emerged. In the first, typical of the roots, Cd concentration increased as Cd availability in the external medium did; by contrast, in the shoots, the metal concentration increased moving from 0.01 to 0.1 μM Cd^{2+}, and then did not significantly change moving toward the highest concentration analyzed (Fig. 1a). From these data, we calculated that the root capacity to retain Cd ions (i.e. the percentage of the total Cd retained in the roots; Fig. 1b) rose from 49% ± 1.5 (0.01 μM Cd^{2+}) up to 79% ± 4.7 (1 μM Cd^{2+}).

2.2. Effect of Cd exposure on thiol biosynthesis and expression of genes involved in Cd chelation and vacuolar sequestration

Since the activity of homeostatic mechanisms based on thiol biosynthesis (*i.e.* PCs and related peptides) and direct vacuolar sequestration may potentially allow a greater proportion of Cd to be retained in roots, we studied the effects of the different Cd concentrations on both GSH and NPT levels of the root and on the expression of genes related to PC biosynthesis (*OsPCSs*) and Cd vacuolar sequestration (*OsHMA3*). Following Cd exposure, the NPTs of rice roots progressively increased from levels equivalent to those of GSH in untreated control roots (120 ± 4.3 versus 115 ± 5.2 nmol GSH eq. g^{-1} FW, respectively) up to values 10-fold higher (Fig. 2). However, by contrast, the GSH levels of the roots significantly decreased as Cd concentration in the external medium increased: a maximum twofold decrease in GSH was observed at the highest Cd concentration administered (Fig. 2).

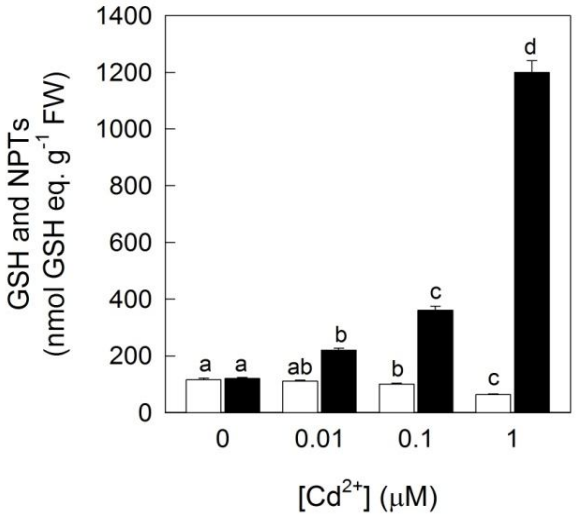


Fig. 2 : Total glutathione (GSH) and non-protein thiols (NPTs) in rice roots. Plants were exposed to different Cd concentrations (0.01, 0.1 and 1 μM) for 10 d. Total GSH (white bars) and NPT (black bars) levels are expressed as GSH equivalents. Bars and error bars are means and SE of three experiments run in triplicate (n = 9). Different letters indicate significant differences between Cd treatments ($P < 0.05$).

RT-PCR analysis performed on cDNA obtained from roots showed that the levels of the transcripts of the two *OsPCS* genes mainly expressed in roots were significantly higher in Cd-exposed than in control plants; moreover, the steady-state levels of the transcripts appeared similar under all the Cd exposure conditions analyzed (Fig. 3). A similar behavior was observed for *OsHMA3*, although less significant changes in the transcript levels of this gene were detectable (Fig. 3).

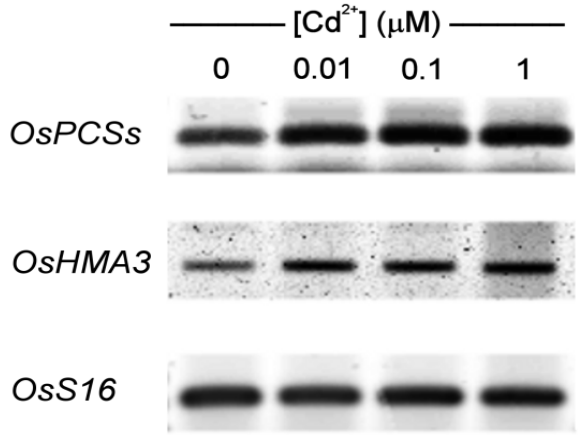


Fig. 3 : RT-PCR analysis of *OsPCS* and *OsHMA3* gene expression in rice roots. PCRs were carried out for 30 cycles where cDNAs were exponentially amplified. For *OsPCSs*, primers were designed on conserved sequences of the two *OsPCS* loci (LOC_Os05g34290 and LOC_Os06g01260) expressed in roots, and gave overlapping amplification products of 276 bp. PCR products were separated in agarose gel and stained with synergy brands (SYBR) Green I. Signals were detected using a laser scanner with 532 nm laser and 526 nm filter. *OsPCSs*, phytochelatin synthases; *OsHMA3*, heavy metal P_{1B}-ATPase 3; *OsS16*, S16 ribosomal protein.

2.3. Fractioning of Cd in rice roots

Fractioning of Cd retained by roots was carried out using the sequential extraction procedure with buffer and acid previously described by Rauser & Meuwly (1995). Table 1 summarizes results obtained in representative experiments where the sum of Cd ions recovered in the different fractions accounts for at least 97% of the total Cd content of the roots.

Tab. 1 : Fractioning of Cd ions retained in rice roots.

	Cd content (nmol g^{-1} FW)				
	Buffer soluble (1–6)				
	Anionic	**Cationic**	**Acid soluble (7–9)**	**Ash**	**Total**
0.01 µM Cd^{2+}	2.96 ± 0.06 (a)	7.90 ± 0.18 (a)	22.44 ± 0.70 (a)	3.16 ± 0.08 (a)	36.46 ± 1.02 (a)
0.1 µM Cd^{2+}	18.14 ± 0.48 (a)	44.08 ± 1.1 (b)	118.62 ± 3.44 (b)	15.04 ± 0.48 (b)	195.88 ± 5.50 (b)
1 µM Cd^{2+}	215.64 ± 7.34 (b)	120.12 ± 3.48 (c)	141.82 ± 3.40 (c)	19.84 ± 0.46 (c)	497.42 ± 14.68 (c)

Plants were exposed to different Cd concentrations (0.01, 0.1 and 1 µM) for 10 d. Cd retained by roots was extracted with buffer and acid using the sequential procedure described in Materials and Methods section. Data are means and SE of three experiments, each performed with eight plants (n = 3). Different letters indicate significant differences between Cd treatment (P < 0.05).

Following extraction, three main Cd fractions were obtained from roots: (1) buffer soluble (extracts 1–6); (2) acid soluble (extracts 7–9); (3) ash (non-soluble Cd). Extracts 1 to 6 were further resolved into two fractions, named anionic and cationic, by anion-exchange chromatography. Such a separation allowed us to distinguish Cd ions potentially free (cationic) from those tightly retained in complexes with thiol-peptides or other soluble molecules negatively charged in the extraction buffer (anionic). The buffer extracts (1–6) accounted for 29.8, 31.8 and 67.5% of Cd ions retained by roots of plants exposed to 0.01, 0.1 and 1 µM Cd^{2+}, respectively. Concerning Cd remaining in the pellets, it was largely extracted in ice-cold 100 mM HCl (extracts 7–9) and then a small amount of the metal was found in the ashes. Passage of extracts 1–6 through an anion-exchange chromatography column concentrated the anionic forms of buffer-soluble Cd for gel filtration. With the rise from 0.01 to 1 µM Cd^{2+} in external concentration, the percentage of total Cd not bound to the anion exchanger did not significantly change (a small increase from 21.6 to 24.1% was observed), whereas that of Cd in anionic form significantly increased from 8.1 to 43.4%. Anionic buffer-soluble Cd was further resolved by gel filtration on a Sephadex G-50 column into two peaks, referred to as HMW and LMW Cd-binding complexes, centred before the V_t of the column (Fig. 4). The amounts of NPTs recovered in these fractions accounted for at least 95% of the GSH

equivalents resulting from the difference between the levels of NPTs and GSH measured in the roots (data not shown); this result allowed us to identify Cd-binding complexes as the classical HMW and LMW Cd-PCs complexes. Peaks of HMW and LMW Cd-binding complexes were centred around K_{av} = 0.41 and *K*av = 0.61, respectively; no Cd ions were found at both void (K_{av} = 0) and total (K_{av} = 1) volumes. In roots of plants exposed to 0.01 µM Cd^{2+}, the LMW Cd-binding complex was the sole observed (Fig. 4a), whereas the exposure to 0.1 µM Cd^{2+} resulted in a near equal distribution of Cd ions between the two complexes (Fig. 4b). Finally, the HMW Cd-binding complex became the most relevant one in the roots of plants exposed to 1 µM Cd^{2+}, as it accounted for 84% of Cd ions in the anionic fraction (Fig. 4c).

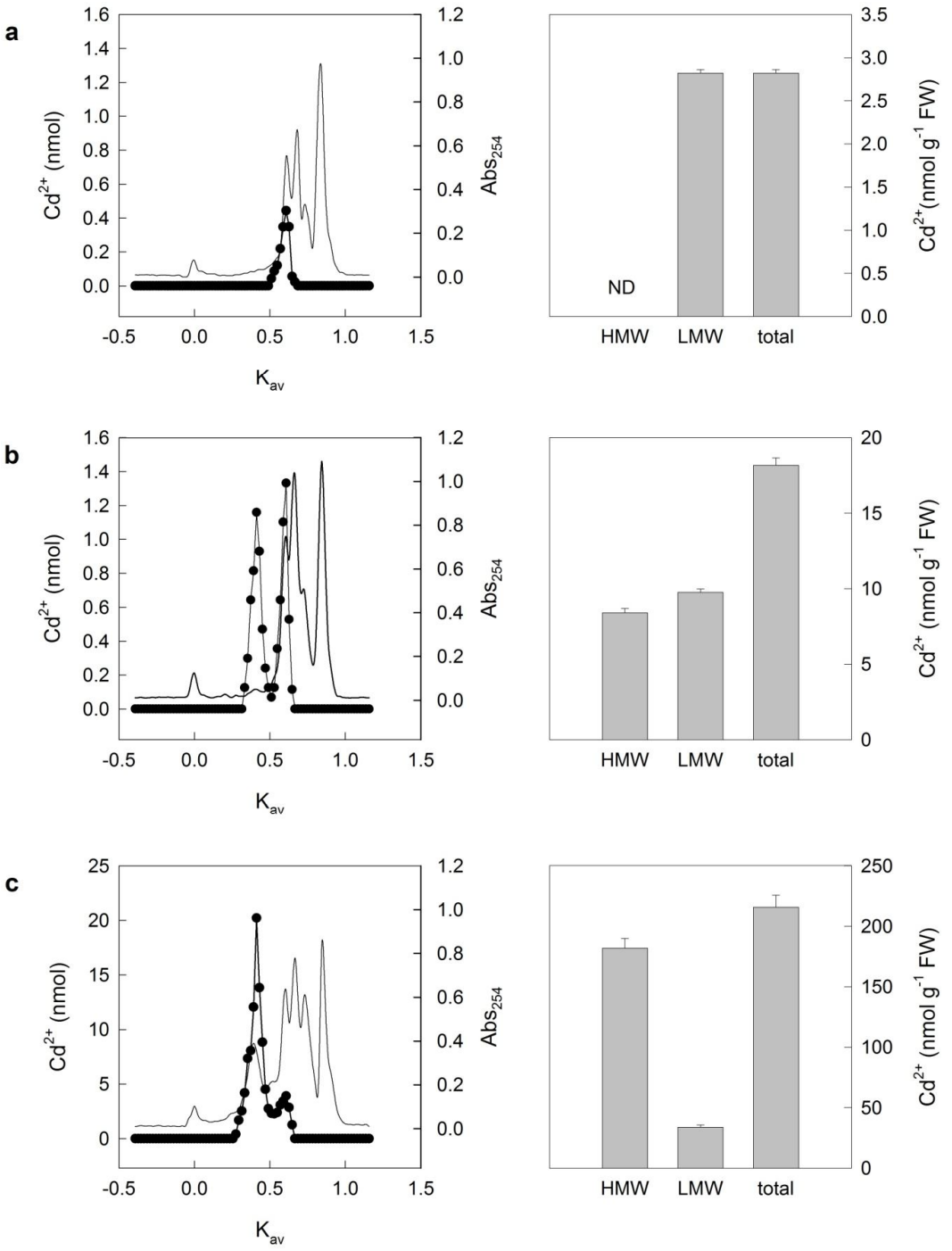


Fig. 4 : Cd-binding complexes of rice roots detected by gel filtration chromatography. Plants were exposed for a 10 d period to 0.01 µM Cd^{2+} (a), 0.1 µM Cd^{2+} (b) and 1 µM Cd^{2+} (c). The anionic fraction from buffer extracts 1–6 was chromatographed on a Sephadex G-50 column. Cd eluted (black circles) was measured by ICP-MS. The continuous trace indicates the absorbance at 254 nm.Void and total volume peaks are centred at *K*av = 0 and K_{av} = 1, respectively. Peaks of high-molecular-weight (HMW) and low-molecular-weight (LMW) Cd-binding complexes are centred around K_{av} = 0.41 and K_{av} = 0.61, respectively. Data are representative of one typical experiment repeated three times with similar results. The histograms associated to each elution profile show the amount of Cd ions in HMW and LMW Cd-binding complexes. Bars and error bars are means and SE of three experiments each performed with eight plants (*n* = 3). ND, not detectable; FW, fresh weight.

2.4. Isolation and partial characterization of OsHMA2v

OsHMA2v (GeneBank accession HQ646362) was amplified by RT-PCR from total RNA isolated by rice roots. Primers were designed on the deduced ORF registered in PlantsT (PlantsT 64490; http://plantst.genomics.purdue.edu/), since the deduced ORF for cv. Nipponbare (LOC_Os06g48720) – reported in the MSU Rice Genome Annotation Project Database and Resource (http://rice.plantbiology.msu.edu/) – codifies for a shorter polypeptide of 156 amino acids. The amplified *OsHMA2v* cDNA encodes a polypeptide of 1067 amino acids, with a predicted mass of 116 kDa, sharing 95% identity with the 1060 amino acid-long protein reported in PlantsT (see Appendix Fig. 1). Sequence analysis confirmed the identity of OsHMA2v as a member of the P_{1B}-ATPase transporter subfamily (Fig. 5a), as indicated by the presence of: (1) eight predicted transmembrane domains (TMs), according to the topology prediction software MEMSAT3; (2) a typical CPC (351CysProCys353) domain in the TM6; (3) the characteristic signature sequences found in all P-typeATPases (DKTGT; GDGxNDxP and PxxK motifs). Signature sequences corresponding to $Zn^{2+}/Cd^{2+}/Pb^{2+}/Co^{2+}$ ATPases were also found inTM6 [CPC(x)4SxP],TM7 [N(x)7 K] andTM8 [DxG] (Williams & Mills 2005). Sequence analysis revealed OsHMA2v as a member of cluster 2 in the phylogenetic tree of the P_{1B}-ATPase subfamily (see Appendix Fig. 2); such a group is thought to include all $Zn^{2+}/Cd^{2+}/Pb^{2+}/Co^{2+}$ ATPases (Williams & Mills 2005). Moreover, OsHMA2v 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 (17GlyIleCysCysThrSerGlu23), found in the *Arabidopsis* HMA2, HMA3 and HMA4 Zn^{2+}-ATPases (Eren *et al*. 2007). Finally, OsHMA2v 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.

To determine whether OsHMA2v plays a role in Cd transport, we performed a functional assay in yeast. OsHMA2v conferred strong Cd tolerance when expressed in the *S. cerevisiae* INVSc1 strain under the control of the *GAL1* promoter (Fig. 5b). Such a finding strongly suggests that this protein could function as a detoxification system in yeast by pumping excess of cytosolic Cd ions into the apoplast. Similar results were obtained by comparing the duplication times of the yeast cells incubated in SG liquid media supplemented with 75 µM Cd^{2+}, which resulted 770 ± 35 and 408 ± 20 min for control cells and OsHMA2v expressing cells, respectively. The duplication times of the two recombinant yeast clones measured in the same medium but in the absence of Cd were similar (318 ± 13 min; data not shown).

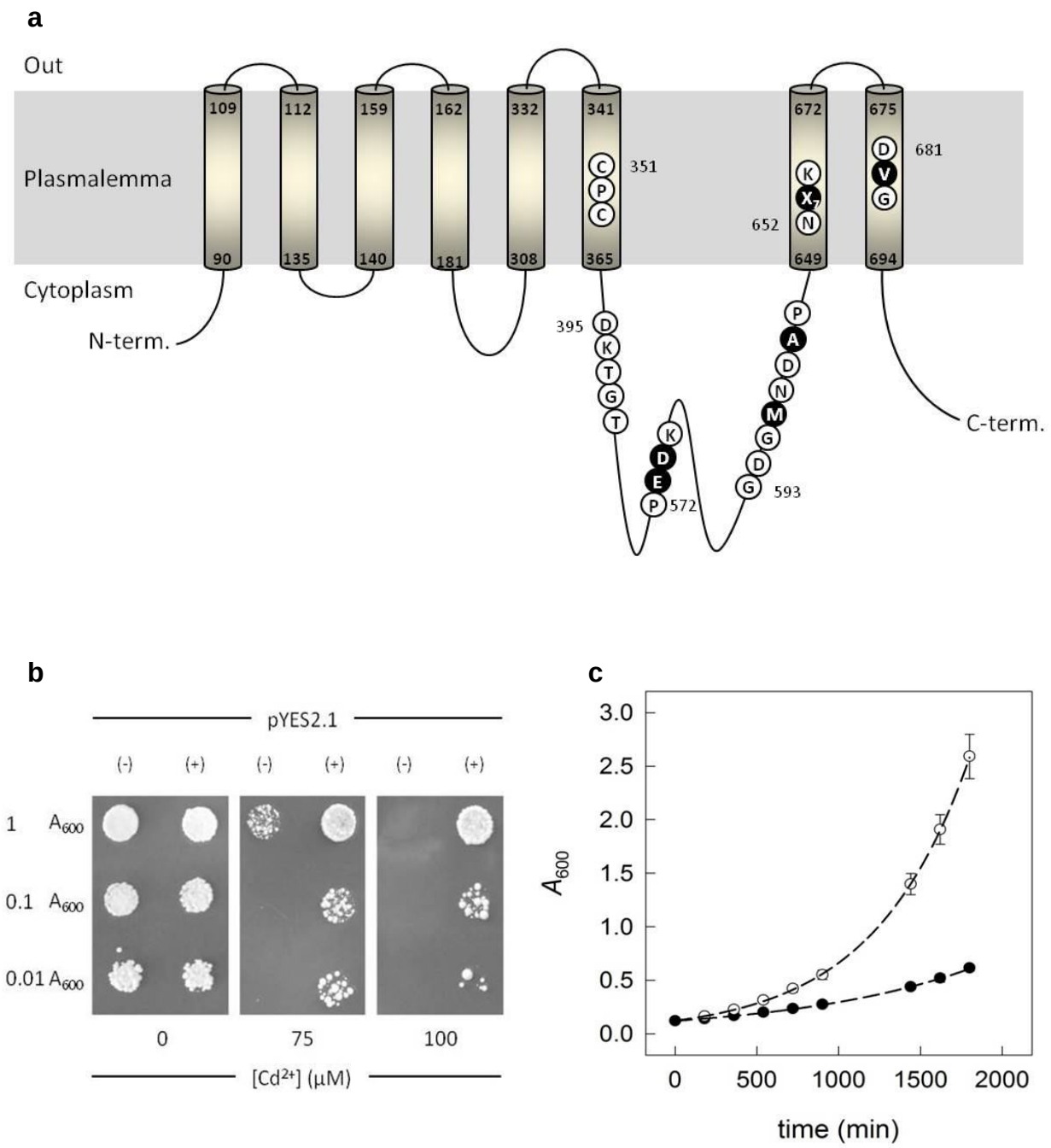


Fig. 5 : Membrane topology and functional characterization of OsHMA2v. (a) Eight TM helices are predicted in OsHMA2v using MEMSAT3. Numbers in bold indicate the position of TM segments within the OsHMA2v sequence. The small cytoplasmic loop is between helices 4 and 5 and the large cytoplasmic loop is between helices 6 and 7. Signature sequences in TM6 [^{351}CPC^{353}], TM7 [^{652}N(x)7K^{660}] and TM8 [^{681}DxG^{683}] are conserved in all Zn_{2+}/Cd_{2+}/Pb_{2+}/Co_{2+} ATPases. (b) Yeast cells expressing OsHMA2v under the control of the inducible *GAL1* promoter (+) or harboring the empty pYES2.1 vector (-) were grown at 30 °C for 3 d on SG media supplemented or not with 75 or 100 mM $CdCl_2$. (c) Growth of yeast cells expressing OsHMA2v (white circles) or harboring the empty pYES2.1 vector (black circles) in SG media supplemented with 75 mM $CdCl_2$. Bars and error bars are means and SE of two experiments performed in triplicate (n = 6).

2.5. Analysis of root-to-shoot Cd translocation

The dynamic of root-to-shoot Cd translocation was examined by measuring Cd concentration in the xylem sap of rice plants exposed to different Cd concentrations. In these experiments, Cd translocation was estimated as the amount of Cd ions loaded and transported in the xylem sap for 1.5 h. As reported in Fig. 6a, the translocation isotherm of Cd was a saturating curve. The amount of Cd ions transported in the xylem sap seemed to have already reached saturation at 0.1 µM Cd^{2+} external concentration, and was linearly related (r^2 = 0.996) to the total amount of Cd accumulated in the shoots over a 10 d period (Fig. 6b).

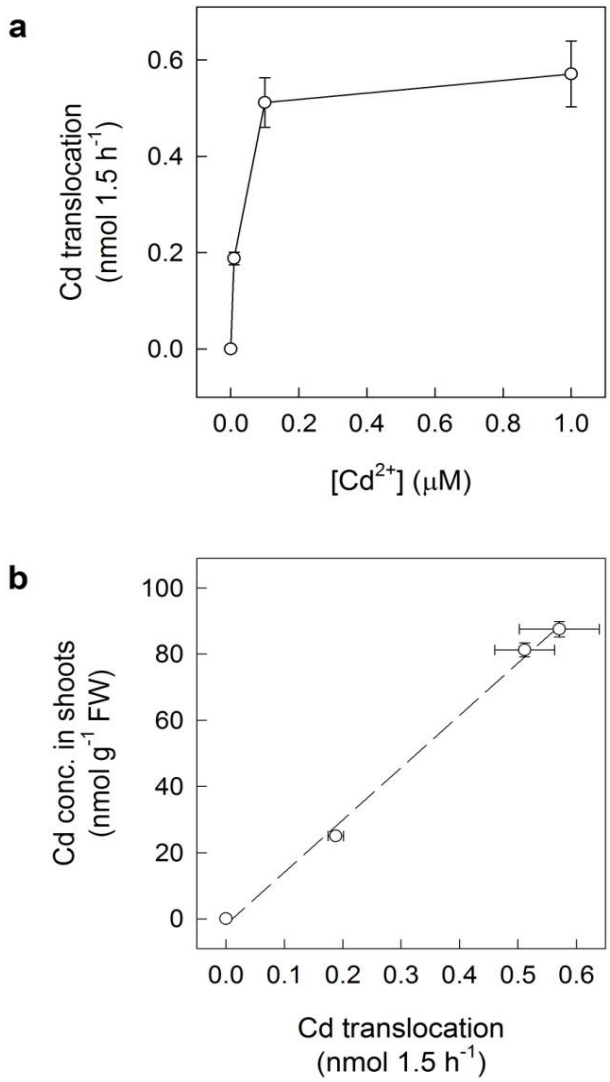


Fig. 6 : Analysis of Cd translocation in rice plants. Plants were exposed to different Cd concentrations (0.01, 0.1 and 1 µM) for 10 d. At the end of the exposure period, shoots were separated from roots and the xylem sap exuded from the cut (root side) surface was collected. (a) Cd ions loaded and transported in the xylem sap during 1.5 h. Data are means and SE of two experiments each performed with 16 plants (n = 32). (b) Relationship between Cd ions loaded in the xylem sap and Cd concentration in shoots after a 10 d period of Cd exposure. FW, fresh weight.

Finally, to investigate the possible role of OsHMA2v in Cd translocation, we studied the effect of different Cd exposures on the steady-state levels of its relative mRNA. RT-PCR analysis performed on root cDNA showed that the transcript levels of *OsHMA2v* were significantly higher in Cd-exposed than in control plants (Fig. 7); however, also in this case, the steady-state levels of the transcripts did not appear to vary under all the Cd exposure conditions analyzed.

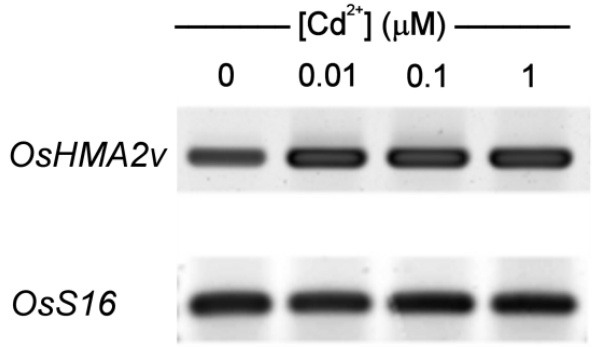


Fig. 7 : RT-PCR analysis of *OsHMA2v* gene expression in rice roots. PCRs were carried out for 30 cycles where cDNAs were exponentially amplified. PCR products were separated in agarose gel and stained with synergy brands (SYBR) Green I. Signals were detected using a laser scanner with 532 nm laser and 526 nm filter. *OsHMA2v*, heavy metal P_{1B}-ATPase 2; *OsS16*, S16 ribosomal protein.

3. Discussion

The data presented in this work have been obtained by using rice plants exposed to a broad range of relatively low Cd concentrations (from 0.01 to 1 μM), with the specific aim of simulating the real Cd concentrations experienced by roots in contaminated paddy field soils. As expected, rice capacity to absorb Cd was dependent on the metal concentration in the hydroponic solution (Fig. 1a); however, data clearly indicate that Cd partition between roots and shoots significantly changed in the different conditions. Such a behavior seriously affected the percentage of Cd ions retained by roots (*i.e.* Cd root retention; Fig. 1b), suggesting that some of the biochemical events or physiological processes controlling metal-ion systemic homeostasis may become limiting for Cd translocation when Cd concentrations rise. To date, different mechanisms involving the metal chelation and sequestration pathways (Rauser 1995; Clemens 2006b), as well as a wide range of transport proteins belonging to various families (Williams, Pittman & Hall 2000; Hall &Williams 2003; Colangelo & Guerinot 2006; Grotz & Guerinot 2006; Ueno et al. 2010a), have been described as implicated in metal-ion homeostasis. Among these, the Cd detoxification mechanism based on the synthesis of PCs and the activity of metal transporters sequestering Cd into the vacuoles would seem the major players acting in a complex 'firewall system' which retains Cd in the roots and

limits its translocation to the shoots. Large numbers of plant species have been shown to respond to Cd and other heavy metals by producing PCs (Rauser 1995; Zenk 1996; Cobbett & Goldsbrough 2002). Such thiol compounds have largely been shown to be involved in Cd chelation and vacuolar sequestration (Clemens 2006b; Nocito et al. 2007). The isolation of two *Arabidopsis* mutants, *cad1* and *cad2*, which are deficient in PC and GSH biosynthesis, respectively, and consequently Cd hypersensitive, contributed to clarify the significance of PCs and related thiol-peptides for Cd detoxification in plants (Howden et al. 1995a,b; Cobbett et al. 1998; Ha et al. 1999). Although new and intriguing roles for PCs have been hypothesized in Cd mobility (Gong et al. 2003), a recent paper – comparing *Arabidopsis* wild-type and *cad1* genotypes grown under low Cd concentration – clearly showed that PCs allow a greater proportion of Cd to be retained in roots rather than being translocated to shoots, confirming the essential role of these peptides also in Cd root retention (Wong & Cobbett 2009). In our conditions, rising Cd exposure progressively increased the levels of NPTs of the roots and concomitantly decreased those of GSH (Fig. 2). Since GSH represents the main non-protein thiol in non-Cd-stressed root cells, we can reasonably suppose that the increase in NPT levels was ascribable to the synthesis of PCs, which, conversely, represent the most abundant class of NPTs in Cd-stressed root cells (Nocito et al. 2002, 2007). It is generally assumed that PC biosynthesis may be regulated in several ways, through a direct control of PCS level and activity or, indirectly, by a fine tuning of biosynthetic pathways leading to GSH production (Clemens 2006b; Nocito et al. 2007). In our experiments, the activation of PC biosynthesis was further supported by the RT-PCR analysis of *OsPCS* genes, whose transcript steady-state levels in the root tissues were significantly higher in Cd-exposed plants as compared to control plants (Fig. 3). However, although transcriptional control of PCS expression has been previously reported in wheat roots (Clemens et al. 1999), the induction of PC biosynthesis in rice roots seemed to be a post-translational response to Cd accumulation in the root cells (*i.e.* activation by metal-ions and/or metal-GSH complexes; Vatamaniuk et al. 2000), as the steady-state levels of the *OsPCS* transcripts in the roots were comparable under all Cd exposure conditions analysed and therefore did not account for the observed changes in NPT levels. PC-independent mechanisms of vacuolar sequestration, preventing high concentrations of free metal-ions from persisting in the cytosol, are thought to be central to the tolerance of plants to high levels of divalent cation metals. To date, several tonoplast-localized transporters have been supposed to be involved in Cd transport into the vacuole (Korenkov et al. 2007a,b), but the importance of these activities is not clear and still remains to be investigated in rice. Recently, Ueno et al. (2010a) described a member of the P_{1B}-ATPase transporter family, OsHMA3, as the main candidate for direct Cd sequestration into the vacuoles. Our results indicate that rising Cd exposures poorly influenced the transcript levels of *OsHMA3* in the roots (Fig. 3),

suggesting that the transcriptional regulation of this gene is not involved in the observed changes in Cd root retention. Since the natural variation occurring in *OsHMA3* may account for differences in Cd root retention among rice cultivars (Ueno et al. 2010a) but not for changes within the same cultivar under different Cd exposures, it is reasonable to suppose that this gene codifies for a structural component of a complex 'firewall system' which, in concert with other structural genes, constitutively acts in sequestering an excess of Cd ions into vacuoles of the root cells. Moreover, since direct Cd sequestration into the vacuole seems to be essential for the evolution and stabilization of Cd-PC complexes in the vacuole (Ortiz et al. 1995), it is possible to hypothesize a role for this transporter in the evolution of Cd-PCs complexes. Taken as a whole, as so far presented, the data strongly support the hypothesis that changes in Cd root retention were, at least in part, due to the synthesis of thiol-rich peptides. However, since studies on Cd chelation and sequestration have shown that the relative composition of Cd-PC complexes, and then their –SH : Cd^{2+} ratios, may change depending on both time of exposure and Cd concentration in the external medium (Murasugi, Wada & Hayashi 1981; Leopold, Günther & Neumann 1998; Leopold et al. 1999; Rauser 2003), we cannot conclude that a higher amount of thiol-rich peptides necessarily results in an enhanced root capacity to chelate, sequestrate and retain Cd in the roots. To solve this impasse, we performed a cellular Cd fractioning aimed at measuring the amount of Cd ions retained in thiol-peptide complexes. Results indicated that the percentages of the total Cd recovered in the three main fractions changed under the different exposure conditions (Table 1), suggesting that profound variations in the cellular state of Cd ions retained by roots occurred. Interestingly, buffer extracts (1–6) accounted for 29.8, 31.8 and 67.5% of Cd ions retained by roots of plants exposed to 0.01, 0.1 and 1 μM Cd^{2+}, respectively; as a consequence, the percentage of non-soluble Cd ions remaining in the pellet decreased as Cd external concentration increased. Such a finding strongly suggests that the observed changes in the root capacity to retain Cd ions were not ascribable to an increase of any form of Cd insolubilization into the root apoplast. Finally, gel filtration analysis of the anionic fractions revealed that the amount of Cd retained in the HMW and LMW complexes, as well as its partition between these, profoundly changed in the different conditions, indicating that, as in other cereals (Rauser 2003), HMW and LMW Cd-binding complexes of rice roots were in a dynamic state depending on Cd external concentrations (Fig. 4). It is generally assumed that the major sites of Cd sequestration are the vacuoles of the root cells. Ortiz et al. (1992, 1995) proposed that in *Schizosaccharomyces pombe*, the first products of Cd chelation are cytosolic LMW Cd-PCs complexes, which are compartmentalized into the vacuole by means of a specific ABC-type transporter. Once inside the vacuole, the LMW complexes incorporate S^{2-} and other Cd ions, evolving into the more stable HMW Cd-PCs complexes. Thus, in a population of dividing cells, the

two complexes are part of a dynamic process in which LMW Cd-PCs complexes function as cytosolic carriers, while the vacuolar HMW Cd-PCs complexes represent the major Cd storage forms in the cells. Several features of this model have also been found in higher plants (Vögeli-Lange & Wagner 1990; Salt & Rauser 1995; Rauser 2003; Clemens 2006b). Starting from these findings, we can reasonably assume that Cd ions in the HMW complexes of the root cells may not be available to be translocated from roots to shoots. 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). For these reasons, it seems reasonable to suppose that Cd ions in cytosolic LMW complexes are not available for long-distance root-to-shoot transport via the xylem. Another important aspect to be considered concerns the cellular state of Cd ions contained in the ashes and in the acidic fractions. Since these metal-ions are probably tightly adsorbed to cellular matrices or apoplast components (Weigel & Jäger 1980; Khan et al. 1984), they also should be considered as not available to be translocated. Indeed, the protocol used to desorb rice roots (washing in 5 mM $CaCl_2$; see Materials and Methods section) removes Cd ions from the root surface and the extracellular spaces of the root cortex, but does not allow the removal of Cd ions from the extracellular spaces in the stele (Rauser 1987, 2003), which should be considered as an important component of the total Cd retained by the root. Finally, the fraction of buffer-soluble Cd not immobilized by the anion exchanger may have come from Cd ions in free cytosolic form and/or aspecifically bound with organic or inorganic ligands to form cellular complexes of relatively low thermodynamic stability. These Cd ions appear to have all the requisites to be considered the major Cd pool with relatively high mobility in the root cells and, then, potentially available for long-distance transport from roots to shoots.

Figure 8a reports data about Cd fractioning expressed as a function of the total amount of Cd retained by roots. From this plot, we can easily show the changes in the percentages of Cd ions in each fraction in relation to the severity of the Cd stress imposed. The proportion of Cd ions retained in Cd-binding complexes was similar in the roots of plants exposed to 0.01 and 0.1 μM Cd^{2+}, and then dramatically increased at the highest concentration analysed; conversely, the proportion of Cd ions tightly bound to cellular components (acid soluble + ash) increased showing a saturation-like curve. Thus, aspecific adsorption to cellular components strongly contributed to Cd root retention at

the two lowest Cd concentrations analysed. The amount of potentially free Cd ions necessarily results from a complex equilibrium between Cd chelation, sequestration and adsorption. In this view, the gradual saturation of cellular sites with potential for Cd adsorption may lead to a transient increase in the activity of free Cd ions, which, in turn, stimulates PC biosynthesis and then Cd sequestration in Cd-binding complexes (Fig. 8a). Thus, it is interesting to note that the proportions of both potentially mobile (cationic) and nonmobile (complexed + acid soluble + in the ashes) Cd ions in the roots did not change under all the analysed conditions (Fig. 8b). Such a finding strongly suggests that other mechanisms – other than those involved in metal-ion sequestering and immobilization in root cells – may play a key role in limiting the systemic allocation of Cd ions especially at higher external concentrations, resulting in an increase in the root capacity to retain Cd. Otherwise, if this were not the case, a constant root retention should be expected under all the analysed conditions. The mechanisms involved in metal-ion systemic allocation are thought to be good candidates to account for this limit, since Cd translocation via the xylem was indicated as one of the major and common physiological process determining Cd accumulation in shoots and grains of rice plants (Uraguchi et al. 2009). Our results on Cd concentration in the xylem sap revealed that the amount of Cd ions loaded into the xylem had already approached saturation at 0.1 μM Cd^{2+} external concentration (Fig. 6a). Since free Cd ions in the cationic fraction of the roots should be considered as potentially available to be translocated to the shoots, it seems interesting to analyze data in relation to the amounts of Cd ions found in this fraction. Such an analysis returned a translocation isotherm properly describable by a single hyperbolic Michaelis–Menten curve (r^2 = 0.993, $K_{1/2}$ = 17.79 ± 4.27 nmol g^{-1} FW, and V_{max} = 0.67 ± 0.04 nmol 1.5 h^{-1}) probably resulting from the activities of transport systems which reached a saturation-like condition when moving towards higher Cd concentrations (Fig. 8c). Although the identity of these systems is still unknown in rice, we can reasonably suppose that some transporters governing the translocation of chemically related essential metals (*i.e.* Zn^{2+}) throughout the plant may also play a pivotal role in determining the amount of Cd ions loaded into the xylem and then the 'passive' capacity of the roots to retain them, especially at the highest external concentrations. In other words, the amount of Cd ions escaping the 'root firewall' and not loaded into the xylem vessels may be predicted as a component of a 'passive system' resulting in Cd root retention (see Fig. 8d). Similar conclusions have been reported in work aimed at comparing genotypes of wheat and rice showing different tendencies to accumulate Cd in shoots and grains (Harris & Taylor 2004; Uraguchi et al. 2009). Good candidate genes for the control of Cd translocation in rice are the orthologs of *AtHMA2* and *AtHMA4*, which have been shown as the main genes controlling Cd systemic allocation in *Arabidopsis* (Mills et al. 2003, 2005; Eren & Argüello 2004; Verret et al. 2005; Wong & Cobbett 2009). Since the *in silico*

search for functionally related proteins in rice showed that OsHMA2 and OsHMA3 are the unique rice P_{1B}-ATPases belonging to the same cluster as AtHMA2, AtHMA3 and AtHMA4 in the P_{1B}-ATPase phylogenetic tree (see Appendix Fig. 2) – and OsHMA3 being a vacuolar transporter (Ueno et al. 2010a) – it appears reasonable to consider *OsHMA2* as the only putative ortholog of the *Arabidopsis* genes codifying for the Zn^{2+}/Cd^{2+} ATPases involved in Cd translocation. Although additional evidence is required to fully support this conclusion, the preliminary functional analysis of OsHMA2v in yeast confirms the involvement of this protein in mediating Cd efflux from the yeast cells, suggesting a putative role of OsHMA2v as Cd xylem-loading system in rice (Fig. 5). Finally, our results indicate that the steady-state levels reached by the *OsHMA2v* transcript under Cd exposure were independent of the Cd concentrations in the external medium and thus could account for the observed saturating kinetic of Cd translocation (Fig. 7).

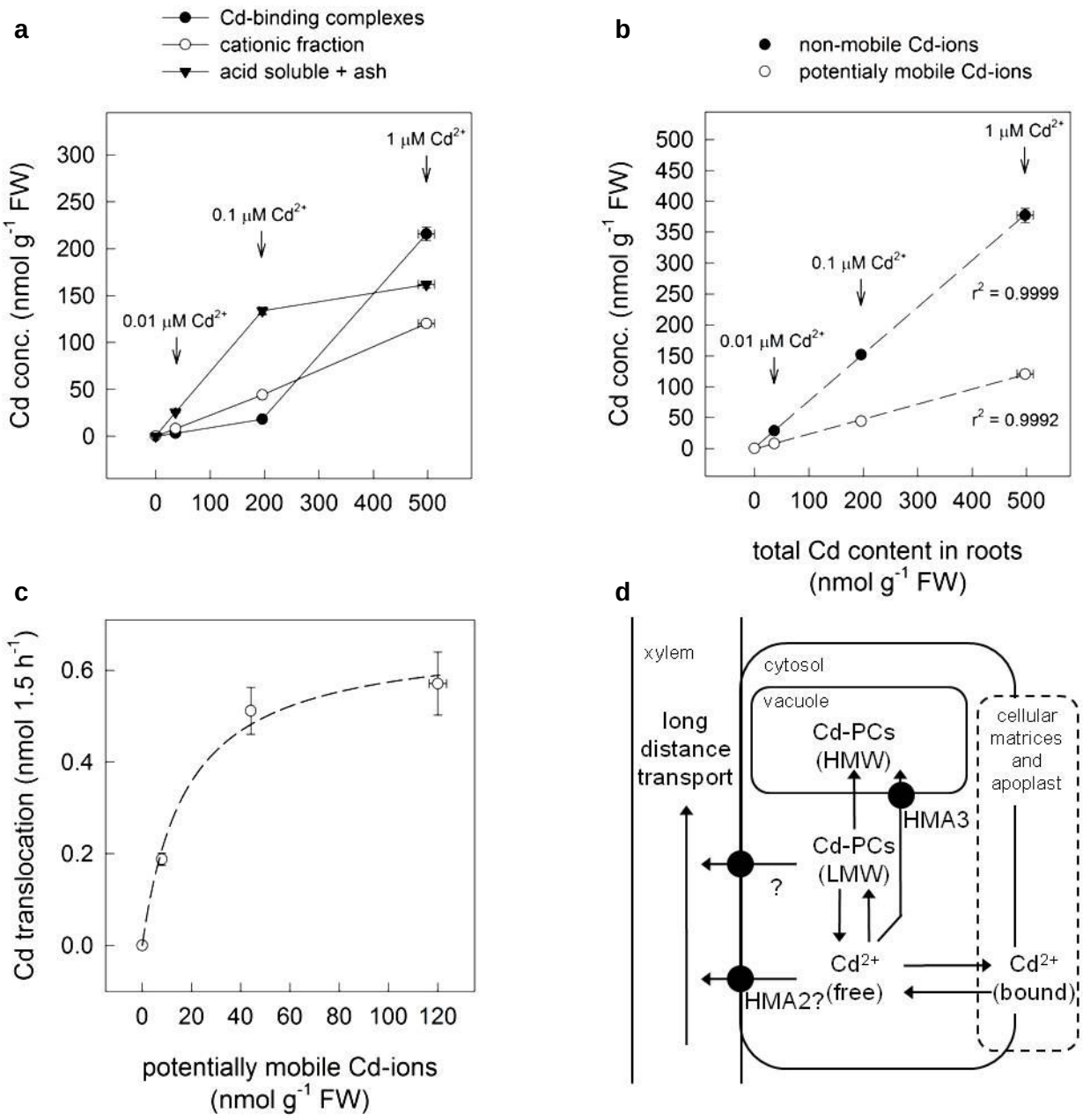


Fig. 8 : Integrated analysis of data about Cd fractioning and translocation and schematic representation of the root "firewall system" involved in Cd root retention in rice. (a) Partitioning of Cd-ions among the different fractions. (b) Partitioning of Cd-ions between potentially mobile and non-mobile fractions. (c) Cd translocation as a function of the amount of potentially mobile Cd-ions in the roots. Data are fitted with the kinetic equation: $y = (V_{max}\ x) / (K_{1/2} + x)$. (d) The amount of potentially mobile Cd-ions results from a complex equilibrium between biochemical events and physiological processes involved in Cd chelation, sequestration, adsorption and translocation. FW, fresh weight; HMW, high molecular weight; LMW, low molecular weight; PC, phytochelatin.

In conclusion, data presented in this work indicate that Cd root retention capacity in rice results from several different processes. In particular, we showed Cd chelation, compartmentalization, adsorption and translocation processes as components of a complex 'firewall system' which acts in limiting Cd translocation from the root to the shoot, reaching different equilibrium positions depending on Cd external concentration. The contribution of each of these processes to Cd immobilization in the root system seems to be dependent on the amount of Cd taken up by plants; thus, changes in Cd external availability result in different Cd root retention capacities, which do not always seem to be high enough to limit Cd translocation to shoots. Considering the importance of this trait in limiting the amount of Cd that may enter the food chain, it is important also to note that low levels of Cd contamination in paddy field soil may potentially result in worrying Cd accumulation in the shoots, and thus in edible parts of the plants, since in these conditions, the minimal Cd root retention capacity may be expected.

Chapter 2:

Cadmium translocation in rice (Oryza sativa L.*): towards the definition of a Zn independent pathway*

ABSTRACT

An advancement in the characterization of OsHMA2, recently found to be able to transport Cd, provided evidence also of its ability to transport Zn^{2+}, thus confirming it is a Zn^{2+}/Cd^{2+}ATPase that is likely to be involved in the xylem loading of both metals. Such a finding led to identify a transport pathway Zn and Cd have in common for which they could undergo competition effects. In order to investigate such an occurrence, rice plants were differentially exposed to increasing Cd and Zn external concentrations, while maintaining a steady Zn and Cd external level, respectively. The analysis of the xylem sap concentration of both metals revealed that the Zn xylem content significantly decreased as far as the external Cd concentration increased. By contrast, the Cd xylem content resulted mostly unaffected by the increasing external Zn concentration. These results suggested Cd competes with Zn for a xylem loading system but not *vice versa*, so it is reasonable to suppose the existence of another transport system responsible for the xylem loading of Cd which is Zn-indipendent. A good candidate for such a task is OsHMA4 which the *in silico* analysis indicated as a putative Cu^{+}/Ag^{+} ATPase. Preliminary assays in yeast showed it is able to transport Cd but not Zn, supporting the hypothesis that root-to-shoot translocation of Cd and Zn occur via differential pathways, just partially shared.

Key-words: cadmium, zinc, xylem loading, P_{1B}-ATPase transporters, *Oryza sativa* L.

1. Introduction

Zinc (Zn) is an essential nutrient for plants, playing several roles in cellular processes, which highlights the diverse biological functions of metal ions. Zn is in fact involved in protein, nucleic acid, carbohydrate and lipid metabolism. In addition, it is critical for the control of gene transcription and the coordination of other biological processes regulated by proteins containing DNA-binding Zn-finger motifs (Rhodes & Klug 1993), RING fingers and LIM domains (Vallee & Falchuk 1993). Several molecules associated with DNA and RNA synthesis are also Zn metalloenzymes, such as RNA polymerases (Wu et al. 1992), reverse transcriptases and several transcription factors (Wu & Wu 1989). The relevance of these processes for cell metabolism makes clear the need for them to be tightly regulated, in order to provide the required amount of Zn and to prevent the toxic effects caused by its excess (Ishimaru et al. 2011). However, it is Zn deficiency that nowadays is a serious agricultural problem as almost one half of the cereal-growing soils in the world contain low Zn (Graham & Welch 1996; Cakmak et al. 1996, 1999). This cause cereals to be inherently low in Zn concentrations to meet daily requirement of humans thus causing Zn deficiency symptoms and pathologies, especially in developing world where cereal grains, especially wheat and rice, contribute to about 70 % of the daily calorie intake (Cakmak 2008). 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). Under Zn-deficient soil conditions, plants show a high susceptibility to environmental stress factors such as drought stress and pathogenic infections, and develop severe symptoms such leaf necrosis and stunting growth (Alloway 2007).

Interestingly, the movement of Zn through the plant has been seen to be often coupled with Cd, both at the uptake and the translocation level. Gene expression analysis revealed that a high number of genes belonging to the ZIP (Zinc/Iron regulated Proteins) family are constitutively expressed in the zinc-hyperaccumulator *Arabidopsis halleri*. These genes encode metal transporter proteins responsible for the uptake of Zn but also Cd, as demonstrated by the inhibition of both short-term Cd influx and long-term Cd accumulation caused by Zn treatment (Ueno et al. 2008; Zhao et al. 2006; Pence et al. 2000). Bert and coworkers (2003) also obtained a significant positive correlation between the accumulation of Zn and Cd in the backcross progeny of *A. halleri* and the non-accumulator *A. lyrata* spp. *petraea*. Competitive interaction between Cd and Zn in the uptake processes has also been proved in non-accumulator plants (Cataldo et al. 1983), including crop species (Hart et al. 2002, 2005). In addition to the uptake processes, Zn and Cd have been proven to have similar translocation dynamics, probably due to shared transport systems responsible for the xylem loading of both metal ions. Under Zn deficiency conditions, for instance, the low Cd trait has

been associated with low Zn concentration in the flag leaf in wheat, therefore it is likely that the low grain Cd trait may be connected with decreased Zn accumulation in grains (Hart et al. 2005). Same wise, a strong and positive correlation has been observed between Cd and Zn shoot concentration in rice, where the root-to-shoot translocation via the xylem has been proved to be the major physiological process determining Cd distribution and thus accumulation (Uraguchi et al. 2009).

In *A. thaliana* two members of the P_{1B}-type ATPase family, AtHMA2 and AtHMA4, were found to play an important role in root to shoot Zn translocation (Hussain et al. 2004; Verret et al. 2005). Interestingly, the heterologous expression of AtHMA4 in yeast increased its resistance to Zn and Cd, whereas disruption in AtHMA4 function resulted in increased levels of such metals (Mills et al. 2005). Both AtHMA2 and AtHMA4 are localized on the plasma membrane and the genes encoding them result predominantly expressed in the vascular bundles (Verret et al. 2005; Mills et al. 2003, 2005; Hussain et al. 2004), which suggests their function as efflux pump to extrude the excess of the metal 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). Moreover, the *Athma2-Athma4* double mutant showed a strongly reduced root-to-shoot Cd translocation, confirming that AtHMA2 and AtHMA4, classified as Zn^{2+}ATPases, are also able to move Cd and are responsible for its xylem loading and thus translocation (Hussain et al. 2004). The role of HMA4 in Cd and Zn movement has also been confirmed in hyperaccumulator species as *T. caerulescens*, where HMA4 was seen to be involved in the xylem loading of both Zn and Cd (Papoyan & Kochian 2004), and *A. halleri*, where the decreased level of transcript of *HMA4* by RNA interference resulted in increased Cd root retention capacity (Hanikenne et al. 2008).

The well documented correlation existing between Cd and Zn transport pathways is the basis for addressing the problem of excessive grain Cd concentrations by applying agronomic practices aimed at reducing Cd accumulation. However, divergent results have been obtained. 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 & Bailey 1998). More recently, studies on wheat grown under Zn deficiency have shown that Zn fertilization is effective in reducing Cd accumulation in the grains when applied during the vegetative phase (Hart et al. 2005).

The contrasting effects of Zn application on Cd accumulation may depend on the several factors, not clearly understood yet, that interact both in the soil and within the plant. Studies conducted on

soybean report that the combination of Cd and Zn in the nutrient solution caused a significant decrease of Zn content in the shoots in case of long-term exposure. On the contrary, Cd shoot concentration slightly increased in these experimental conditions. Interestingly this finding seems to suggest that Cd competes with Zn in the root-to-shoot translocation but not *vice versa*. In addition, a remarkable difference was observed in the distribution of these metals through the whole plant: Cd was in fact almost entirely retained in the roots, whereas a large amount of Zn resulted allocated to the shoots (Chaoui et al. 1997). Differences in Cd/Zn transport and allocation dynamics have also been proved in cereal crops. Comparing two near isogenic lines (NILs) in wheat, Cd resulted highly concentrated in the bracts of the low Cd accumulating cultivar and in the developing grains of the high accumulating Cd cultivar instead. By contrast, no large difference between the two isolines was observed in the pattern of Zn distribution among spike components (Hart et al. 2005). These results highlight that although Zn and Cd have similar chemical properties, and plasma membrane transport of the two metals is likely to take place via shared transport systems (Pence et al. 2000; Clemens 2001; Hart et al. 2002), their translocation from root to shoot occur via differential pathways, just partially shared, that still need to be identified and fully understood.

Despite the economical and agricultural importance of rice, there is still a considerable lack of knowledge on these topics. Recently, OsHMA2, a putative Zn^{2+}/Cd^{2+}ATPase, has been partially characterized and found to be able to transport Cd. This suggested it could be involved in root-to-shoot Cd translocation, taking part in the complex network of physiological processes determining Cd distribution (Nocito et al. 2011).

Therefore, the objective of this study was to further explore and characterize Cd translocation dynamics. With this aim, we focused our attention on the identification of transporters likely to be involved in Cd xylem loading and investigated possible interactions with other metals Cd might compete with for the same transport system(s). Hence, in the present study we present an advancement in the characterization of OsHMA2, providing evidence it is a Zn^{2+}ATPase and thus identifying a transport pathway Zn and Cd have in common. Also, we identified in OsHMA4 an additional Cd transport system which, interestingly, is Zn-independent.

2. Results

2.1. OsHMA2v heterologous expression in yeast

The heterologous expression of OsHMA2v in yeast showed an enhanced growth upon Zn exposure of the *S. cerevisiae* INVSc1 strain expressing OsHMA2v under the control of the *GAL1* promoter (Fig.1). An increased tolerance of OsHMA2v expressing yeast to Zn was observed at 20 mM $ZnCl_2$, whereas almost no growth of the yeast cells harboring the empty vector could be detected at 22.5 mM $ZnCl_2$. These results confirmed the role of OsHMA2v as a transport system of both toxic metals (Cd) (Nocito et al. 2011) and physiological metals (Zn).

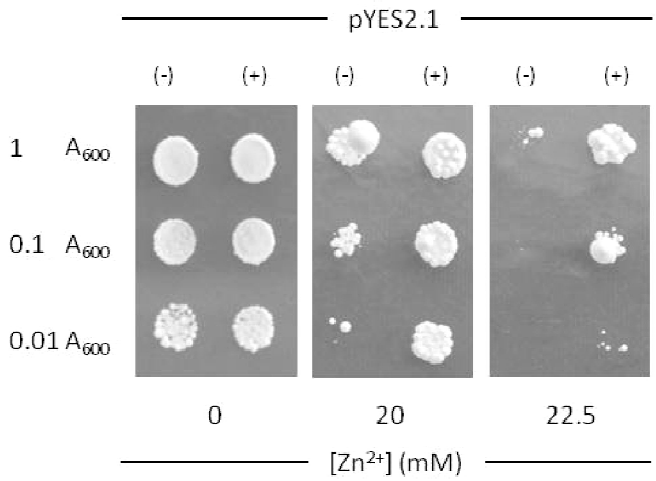
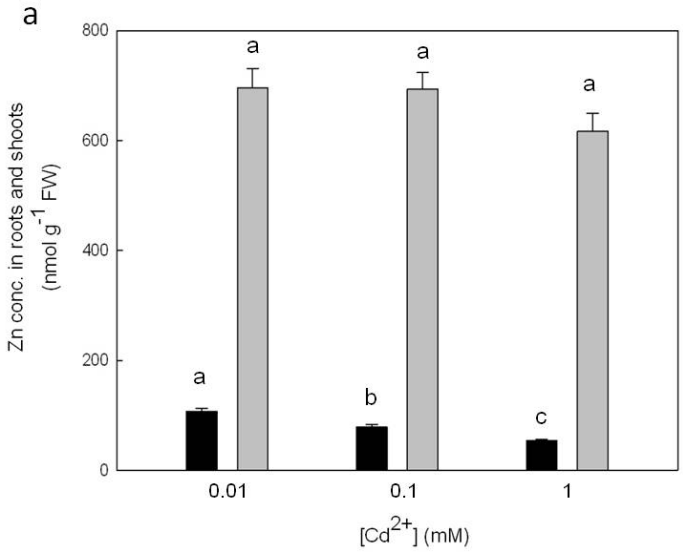


Fig. 1 : Heterologous expression analysis of OsHMA2v. Yeast cells expressing OsHMA2v under the control of the inducible *GAL1* promoter (+) or harboring the empty pYES2.1 vector (-) were grown at 30 °C for 3 d on SG media supplemented or not with 20 or 22.5 mM $ZnCl_2$.

2.2. Zn partitioning between root and shoot in increasing Cd concentration

In order to investigate the effect of Cd exposure on Zn distribution through the plant, Zn concentration was measured in roots and shoots of rice plants treated with increasing Cd concentrations (0.01 – 0.1 – 1 µM) for a10 days period.

a



b

	Zn content (nmol g^{-1} FW)	
	Buffer soluble - cationic fraction	**Total root content**
0.01 µM Cd^{2+}	77.64 ± 3.96 (a)	695.92 ± 35.04 (a)
0.1 µM Cd^{2+}	75.74 ± 3.33 (a)	693.42 ± 30.11 (a)
1 µM Cd^{2+}	111.45 ± 6.13 (b)	616.41 ± 32.92 (a)

Fig. 2 : Zn partition in rice plants and determination of the Zn cationic fraction in increasing Cd concentration. Plants were exposed to different Cd concentrations (0.01, 0.1 and 1 µM) maintaining a steady concentration of Zn (1 µM) for 10 d. (a) Zn partition between roots (grey bars) and shoots (black bars). (b) Zn cationic fraction determined in rice roots. Bars and error bars are means and SE of three experiments run in triplicate (n = 9). Different letters indicate significant differences between Cd treatments (P < 0.05). FW, fresh weight.

Two different trends could be identified (Fig. 2a): the former characterizing the shoots, showed a significant decrease as far as the external Cd concentration increased. The latter instead, characterizing the roots, did not present any significant change moving to the highest Cd concentration analyzed.

The amount of Zn retained in the roots was further analyzed by a fractioning experiment (for further details, see paragraph 2.5) that allowed the determination of the potentially free (cationic) Zn fraction (Fig 2b). This was found to maintain a steady level moving from 0.01 to 0.1 µM Cd^{2+}, accounting for 11.15 and 10.92% of Zn ions retained in the roots respectively, and then to significantly increase moving to the highest Cd concentration analyzed (18.08%).

2.3. Analysis of root-to-shoot Cd and Zn translocation in increasing Cd concentration

With the aim of exploring possible competition effects between Cd and Zn when both present in the nutrient solution, the concentration of the two metals was measured in the xylem sap of rice plants exposed to increasing Cd concentrations. Cd and Zn root-to-shoot translocation was thus derived by measuring the amount of Cd and Zn loaded and transported in the xylem for 1.5 h, as reported in Fig. 3.

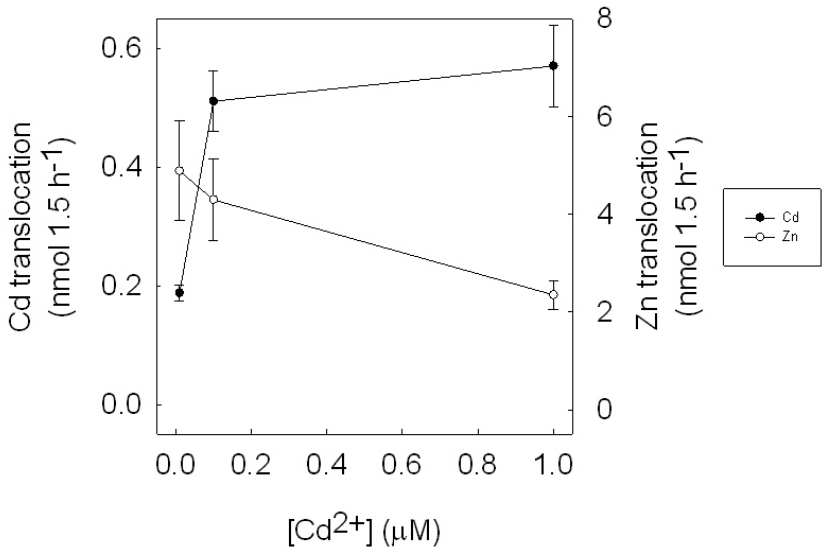


Fig. 3 : Analysis of Cd and Zn translocation in rice plants in increasing Cd concentration. Cd and Zn ions loaded and transported in the xylem sap during 1.5 h of plants exposed to different Cd concentrations (0.01, 0.1 and 1 µM) maintaining a steady concentration of Zn (1 µM) for 10 d. Data are means and SE of two experiments each performed with 16 plants (n = 32). FW, fresh weight.

As already noted by Nocito and coworkers (2011), the translocation isotherm of Cd showed a trend that can be described by a saturating curve (Fig. 3). In particular, the amount of Cd that could be transported into the xylem sap was found to approach the saturation at an external concentration of 0.1 µM Cd^{2+}. Interestingly, the Zn content in the xylem sap gradually decreased as far as the external Cd concentration moved to 1 µM Cd^{2+}, presenting the same trend observed in Zn accumulation in the shoots over the treatment period (Fig. 2a).

2.4. Cd and Zn partitioning between root and shoot in increasing Zn concentration

After investigating the potential effects of increasing Cd treatment on Zn retention and accumulation dynamics, we examined whether Cd distribution through the plant was affected by increasing Zn in the nutrient solution. Therefore, Cd and Zn contents were measured in roots and shoots of rice plants treated with increasing Zn concentrations (0.1 – 1 – 10 μM) while maintaining the same Cd treatment (0.1 μM) for a 10 days period.

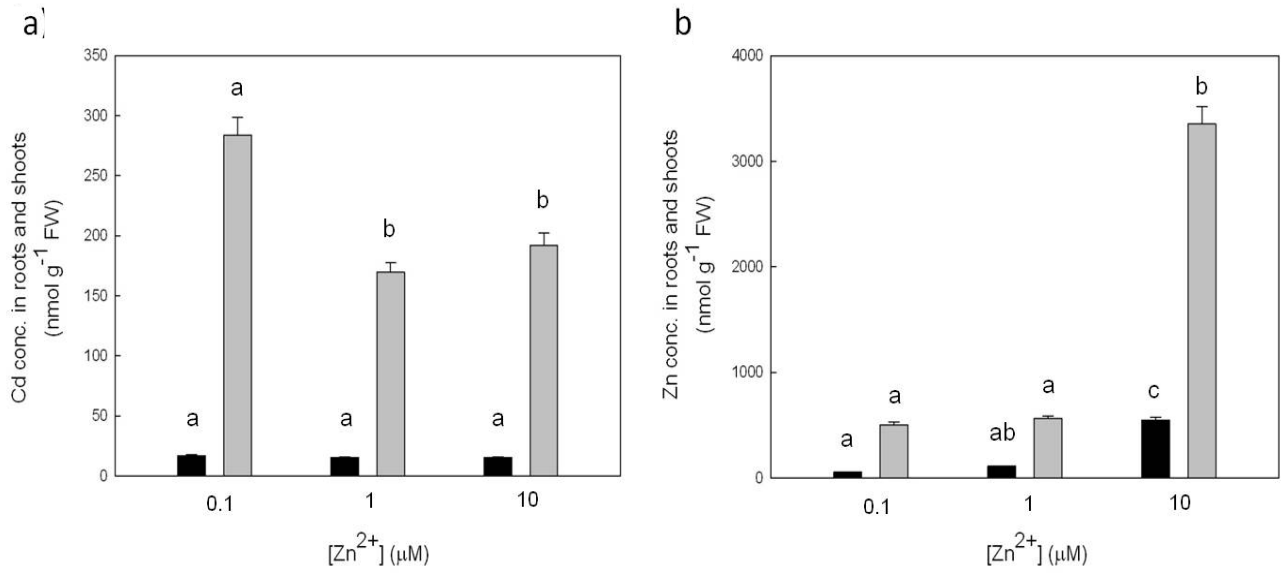


Fig. 4 : Cd and Zn partition in rice plants in increasing Zn concentration. (a) Cd partion between roots (grey bars) and shoots (black bars) in plants exposed to different Zn concentrations (0.1, 1 and 10 μM) maintaining a steady concentration of Cd (0.1 μM) for 10 d. (b) Zn partion between roots (grey bars) and shoots (black bars) in plants exposed to different Zn concentrations (0.1, 1 and 10 μM) maintaining a steady concentration of Cd (0.1 μM) for 10 d. Bars and error bars are means and SE of three experiments run in triplicate (n = 9). Different letters indicate significant differences between Cd treatments (P < 0.05). FW, fresh weight.

Considering Cd (Fig. 4a), the increasing external Zn concentration resulted in a significantly decreasing trend in the roots, whereas did not affect the shoot concentration. By contrast, the Zn content resulted significantly enhanced both in roots and shoots moving to the highest Zn concentration analyzed (Fig. 4b). In the former case, a remarkable increase was observed moving from 1 to 10 μM Zn^{2+}, whereas in the latter, significant changes were observed as far as the Zn treatment increased.

2.5. Fractioning of Cd and Zn in rice roots in increasing Zn concentration

According to the method proposed by Rauser & Mewly (1995) basing on sequential extractions in buffer and acid, a fractioning experiment was carried out in rice roots of plants exposed to increasing Zn concentrations. The extraction procedure resulted in three main fractions: the buffer soluble (extracts 1 - 6), the acid soluble (6 - 9) and the ash, consisting in the exhausted pellet. The buffer extract was further processed by means of anion exchange chromatography. The different affinity for the positively charged resin of Zn/Cd ions in the free ionic form on one hand, and complexed in thiolate compounds or to other organic molecules on the other, allowed the separation of the potentially mobile cationic fraction from the anionic fraction that is likely to be stably retained in the roots.

In Tab.1 the results from representative experiments are reported as well as the relative SE.

Tab 1: Fractioning of Cd and Zn ions retained in rice roots in increasing Zn concentration.

(I)

	Cd content (nmol g^{-1}FW)				
	Buffer soluble (1-6)				
	Anionic	**Cationic**	**Acid soluble (7-9)**	**Ash**	**Total**
0.1 μM Zn^{2+}	42.64 ± 2.13 (a)	19.74 ± 1.03 (a)	206.57 ± 10.53 (a)	14.88 ± 0.74 (a)	283.83 ± 14.44 (a)
1 μM Zn^{2+}	29.87 ± 1.28 (b)	16.08 ± 0.90 (a)	115.21 ± 5.07 (b)	8.57 ± 0.37 (b)	169.74 ± 7.62 (b)
10 μM Zn^{2+}	24.38 ± 1.27 (b)	28.23 ± 1.58 (b)	128.29 ± 7.06 (b)	10.81 ± 0.56 (b)	191.71 ± 10.47 (b)

(II)

	Zn content (nmol g^{-1}FW)				
	Buffer soluble (1-6)				
	Anionic	**Cationic**	**Acid soluble (7-9)**	**Ash**	**Total**
0.1 μM Zn^{2+}	32.60 ± 1.76 (a)	55.08 ± 2.97 (a)	243.13 ± 13.85 (a)	172.70 ± 9.50 (a)	503.52 ± 28.09 (a)
1 μM Zn^{2+}	29.87 ± 1.28 (a)	61.58 ± 3.63 (a)	279.29 ± 12.01 (a)	180.25 ± 7.39 (a)	563.75 ± 24.87 (a)
10 μM Zn^{2+}	163.91 ± 11.11 (b)	377.63 ± 18.13 (b)	2405.87 ± 115.48 (b)	404.60 ± 19.42 (b)	3352.08 ± 164.18 (b)

(I) Cd fractioning in plants exposed to different Zn concentrations (0.1, 1 and 10 μM) maintaining a steady concentration of Cd (0.1 μM) for 10 d. (II) Zn fractioning in plants exposed to different Zn concentrations (0.1, 1 and 10 μM) maintaining a steady concentration of Cd (0.1 μM) for 10 d. Cd and Zn retained in roots were extracted using the sequential procedure described in the 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 Cd treatment (P < 0.05).

Concerning Cd content in roots (I), an overall decrease was observed while increasing the external Zn concentration. However, it is interesting to note that the three fractions obtained by the extraction procedure differently contributed to such a decrease. The buffer extract (1 - 6), in fact, showed a significant increase as it accounted for 21.98, 27.07 and 27.44% of Cd ions retained by roots of plants exposed to 0.1, 1 and 10 µM Zn^{2+}, respectively. By contrast, both Cd extracted in ice-cold 100 mM HCl (extracts 7 – 9) and Cd measured in the exhausted pellet (ash) were found to be significantly reduced as far as the external Zn concentration increased, particularly moving from 0.1 to 1 µM Zn^{2+}. Moreover, analyzing in details the buffer extract by means of anion exchange chromatography, it appeared that the enhancement of Cd in such an extract was given by the increase in the potentially mobile Cd cationic fraction, which significantly rose moving to the highest Zn concentration analyzed.

The determination of Zn root content (II), instead, showed an overall increase while moving to higher Zn concentrations in the nutrient solution. Such a trend appeared to be equally determined by the three fractions obtained by the fractioning procedure, in that all of them presented a significant increase moving from 1 to 10 µM Zn^{2+}. In particular, taking into account the increase of Zn in the buffer extract, it was observed that both the anionic and the cationic fractions positively contributed to it, showing again a significant increase moving to the highest Zn concentration analyzed.

2.6. Analysis of root-to-shoot Cd and Zn translocation in increasing Zn concentration

In order to verify whether Zn treatment had an effect over Cd translocation dynamics, the concentration of both Cd and Zn was measured in the xylem sap of rice plants exposed to increasing Zn concentrations. As abovementioned, Cd and Zn root-to-shoot translocation was estimated by measuring the amount of Cd and Zn loaded and transported in the xylem for 1.5 h.

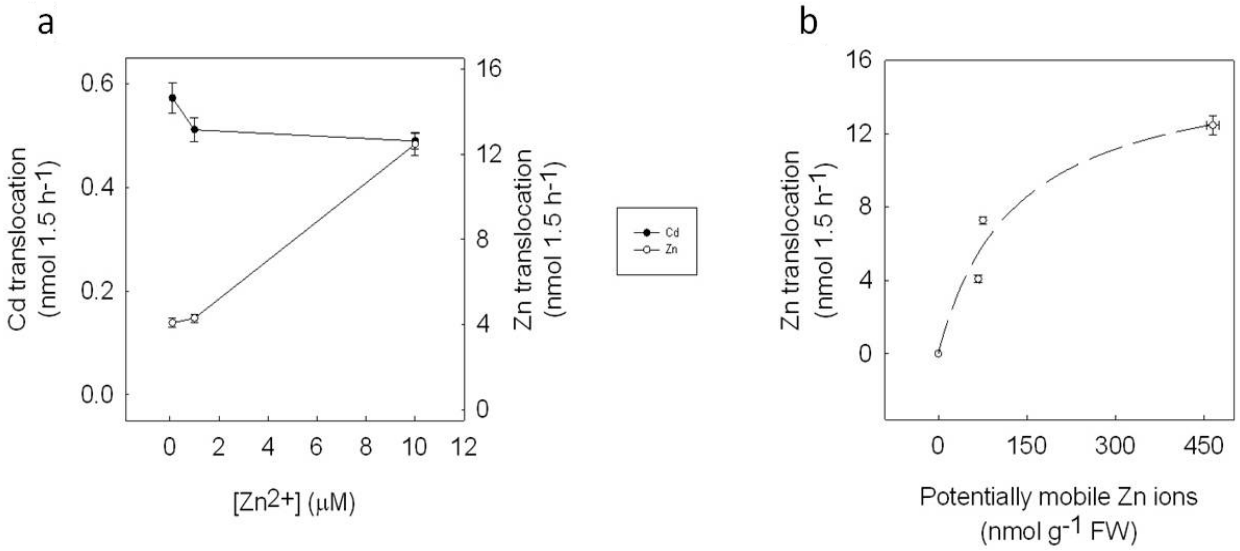


Fig. 5 : Analysis of Cd and Zn translocation in rice plants in increasing Zn concentration. (a) Cd and Zn ions loaded and transported in the xylem sap during 1.5 h of plants exposed to different Zn concentrations (0.1, 1 and 10 µM) maintaining a steady concentration of Cd (0.1 µM) for 10 d. Data are means and SE of two experiments each performed with 16 plants (n = 32). FW, fresh weight. (b) Zn translocation as a function of the amount of potentially mobile Zn ions in the roots. Data are fitted with the kinetic equation: $y = (V_{max}\ x) / (K_{1/2} + x)$.

In these experimental conditions (Fig. 5a), the amount of Zn loaded and transported in the xylem resulted enhanced, showing a remarkable increase moving from 1 to 10 µM Zn^{2+}. By contrast, the amount of Cd translocated resulted mostly unaffected, as just a very slight decrease could be observed while increasing the Zn^{2+} treatment.

Since the cationic fraction determined by anion exchange chromatography following sequential extractions can be considered as potentially mobile and thus available to be translocated, the amount of Zn ions found in such a fraction can be related to the Zn content detected in the xylem sap (Fig. 5b). This analysis resulted in a translocation isotherm that can be described by a single hyperbolic Michaelis–Menten curve, characterized by the following parameters: r^2 = 0.986; $K_{1/2}$ = 129.83 ± 0.62 nmol g^{-1} FW; and V_{max} = 15.99 ± 3.04 nmol 1.5 h^{-1}. Such a curve is likely to result from the activity of transport system(s) that approaches the saturation as far as the external Zn concentration increases.

2.7. Isolation and partial characterization of OsHMA4v

The full length gene encoding OsHMA4v was amplified by RT-PCR from total RNA extracted from rice roots. The amplification was carried out by using primers designed on the deduced ORF for *cv.* Nipponbare (LOC_Os02g10290) reported in the MSU Rice Genome Annotation Project Database and Resource (http://rice.plantbiology.msu.edu/). The resulting amplicon codifies a polypeptide of 978 amino acids, characterized by a predicted mass of 105.2 kDa. Moreover, the OsHMA4v aminoacidic sequence shares 99% identity with the protein predicted for OsHMA4 *cv.* Nipponbare.

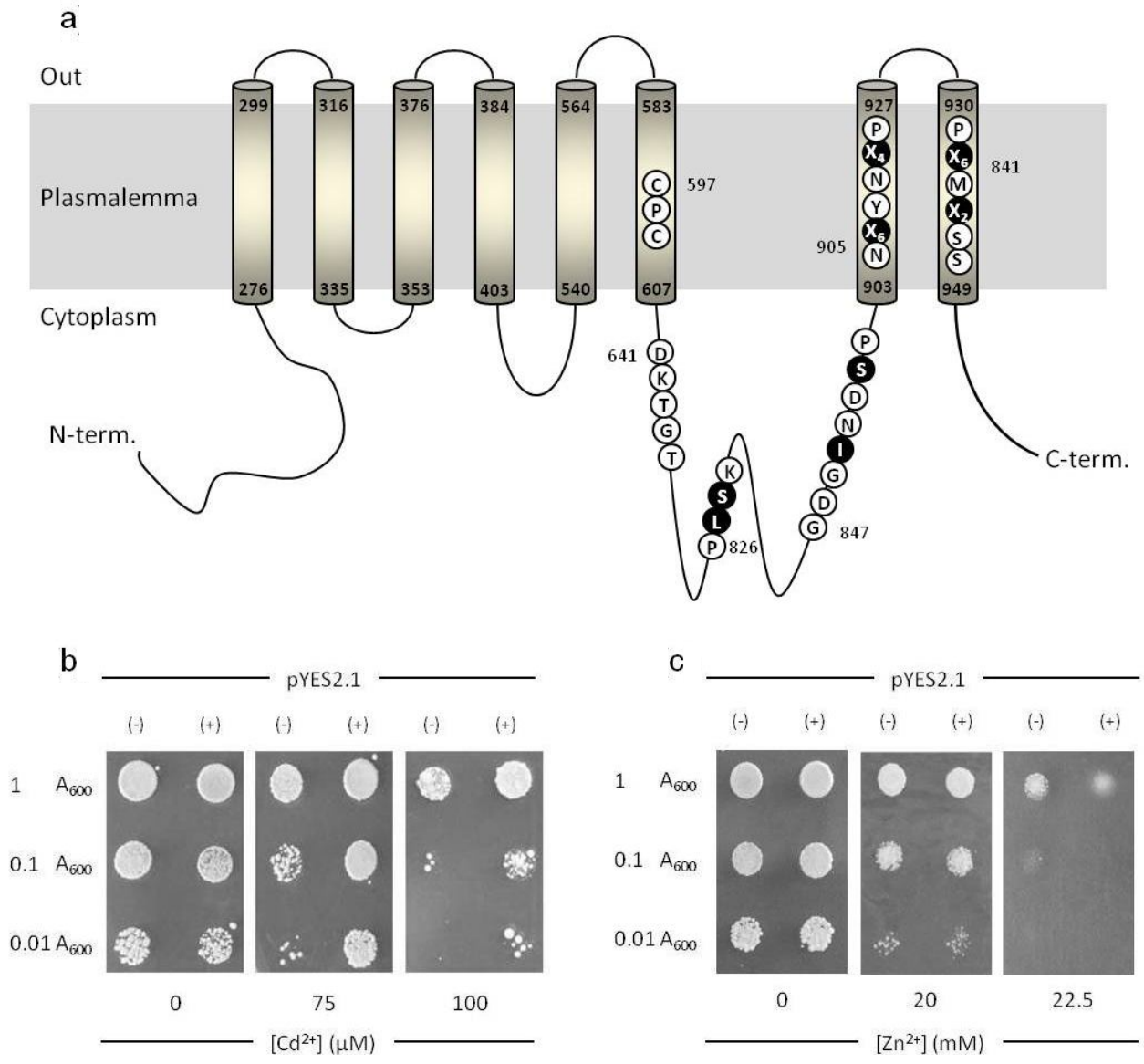


Fig. 6 : Membrane topology and functional characterization of OsHMA4v. (a) Eight TM helices are predicted in OsHMA4v using MEMSAT3. Numbers in bold indicate the position of TM segments within the OsHMA4v sequence. Signature sequences in TM6 [^{351}CPC^{353}], TM7 [^{905}N(X_6)YN(X_4)P^{918}] and TM8 [^{841}P(X_6)M(X_2)SS^{852}] are typical of all Cu^+/Ag^+ ATPases. (b) Yeast cells expressing OsHMA4v under the control of the inducible *GAL1* promoter (+) or harboring the empty pYES2.1 vector (-) were grown at 30 °C for 3 d on SG media supplemented or not with 75 or 100 mM $CdCl_2$ or (c) with 20 or 22.5 mM $ZnCl_2$.

The membrane topology prediction analysis (Fig. 6a) performed by software MEMSAT3 confirmed OsHMA4v as a transporter belonging to the P_{1B}-type ATPase family, due to the presence of: (a) eight predicted transmembrane domains (TMs); (b) the CPC (^{597}CysProCys^{599}) 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 functioning of the transporter as it contains the phosphorylatable aspartic residue. In addition, the signature sequences characteristic of the Cu^+/Ag^+ ATPase were also identified, *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 & Mills 2005). The analysis of the amino acidic sequence of OsHMA4v also revealed the presence of a short C-terminal end (29 amino acids) where, contrary to OsHMA2 (Nocito et al. 2011), no Cys-Cys repeated sequences nor His stretches could be identified. By contrast, OsHMA4 presented a relatively long N-terminal end (275 amino acids), where two repetitions of the highly conserved CysXXCys (^{48}CysXXCys^{51}, ^{122}CysXXCys^{125}) metal binding sequence (Lutsenko et al. 2003) were found. The length of the N-term of OsHMA4v, as well as the presence and the position of the first CysXXCys conserved motif, resembles very much the structure of AtHMA5, which is the Arabidospis P-type ATPase OsHMA4v shares the highest identity with (57%). In addition, OsHMA4v is also highly similar (47% identity) to AtHMA7 which, interestingly, presents the same pattern in tandem of the conserved CysXXCys (^{66}CysXXCys^{69}, ^{144}CysXXCys^{147}) motif.

In order to clarify the role of OsHMA4v in Cd and/or Zn transport, the protein was analyzed by means of heterologous expression in yeast exposed to Cd and Zn, respectively. A stronger tolerance to Cd was clearly observed in *S. cerevisiae* INVSc1 strain expressing OsHMA4v (Fig. 6b), whereas no evidence for enhanced tolerance to Zn was found (Fig. 6c). These results support the hypothesis that OsHMA4 functions as a pump and takes part in the detoxification process of Cd ions by extruding them into the apoplast. By contrast, it does not seem to be involved in Zn movement, and thus in its extrusion, when present in excess.

2.8. Expression profiles of *OsHMA2*, *OsHMA3* and *OsHMA4* in relation to Zn and Cd treatments

In order to verify whether the expression level of *OsHMA2*, *OsHMA3* and *OsHMA4* is affected by Cd and/or Zn treatment, their expression profiles were relatively quantified by performing a quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis in root.

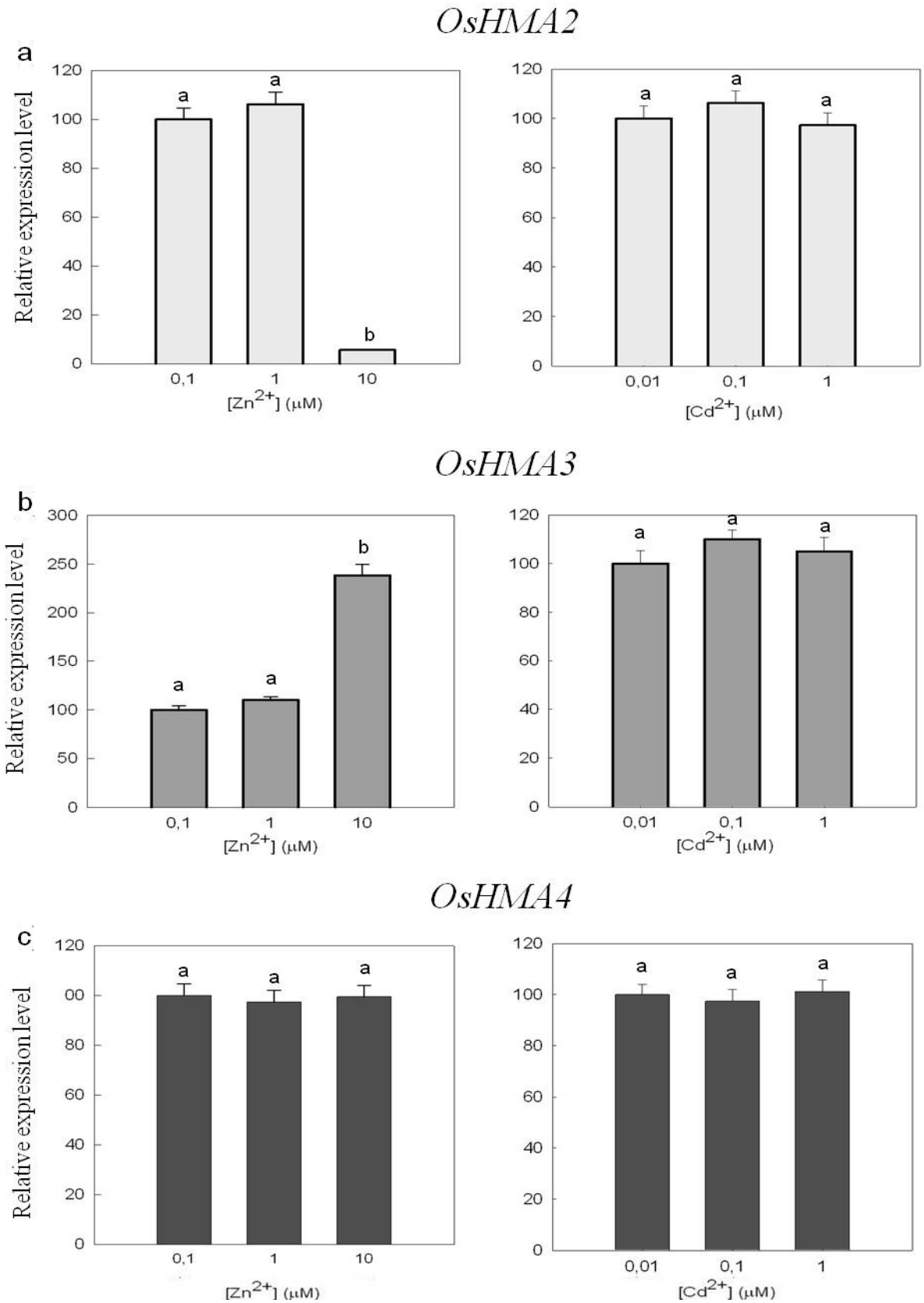


Fig. 7 : Relative quantification of *OsHMA2* (a), *OsHMA3* (b) and *OsHMA4* (c) expression level. The analysis was performed by TaqMan® gene expression assays in roots of rice plants exposed to: (left) increasing Zn concentrations (0.1, 1 and 10 µM) maintaining a steady concentration of Cd (0.1 µM) ; (right) increasing Cd concentrations (0.01, 0.1 and 1 µM) maintaining a steady concentration of Zn (1 µM) Cd (right). The housekeeping gene used as endogenous control was *OsUBC*.

A significant decrease in the *OsHMA2* transcript level was observed moving to the highest external Zn concentration analyzed (Fig. 4a). Indeed, whereas at 0.1 and 1 μM Zn^{2+} the transcript level was seen to maintain a steady-state, at 10 μM Zn^{2+} a considerable fall was observed, so that *OsHMA2* appears to be strongly downregulated by increasing the external Zn concentration. By contrast, when analyzed with respect to Cd treatment, *OsHMA2* expression level resulted basically unaffected, as it did not vary under the different concentrations analyzed. Differently from *OsHMA2*, *OsHMA3* gene expression resulted induced by Zn treatment (Fig. 4b). Again, only a slight difference was detected moving from 0.1 to 1 μM Zn^{2+}, whereas a remarkable increase was observed at 10 μM Zn^{2+}. Same as *OsHMA2*, *OsHMA3* transcript level did not change depending on Cd external concentration. Finally, when analyzing *OsHMA4*, it appeared that neither Zn nor Cd treatments induce significant differences in its expression level (Fig. 4c), as in both experimental conditions the gene expression profile maintained a steady-state.

3. Discussion

Most of the evaluation of the physiological effects of heavy metals excess on plants have been made for single elements, even though real cases of contamination, involving both soils and plants, are multiple rather than simple. For example, sewage sludge, mining and mine wastes, processed waste water and phosphate fertilizers are commonly contaminated with several heavy metals simultaneously (Sommers 1980; Mortvedt et al. 1981; Wallace & Berry 1989). In such cases, metals can mutually interact in a synergistic, additive and protective way in plants. Such an occurrence could actually take place also considering heavy metals and essential nutrients, especially in case of high chemical similarities between essential and non-essential elements (Wallace 1982; Wallace & Berry 1989).

Indeed, our study takes place in this context with the aim of examining the well documented interaction between Cd and Zn and investigating its possible effects on Cd accumulation. Such an interaction has been proved both at the uptake and the translocation level, in that Zn movement and distribution through the plant has been seen to be often coupled with Cd both in hyperaccumulator and non-hyperaccumulator species (Ueno et al. 2008; Zhao et al. 2006; Pence et al. 2000; Cataldo et al. 1983; Hart et al. 2002, 2005; Hussain et al. 2004). To the combined movement of Cd and Zn contribute different classes of transporters either responsible for their influx or efflux. Concerning the uptake of both metals, the ZIP (Zinc/Iron regulated Proteins) family has been seen to play a major role as it includes proteins that contribute to metal-ion homeostasis by transporting cations into the cytoplasm. Interestingly, several genes belonging to this family have been proved to be

constitutively expressed in species where increasing Zn concentrations inhibited the uptake and the accumulation of Cd, providing evidence that they are involved in the movement of both metals (Pence et al. 2000; Cataldo et al. 1983; Hart et al. 2002, 2005; Ueno et al. 2008). However, the interaction between Cd and Zn is of particular concern at the translocation level, especially in crop species. The root-to-shoot translocation via the xylem, in fact, has been proved to be the major physiological process determining Cd distribution and thus accumulation in rice, where a strong and positive correlation has been observed between Cd and Zn shoot concentration (Uraguchi et al. 2009). Same wise in wheat plants grown under Zn deficiency, the low Cd trait has been associated with low Zn concentration in the flag leaf suggesting that the low grain Cd trait may be connected with decreased Zn accumulation in grains (Hart et al. 2005). Such an outcome suggests that there might be a competition between these two metals which may be positively exploited in order to reduce Cd accumulation in the grains of cereal crops. Basing on the same premises, in fact, the application of Si to the growth medium was proved to markedly decrease As concentrations in both roots and shoots as well as the total As uptake in rice seedlings (Guo et al. 2005). Particularly relevant in the determination of Cd and Zn shoot accumulation are transporters belonging to the P_{1B}-type ATPase subfamily, which present several functions in the cell, ranging from metal delivery to cellular compartment to detoxification processes (Colangelo & Guerinot 2006; Williams & Mills 2005). In particular, in *Arabidopsis thaliana* AtHMA2 and AtHMA4 were found to be responsible for root-to-shoot Zn translocation, since the *Athma2-Athma4* double mutant showed a strongly reduced Zn concentration in the shoots (Hussain et al 2004). Moreover, both *AtHMA2* and *AtHMA4* resulted predominantly expressed at the root level in correspondence of the vascular bundles and the encoded proteins were found to be localized on the plasma membrane, which provided evidence they are involved in the xylem loading of Zn (Hussain et al. 2004; Verret et al. 2005; Mills et al. 2003, 2005). Interestingly, the abovementioned *Athma2-Athma4* double mutant also presented almost no Cd accumulation in the shoots and a significantly increased Cd content in the roots, suggesting that both AtHMA2 and AtHMA4, classified as Zn^{2+}ATPases, are also able to move Cd, and are responsible for its translocation (Hussain et al. 2004). Acknowledging the importance of such transporters in the control of Cd translocation, the identification of the orthologs of *AtHMA2* and *AtHMA4* in rice would be of great concern in understanding and characterizing the determinants of Cd accumulation in the grains. To date, the *in silico* analysis conducted on the P_{1B}-type ATPase subfamily in rice and *A. thaliana* led to the identification of two putative orthologs of the Arabidopsis genes codifying for the Zn^{2+}/Cd^{2+} ATPases involved in Cd translocation: *OsHMA2* and *OsHMA3* (Nocito et al. 2011). As OsHMA3 has been recently found to be a vacuolar transporter and take part in Cd root retention (Ueno et al. 2010a), OsHMA2 appears the only transporter in the

same cluster as AtHMA2 and AtHMA4 probably involved in Cd and Zn xylem loading. Such a transporter has been partially characterized in yeast with respect to Cd treatment: this analysis confirmed its role in Cd extrusion thus suggesting it could take part in the xylem loading of Cd (Nocito et al. 2011). In the present study we proved that OsHMA2v also confers increased tolerance to Zn supplied in excess by mediating its extrusion from yeast cells (Fig. 1). This finding provides further evidence that OsHMA2v is a Zn^{2+}/Cd^{2+} ATPase, as also predicted by the membrane topology analysis (Nocito et al. 2011) and thus is likely to be responsible for the xylem loading of both metals. This, in turn, would mean that Cd and Zn share a transport system at the translocation level, which may result in competition effects between the two metals when both present in the nutrient solution. Supporting this hypothesis is the determination of Zn partitioning between roots and shoots in increasing Cd concentrations: the decreasing trend observed in the shoots and the steady level, instead, measured in the roots, strongly suggest that Cd compete with Zn for the transport system that determines the xylem loading of both metals. By contrast, at the uptake level Cd does not seem to affect the amount of Zn that can be absorbed (Fig 2a). As abovementioned, it is well proven that Cd uptake is mediated by transporters that are actually devoted to Zn transport (*i.e.* ZIP family) for which a competition between Cd and Zn has been observed (Ueno et al. 2008; Zhao et al. 2006; Pence et al. 2000; Cataldo et al. 1983). However, there are additional classes of transporters not related to Zn that were proved to be able to move Cd, taking part in its uptake (Chen et al. 1999; Thomine et al. 2000; Clemens at al. 1998; White & Broadley 2003; White 2005; DalCorso et al. 2008; Pedas et al. 2008; Verbruggen et al. 2009, Curie et al. 2001, 2009). Therefore, it is possible that at the experimental conditions analyzed Cd influx takes place through different pathways, causing no pressure on Zn transport system thus resulting in no competition with it at the uptake level. It is interesting to note that even though the root Zn content is not significantly affected by Cd treatment, the different Zn fractions contributing to it are somehow modified, in that the Zn cationic fraction was seen to increase as far as the external Cd concentration increased (Fig 2b). Such a finding could be interpreted as the result of competition effects occurring between Cd and Zn within the cell: the increasing amount of free Cd ions resulting from increasing Cd concentrations in the nutrient solution (Nocito et al. 2011) would probably compete with Zn ions for the adsorption sites in the cellular matrices or apoplast components (Weigel & Jäger 1980; Khan et al. 1984) or may cause the displacement of Zn from Zn metalloenzymes (Wu et al. 1992; Wu & Wu 1989) or other Zn containing proteins (Rhodes & Klug 1993; Vallee & Falchuk 1993), producing an increase in the potentially mobile Zn cationic fraction. The occurrence of a competition of Cd over Zn at the translocation level is further strongly supported by the analysis of the root-to-shoot Zn translocation which revealed a significant decrease of the Zn content in the xylem sap, as far as the

Cd treatment increased (Fig 3). Such a trend is highly similar to the one observed in the shoots, confirming that the Zn contents in the xylem sap and in the shoots are positively correlated, as already observed concerning Cd (Uraguchi et al. 2009).

After verifying the existence of a competition of Cd over Zn and identifying at which level it takes place, our focus then extended to examine whether Zn treatment affects Cd distribution and accumulation processes. The analysis of Cd partitioning between roots and shoots revealed that increasing external Zn concentrations resulted in a significant decrease of Cd root content (Fig. 4a). Such a result shows that the highest Zn concentration analyzed (10 µM Zn^{2+}) produced a significant competition of Zn over Cd in the uptake via the abovementioned transport pathways (Pence et al. 2000; Ueno et al. 2008). Further analysis then revealed that to such a decrease differently contributed the fractions isolated by the fractioning procedure, highlighting that the Zn treatment had an effect on the cellular state of Cd. Indeed, a significant decrease was observed in the acid extract as well as in the ash (Tab. 1-I), but not in the buffer extract that, by contrast, increased accounting for 21.98, 27.07 and 27.44% of Cd ions retained by roots of plants exposed to 0.1, 1 and 10 µM Zn^{2+}, respectively. In particular, this increase is given by the cationic fraction, that significantly rises moving from 1 and 10 µM Zn^{2+}. Such an increase is, again, determined by competition effects between Cd and Zn occurring within the cell once both of them have been taken up by the nutrient solution. While moving to 10 µM Zn^{2+}, in fact, we provided a Zn concentration that is far higher than the physiological range normally required for cell metabolism, which is well simulated by the two first experimental conditions (Hoagland & Arnon 1950). Therefore, such a situation is likely to trigger the physiological response to an excess of metal that could become toxic. In order to limit the toxic effects of heavy metals or excess of essential nutrients, plants respond by producing a class of organic molecules that act as chelators limiting metal availability and thus their toxicity. Such compounds are Cys-rich peptides with the general structure $(\gamma\text{-Glu-Cys})_n$-Gly (n = 2–11) commonly known as phytochelatins (PCs) (Rauser 1995; Zenk 1996; Cobbett & Goldsbrough 2002). The highest Zn concentration analyzed probably activates the synthesis of PCs, as demonstrated by the increase observed in the anionic Zn fraction (Tab. 1-II), and strongly compete with Cd for them. Same wise, such a high Zn concentration is able to displace Cd adsorbed to the cellular components and to the apoplast, which is proved by the decrease of Cd in the acid extract as well as in the ash (Tab.1-I). As a result, these events cause a greater amount of Cd to be in the free ionic form and thus available to be translocated. Interestingly, contrarily to the roots, the shoots did not present significant changes in Cd content as far as the Zn concentration increased (Fig. 4a), suggesting that Zn does not compete with Cd for the transport system responsible for xylem loading. Such a hypothesis is further strongly supported by the analysis of root-to-shoot Cd

translocation which resulted in a slight decrease, not even significant, in the xylem sap Cd content moving to higher Zn concentrations (Fig. 5a).

Taken as a whole, the results reported so far seem to indicate that there is a competition of Zn over Cd at the root level, as increasing Zn treatment prevents and reduces Cd root content overall. However, changes in Cd cellular state induced by Zn treatment also highlighted that it induces an increase in the potentially mobile Cd fraction, which may turn in an enhanced Cd accumulation. Interestingly, almost no competition of Zn over Cd has been proved at the translocation level, whereas a strong one has been observed in the opposite direction, *i.e.* of Cd over Zn. Such an outcome reasonably suggests that the xylem loading of Cd occurs via different transport pathways, just partially shared with Zn. A good candidate to be involved in the xylem loading of both Cd and Zn could be OsHMA2, as it has been proved to be able to move both of them (Nocito et al. 2011; Fig. 1). If that was the case, the translocation isotherms of Cd and Zn would explain the relevance of such a transporter in the transport activities that determine the xylem loading of these metals: indeed, the slight decrease of Cd content in the xylem moving to higher Zn concentrations indicates that most of the xylem loading of Cd occurs via a transport system other than OsHMA2 (Fig. 5a). By contrast, the significant fall observed in the xylem sap Zn content would mean that OsHMA2 plays a major role in its xylem loading (Fig. 3). Therefore, OsHMA2 would constitute itself or at least remarkably contribute to the transport system responsible for the xylem loading of Zn, whose activity seems to approach the saturation moving to higher Zn concentrations (Fig. 5b). These findings open new perspectives in the study of the mechanisms determining Cd xylem loading, as other components seem to contribute to it and thus to Cd accumulation in the grains. The existence of an additional Cd transport pathway *de facto* implies that OsHMA2 is not the only ortholog of *AtHMA2* and *AtHMA4* codifying the ATPases responsible for Cd xylem loading in Arabidopsis. Lee and coworkers (2007) provided an 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 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, there is no evidence that OsHMA7 is involved in Cd movement as no 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. Interestingly, such transporters cluster and thus are functionally related to AtHMA5 and AtHMA7,

classified as Cu^{+}/Ag^{+} ATPase (Mandal et al. 2004; Andrés-Colás et al. 2006; Hirayama et al. 1999; Woeste & Kieber 2000; Williams & Mills 2005). According to these premises, OsHMA4 is likely to take part in the transport of Cd and could be a good candidate to contribute to its xylem loading. Even though further evidence is needed to strengthen our deduction, the preliminary functional analysis of OsHMA4v in yeast we provide in this study confirms the involvement of this protein in mediating Cd efflux from yeast cells (Fig. 6). Such a finding, along with the fact that no evidence of Zn transport ability was found, support the hypothesis that OsHMA4 could be the Zn-independent transport pathway playing a major role in Cd xylem loading.

Finally, the analysis of the expression profiles of *OsHMA2*, *OsHMA3* and *OsHMA4* acknowledged as key elements in Cd distribution by differently contributing to its partitioning through the plant, returned a picture that well fit with the conclusions proposed so far (Fig. 7). OsHMA2, in fact, clearly results to be a Zn^{2+} ATPase supposedly involved in Zn xylem loading, as the gene by which it is encoded resulted strongly deinduced moving to the highest Zn concentration analyzed (Fig. 7a). By contrast, *OsHMA3* was upregulated by such a concentration (Fig. 7b), confirming the role of the related protein as a vacuolar transporter involved in the sequestration of toxic metals as well as essential nutrients when present in excess (Ueno et al. 2010a). The expression of *OsHMA4*, instead, resulted independent from Zn treatment, supporting the hypothesis it is a transport system not involved in Zn movement (Fig. 7c). Interestingly, in our study none of the above transporters resulted transcriptionally regulated by Cd treatment which, in case of OsHMA2 and OsHMA4, would explain the saturating kinetic for Cd translocation (Fig. 3). Such a finding leads to the assumption that OsHMA2, OsHMA3 and OsHMA4 probably transport Cd trough an aspecific recognition of the metal by the their metal binding domains.

In conclusion, according to the data presented in this work, we proved that the presence of both Cd and Zn in the nutrient solutions results in competition effects even at low concentrations of both metals. Particularly, at the translocation level Cd appears to strongly compete over Zn: this is likely to result from the activity of xylem loading systems that Cd just partially share with Zn. As a consequence, Cd movement determining its allocation through the plant is not necessarily combined to Zn, in that their dynamics appear overlapping just to some extent. Such an outcome, although still to be fully explored, should be clearly considered in crop management practices with the aim of preventing that incomplete assumptions have boosting effects on the risk of Cd accumulation in the edible part of the plant.

Concluding remarks

Among trace elements not essential for plant growth and metabolism, Cd is of particular concern as it may exert phytotoxic effects and have direct consequences on human health mainly accumulating in staple food crops which make up a large proportion of dietary intake. Cd is generally present in the soil medium either naturally and/or from anthropogenic sources. In the former case, natural mineral outcrops can been enriched in Cd through the weathering of Cd-rich parent material. More rare is the case in which Cd presence in the soil is due to geochemical occurrence. 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 common sources of Cd contamination are: (a) disposal of Cd containing wastes; (b) deposition of aerosol particles from air pollution; and, more specifically related to agricultural activities, (c) application of sewage sludge and phosphate fertilizers which may contain Cd as an impurity. Notable is also the accidental supply of Cd, due to the use of Cd containing irrigation water. Among other heavy metals, Cd constitutes a big issue in terms of food safety since it tends to be more mobile and thus more available to be translocated to the edible portion of the plant, causing acute or chronic toxicity to humans even at low soil concentrations.

There is a remarkable natural variation in Cd uptake, distribution and accumulation processes both considering crop species and cultivars within the same species. This variation depends on several factors under genetic control that affect the abovementioned processes at different levels. Most of these factors have been widely investigated and characterized in some species, but still very little is known about rice (*Oryza sativa* L.). Rice is, among crop species, the one presenting the highest risk of accumulating Cd in the aerial part of the plant, especially in the grains, so that along with other commercially grown crops destined to international trade such as sunflower, flax and durum wheat, it has been identified as Cd accumulator. Such a tendency is not only due to genetic reasons but also to the agronomic practices normally applied in its cultivation that result in enhancing Cd availability in the soil solution. However, soil management techniques aiming at reducing the risk of Cd accumulation are not always feasible nor cost-effective, thus do not solve the problem especially considering low Cd contaminated soils. More promising is the opportunity to utilize plant breeding to select for genetically low-Cd concentration rice cultivars, taking advantage from the well documented broad variability in the Cd accumulation trait observed in Indica and Japonica cultivars, as well as in hybrids. This genotypic variation provides valuable information that may be used to analyze the physiological and genetic aspects contributing to the low Cd accumulation phenotype. In particular, there are three pivotal physiologic processes most likely to mediate Cd

accumulation in shoots and grains, whose better understanding would shed light on the complex background resulting in the Cd accumulation trait. Such processes are: (a) *uptake by roots*: as Cd does not have a biological function within the organism, no specific transporters have developed through evolution, therefore it is taken up by membrane permeases devoted to the transport of other cations but characterized by broad-range specificity; (b) *xylem-loading mediated translocation to shoots*: Cd is loaded from the symplasm into the xylem vessels by transporters which may aspecifically recognize Cd ions and/or Cd chelates; and (c) *further translocation to grains via the phloem*, even though the exact form in which Cd is translocated, distributed and, eventually, reallocated is still under debate. In order to prevent and possibly limit the toxic effect of Cd, a tight control of these mechanisms is required to keep the concentration of the metal within narrow limits. With regard to this, the first step would be to retain the metal in the root system: as a result, a critical role in Cd retention in the roots is played by phytochelatins (PCs), which are thiolate peptides non-translationally synthesized from glutathione by the enzyme phytochelatin synthase (PCS). They are able to chelate the metal and, in some cases, to mediate its sequestration into the vacuole. If not immobilized, Cd is potentially mobile throughout the plant organism and a pivotal role in the systemic distribution of metal ions is played by transporters mainly belonging to the P_{1B}-type ATPase subfamily.

In such a contest took place the studies conducted during this PhD programme. In particular, the focus has been on Cd root retention and Cd translocation, as they have been seen to be crucial in determining Cd accumulation. Aiming at better understanding these physiological processes, the role of PCs in chelation and subcellular compartimentalization of Cd in the roots was investigated, both by characterizing Cd-PCs complexes with respect of the external Cd concentration and examining the molecular basis of their synthesis. Then, our attention moved on the identification of the genes encoding transporters putatively involved in Cd xylem loading; particularly we looked at the P_{1B}-type ATPase subfamily, acknowledging its major role in Cd translocation. While characterizing such transporters both by molecular and physiological analysis, we also identified the occurrence of clear competition effects of Cd over Zn at the translocation level. Such an outcome highlighted that Cd movement determining its allocation through the plant is not strictly associated to Zn, which is likely to result from the existence of Cd transport pathways that are Zn-independent. All the results that have been presented in this work were obtained by exposing rice plants to a broad range of relatively low Cd concentrations with the specific aim of simulating the real conditions in moderately contaminated soils, due to their common occurrence.

In conclusion, due to our findings we contributed to advance the understanding of the complex network of processes governing Cd accumulation in rice grains which, despite the economical and agricultural relevance of this crop, is still lacking. As a consequence, we opened new perspectives and thus we constitute the basis to further develop molecular and/or physiological markers to early select rice genotypes able to exclude Cd from the grains with the intent of ensuring the food safety of the consumers. Furthermore, it is worth noting that the development of such markers can be also exploited for aims other than preventing Cd accumulation in the edible part of the plant. Indeed, the considerable high biomass of rice plants and the possibility of double cropping and sequential harvesting make rice highly suitable for being implemented in phytoextraction techniques, especially in low contaminated soils, which is the most widespread condition throughout the world.

Materials and methods

1. Plant material, growth conditions and sampling

Rice (*Oryza sativa* L. cv. Volano) 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 (8 seedlings per tank) containing the following complete nutrient solution: 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 500 µM $MgSO_4$, 250 µM $NH_4H_2PO_4$, 25 µM Fe-tartrate, 46 µM H_3BO_3, 9 µM $MnCl_2$, 0.8 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 30 µM Na_2O_3Si, (pH 6.5).

Seedlings were kept for 12 d 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 this period plants were differently treated with Cd and Zn, according to the experimental conditions set up in the two studies carried out: in the former, the abovementioned nutrient solution was supplemented with different amounts of $CdCl_2$ to reach the final concentrations of 0.01, 0.1 and 1 µM. In the latter, two lines of rice plants were separately grown: in the first, $ZnSO_4$ was replaced by $ZnCl_2$ that was provided to maintain a constant concentration of 1 µM, while $CdCl_2$ was supplemented to have the final concentrations of 0.01, 0.1 and 1 µM; in the second, $CdCl_2$ was added maintaining the external concentration of 0.1 µM, whereas $ZnCl_2$ was supplemented to reach the final concentrations of 0.1, 1 and 10 µM. In both studies, the treatment period was 10 day long. 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 analysed immediately.

2. Determination of Cd and Zn in roots and shoots

Samples of ca. 400 mg 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. Cd and Zn content was measured by inductively coupled plasma mass spectrometry (ICP-MS; Varian 820-MS, ICP Mass Spectrometer).

3. Determination of thiols

Roots were pulverized using mortar and pestle in liquid N_2 and stored frozen in a cryogenic tank. For total nonprotein 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 15 min at 13000 *g* and 4°C. Four hundred microliters of supernatant were collected, 66 µl of 37% HCl was added, and then centrifuged again for 10 min at 13000 *g* and 4°C. For the quantification, volumes of 200 µl of the supernatant were collected and mixed with 800 µl of 1M 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.

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

4. Cd and Zn fractioning

Cd and Zn fractioning was carried out essentially as described by Rauser & Meuwly (1995). Briefly, frozen root tissues (ca. 6 g FW) were pulverized in a cold mortar with a pestle and then homogenized with ice-cold N_2-purged 100 mM Tris-HCl (pH 8.6), 1 mM PMSF and 1% (v:v) Tween 20 at the ratio of 1 ml of buffer to 1 g tissue FW. The homogenate was centrifuged at 4°C and 48000 *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, 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 48000 *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 3 cm column of DEAE-Sephadex A-25 equilibrated with 10 mM Tris-HCl (pH 8.6). After loading the column was washed with 25 ml of equilibrating buffer to remove unadsorbed solutes. All the fluid passing through the anion-exchanger was collected for Cd and Zn analysis (cationic fraction). Anionic material was eluted with 4 ml of 10 mM Hepes (pH 8.0) and 1 M KCl. In the first study, the anionic fraction so obtained was further resolved by gel filtration on a Sephadex G-50 column (0.8 x 100 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 2.5 ml were collected for Cd and NPT analysis. The column was calibrated by using 4 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 analysed as above described.
The amount of Cd and Zn ions in mineralised pellets, extracts and column effluents was measured by ICP-MS.

5. Analysis of root-to-shoot Cd and Zn 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 by trapping into a 1.5 ml plastic vial filled with a small piece of cotton for 1.5 h. The amount of collected sap was determined by weighing and both Cd and Zn concentrations were measured by ICP-MS.

6. Cloning of *OsHMA2v* and *OsHMA4v* cDNA

Total RNA was extracted from rice roots using Trizol Reagent (Invitrogen) and first-strand cDNA synthesis was carried out using SuperScript III first-strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen), according to the manufacturer's instructions.
PCR reaction, to amplify the entire coding sequence of cv. Volano heavy metal P_{1B}-ATPase 2 (*OsHMA2v*) and 4 (*OsHMA4v*), was carried out on the first-strand cDNA using Pfu DNA polymerase (Promega).
The PCR primers used for *OsHMA2v* and *OsHMA4v* amplification are reported as follows:

- $OsHMA2_{ATG}$ (5'-GCCACCATGGCGGCGGAGGGAGGGAG-3') and $OsHMA2_{TAG}$ (5'-CTACTCCACTACGATCTCAGGCA-3');
- $OsHMA4_{ATG}$ (5'-GCCACCATGGAGCAGAATGGAGAGAACCAT-3') and $OsHMA4_{TAG}$ (5'-TCACACCAAATCCGGGTCATTCTT-3').

The couples of primers were designed according to *OsHMA2* and *OsHMA4* sequences respectively, referring to the PlantsT database (*OsHMA2* - PlantsT 64490; *OsHMA4* – PlantsT 64504); both forward primers included a consensus sequence for translation initiation (underlined; Kozak 1987, 1990, 1991). The amplification products were cloned into the yeast (*Saccharomyces cerevisiae*) expression vector pYES2.1/V5-His-TOPO (pYES2.1, Invitrogen) under the control of *GAL1*

promoter. The identity of the PCR products were verified by sequencing both the strands, and *OsHMA2v* complete coding sequence was submitted to GenBank (accession HQ646362).

ClustalW program (http://align.genome.jp/) was used to align the OsHMA2v protein sequence (translated ORF) and the sequences of members of the P_{1B}-ATPase sub-family of *Arabidopsis thaliana* and *Oryza sativa*. MEMSAT3 software (http://bioinf.cs.ucl.ac.uk/psipred/) was used to predict the OsHMA2v and OsHMA4v membrane topology.

7. OsHMA2v and OsHMA4v heterologous expression in yeast

Yeast strain INVSc1 MATa his3D1 leu2 trp1-289 ura3-52 (Invitrogen) was transformed with the chimeric OsHMA2v-pYES2.1, OsHMA4v-pYES2.1 or the empty pYES2.1 vector by the standard lithium acetate method (Gietz *et al.* 1992). URA3 recombinant yeast cells were selected on solid synthetic minimal medium (SC) containing 2% (w:v) Glc, 6.7 g l^{-1} yeast nitrogen base, 1.92 g l^{-1} yeast synthetic dropout media without uracil (Sigma).

For the drop test, URA3 recombinant cells were grown to approximately 1 A_{600} unit in the liquid SC medium, washed twice with sterile distilled water and then resuspended in water to final 0.01, 0.1 or 1 A_{600} units. Ten microliters of each cell suspension were dropped on the same solid medium but containing 2% (w:v) Gal instead of Glc (SG medium), in order to induce gene expression. The growing medium was supplemented or not with 75 or 100 μM $CdCl_2$ when testing Cd treatment; instead, it was supplemented with 20 or 22.5 mM $ZnCl_2$ when testing Zn treatment. Yeast cells were incubated at 30°C for 3 d and then analysed for Cd and Zn tolerance.

For the growth analysis, recombinant yeast cells were grown at 30°C in SG liquid medium up to reach a mid-log phase and then diluted to a final absorbance of 0.1 A_{600} in the same SG medium supplemented or not with 75 μM $CdCl_2$.Yeasts were incubated at 30°C and growth was monitored for 30 h 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.

8. Gene expression analysis

8.1. Semiquantitative RT-PCR analysis of *OsPCSs*, *OsHMA2v* and *OsHMA3*

First-strand cDNA, deriving from about 150 ng of total RNA and obtained as described above, was used for the semiquantitative RT-PCR analysis of the transcripts of phytochelatin synthases (*OsPCSs*), heavy metal P_{1B}-ATPase 2 (*OsHMA2v*) and 3 (*OsHMA3*). PCR reactions were carried out using Pfu DNA polymerase and the following couples of oligonucleotide primers: PCS_{for} 5'-

CATCTTCCTCCCAATGCTGG-3' and PCS_{rev} 5'-CCCACTTAGCAATGCGGTTG-3'; $HMA2_{for}$ 5'-GGAGTTCCAGCCAGTTGGTG-3' and $HMA2_{rev}$ 5'-ACCATCAGCGTAGGGCCATC-3'; $HMA3_{for}$ 5'-CTCTGGTGATGCTTGTGAGC3' and $HMA3_{rev}$ 5'-TGATGCCCAGCGATCCAAGC-3'. Concerning *OsPCSs*, primers were designed on conserved sequences of both cDNAs of the two *OsPCS* loci (LOC_Os05g34290 and LOC_Os06g01260 in the TIGR/GenBank database) mainly expressed in roots, to specifically amplify overlapping fragments of 276 bp. Differently, primers for *OsHMA2v* and *OsHMA3* were designed on the *OsHMA2v* (GenBank HQ646362) and *OsHMA3* (LOC_Os07g12900) complete coding sequences, to specifically amplify fragments of 551 bp and 600 bp, respectively. The amplification products were cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen) and their identities were verified by sequencing. cDNA loading was normalized using the *OsS16* (LOC_Os11g03400) ribosomal protein amplicon, as an internal control, obtained with the following primers: $S16_{dir}$ 5'-AGGCACGGTCCAGTGCTTCG-3' and $S16_{rev}$ 5'-CGGTACGACTTCTGGAACCTG-3'. PCRs were carried out for 30 cycles. PCR products were separated in agarose gels and stained with SYBR Green I (Invitrogen). Signals were detected using a laser scanner (Typhoon 9200, GE Healthcare) with a 532-nm laser and a 526-nm filter.

8.2. qRT-PCR of *OsHMA2*, *OsHMA3* and *OsHMA4*

The expression level of *OsHMA2*, *OsHMA3* and *OsHMA4* was relatively quantified by performing TaqMan® gene expression assays. First-strand cDNA was synthesized from 0.5 µg total RNA obtained as previously reported. Reverse transcription was performed using Superscript™ III First-Strand Synthesis Super Mix for qRT-PCR (Invitrogen) according to manufacturer's instructions. The amplification reactions were performed by using the TaqMan® gene expression Master Mix and the FAM™ dye-labeled MGB probes reported in Tab. 1.

Tab. 1 : FAM™ dye-labeled MGB probes used for TaqMan® gene expression assays.

Gene	Locus	Probe ID
OsHMA2	LOC_Os06g48720	Os03631590_m1
OsHMA3	LOC_Os07g12900	Os035484887_g1
OsHMA4	LOC_Os02g10290	Os03582401_m1
OsUBC	LOC_Os02g42314	Os03534000_m1

Genes are indicated by referring to the annotation in the TIGR/GeneBank database. FAM™ dye-labeled MGB probes _m1 tagged are designed to span en exon junction; those _g1 tagged have primers and probe that may be on the same exon, thus may detect genomic DNA.

When possible, the probes were chosen as designed to span an exon junction (type m1), in order to avoid aspecific signals from DNA contamination. The amplification reaction was carried out basing on a two-step protocol set as follows: 1 cycle at 50°C for 2 min then at 95°C for 2 min; 40 cycles at 95°C for 30sec and 60°C for 1 min.

The relative transcript expression of *OsHMA2, OsHMA3* and *OsHMA4* was then obtained normalizing the transcript quantities with respect to *OsUBC* (LOC_Os02g42314) ubiquitin-conjugating enzyme which was used as an endogenous control.

9. Statistical analysis

Statistical analysis was carried out using SigmaPlot for Windows version 11.0 (Systat Software, Inc.). 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 $P<0.05$.

Acknowledgments

It is hard to come to the end of a work three years long and think about all the people you need to thank. Obviously, things change over time making you lose something on the way, but also gain something else. Undoubtedly, many thanks are due to all those have kept pace with my mood, sorrows and gratifications, even though they did not come to the end with me.

Simply, I would like to thank those people that, while taking part in my work, have been patient to me and, above all, taught me how to be patient.

Also, there are just a few people that I feel like mentioning here. A few, but that deserve my biggest thanks indeed.

Dott.ssa Federica Baldin, a.k.a. Zazzà, the one and the only researcher I had the pleasure to work with, my favorite riot ever, irreplaceable travelling companion, coffee-break fellow, roommate and special confidant when needed. To cut a long story short, thank you Fede for being such a good friend, a bit tough at the beginning but incredibly sweet afterwards. After all, it was worthy, don't you think?

My brother Francesco, the grumpiest man on earth but the only one I would ever choose as brother. The passion and the commitment he had while carrying out his PhD and still has in pursuing his career showed me how a real researcher should be and led me to embark on this way, at least for a while.

Barney Panofsky, my beloved Barney.
No words to say thank you: the only thing I can give you to pay you back for the luck I feel like having from now onwards is the deep consciousness that "*this could be the start of something big*".

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Appendix

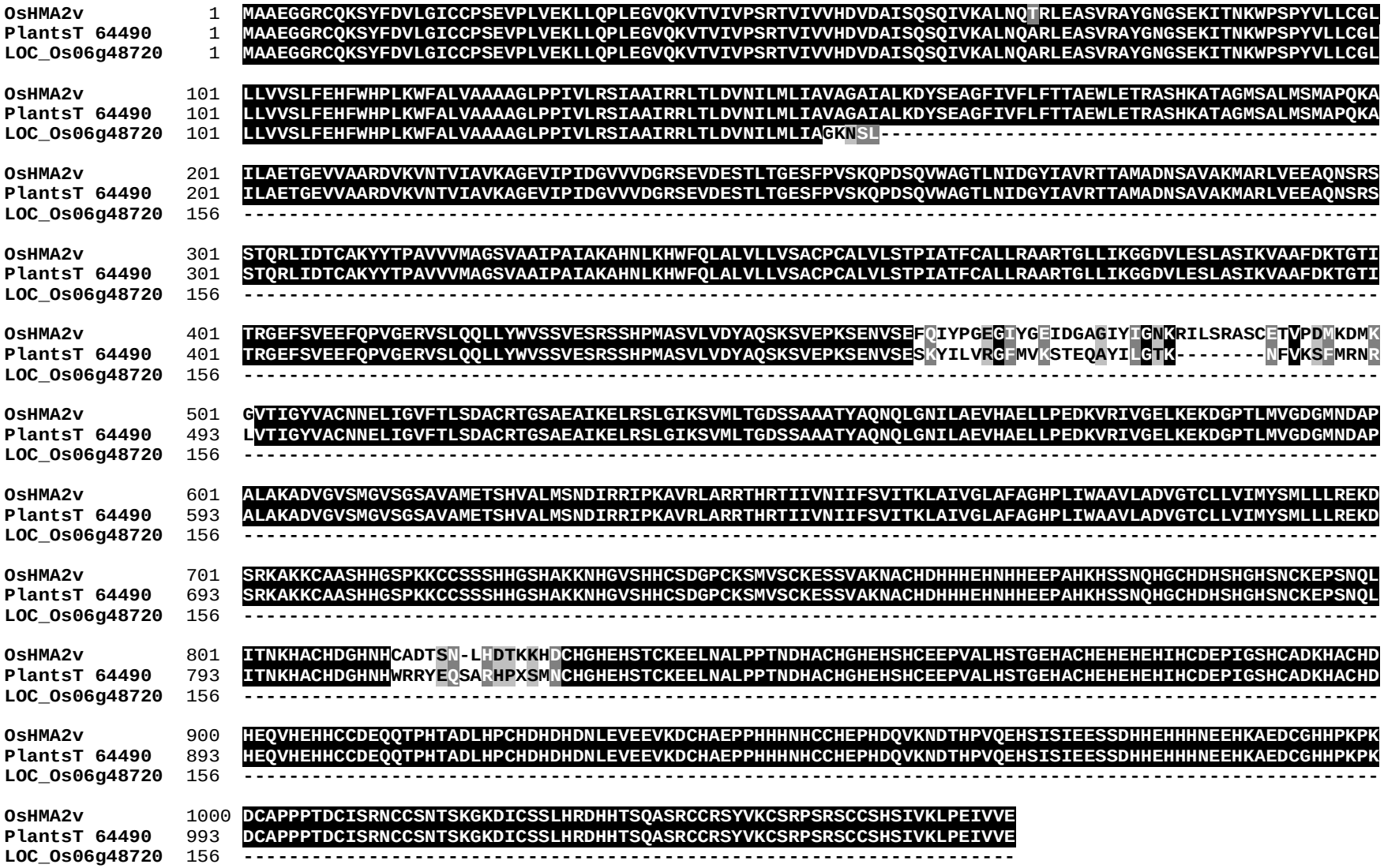

Fig. 1 : Alignment of the amino acid sequence of OsHMA2v (cDNA clone from Volano cultivar) with the OsHMA2 deduced sequences reported in PlantsT (PlantsT 64490) and TIGR (LOC_Os06g48720).

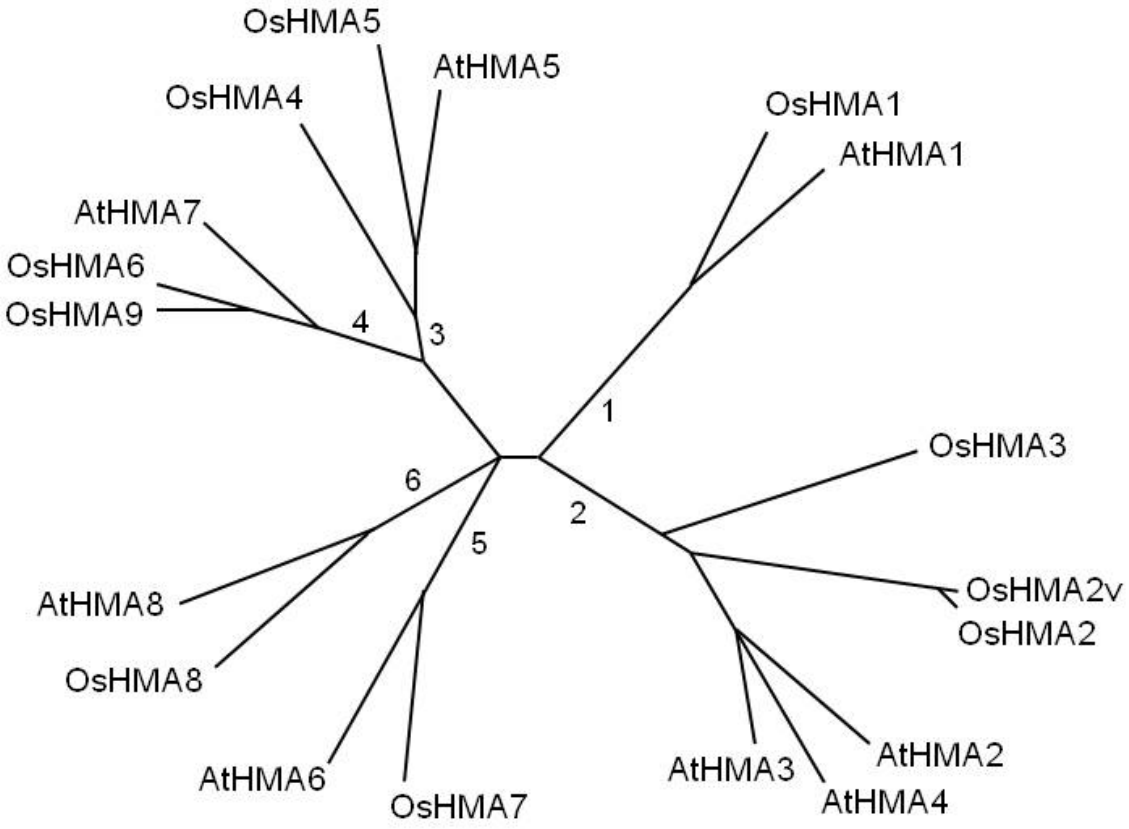

Fig. 2 : Dendrogram showing families of P1B-ATPases in Arabidopsis and rice. The dendrogram was constructed using ClustalW (http://align.genome.jp/). Accession numbers for Arabidopsis thaliana (UniProtKB) are: AtHMA1, Q9M3H5; AtHMA2, Q9SZW4; AtHMA3, Q9SZW5; AtHMA4, O64474; AtHMA5, Q9SH30; AtHMA6, Q3E9R8; AtHMA7, Q9S7J8; AtHMA8, B9DFX7. Accession numbers for Oryza sativa (Rice Genome Annotation Project, GenBank or PlantsT) are: OsHMA1, LOC_Os06g47550; OsHMA2, PlantsT 64490; OsHMA2v, GenBank HQ646362; OsHMA3, LOC_Os07g12900; OsHMA4, LOC_Os02g10290; OsHMA5, LOC_Os04g46940; OsHMA6, LOC_Os02g07630; OsHMA7, LOC_Os08g37950; OsHMA8, LOC_Os03g08070; OsHMA9, LOC_Os06g45500.

Annex 1 : Cloning Reaction and Transformation into the yeast (*Saccharomyces cerevisiae*) expression vector pYES2.1/V5-His-TOPO (pYES2.1, Invitrogen)

1.1. Cloning Reaction

1. After obtaining the PCR product to clone, set the ligation reaction as follows:

Reagent	Chemically competent *E. coli*
Fresh PCR product	0.5 to 4 µL
Salt Solution	1 µL
Sterile water	add to a final volume of 5 µL
TOPO vector	1 µL

2. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).
3. Add 2 µl of the cloning reaction a vial of chemically competent *E. coli* and mix gently. Do not mix by pipetting up and down.
4. Incubate on ice for 5 to 30 minutes.
5. Heat-shock the cells for 30 seconds at 42°C without shaking.
6. Immediately transfer the tubes to ice.
7. Add 250 µl of room temperature SOC medium.
8. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
9. Spread 10-50 µl from each transformation on a pre-warmed selective plate and incubate overnight at 37°C.

1.2. Plasmidic DNA extraction by Wizard Plus SV Minipreps DNA Purification System (Promega)

(a) Production of cleared lysate

1. Pellet 1–10ml of overnight culture for 5 minutes.
2. Thoroughly resuspend pellet with 250µl of cell resuspension solution.
3. Add 250µl of cell lysis solution to each sample; invert 4 times to mix.
4. Add 10µl of alkaline protease solution; invert 4 times to mix. Incubate 5 minutes at room temperature.
5. Add 350µl of neutralization solution; invert 4 times to mix.
6. Centrifuge at top speed for 10 minutes at room temperature.

(b) Binding of plasmid DNA

7. Insert spin column into collection tube.
8. Decant cleared lysate into spin column.
9. Centrifuge at top speed for 1 minute at room temperature. Discard flowthrough, and reinsert column into collection tube.

(c) Washing

10. Add 750µl of wash solution (ethanol added). Centrifuge at top speed for 1 minute. Discard flowthrough and reinsert column into collection tube.
11. Repeat step 10 with 250µl of wash solution.
12. Centrifuge at top speed for 2 minutes at room temperature.

(d) Elution

13. Transfer spin column to a sterile 1.5ml microcentrifuge tube, being careful not to transfer any of the column wash solution with the spin column. If the spin column has column wash solution associated with it, centrifuge again for 1 minute at top speed, then transfer the spin column to a new, sterile 1.5ml microcentrifuge tube.
14. Add 100µl of nuclease-free water to the spin column. Centrifuge at top speed for 1 minute at room temperature.
15. Discard column, and store DNA at –20°C or below.

1.3. Small-Scale Yeast Transformation (adapted from Gietz et al. 1992)

1. Inoculate 10 ml of YPD medium with a colony of the yeast strain to be transformed and shake overnight at 30°C.
2. Determine the OD_{600} of the overnight culture. Dilute culture to an OD_{600} of 0.4 in 50 ml of YPD medium and grow an additional 2-4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 ml 1X TE.
4. Pellet the cells at 2500 rpm and resuspend the pellet in 2 ml of 1X LiAc/0.5X TE.
5. Incubate the cells at room temperature for 10 minutes.
6. For each transformation, mix together 1 µg plasmid DNA and 100 µg denatured salmon sperm DNA with 100 µl of the yeast suspension from previous step.
7. Add 700 µl of 1X LiAc/40% PEG-3350/1X TE and mix well.
8. Incubate solution at 30°C for 30 minutes.
9. Add 88 µl DMSO, mix well and heat shock at 42°C for 7 minutes.
10. Centrifuge for 10 seconds and remove supernatant.
11. Resuspend the cell pellet in 1 ml 1X TE and re-pellet.
12. Resuspend the cell pellet in 50-100 µl 1X TE and plate on a selective plate.