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Plant transporters involved in heavy metal homeostasis

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Abstract. Transition metal ions (predominately manganese, iron, cobalt, nickel, copper and zinc) have an array of catalytic and regulatory roles in the growth and development of all living organisms. However, an excess of these metal ions can also be toxic to any life form and therefore every cell and whole organism needs to maintain the concentration of these essential nutrient metals within a narrow range: a process known as metal homeostasis. Heavy metal ions are taken up into cells by selective transporters and as they cannot be degraded, the "desired" levels of metal ions are achieved by a number of strategies that involve: chelation, sequestration and export out of the cell. Cation Diffusion Facilitators (CDF) is a large family of transporters involved in maintaining the cytosolic metal concentration. They transport different heavy metal divalent ions, but exhibit main affinity for zinc, iron and manganese. Metal Tolerance Proteins (MTPs) are a subfamily of the Cation Diffusion Facilitator (CDF) family found in plants. There has been much interest in these heavy metal transporters in order to provide an insight into plant metal homeostasis, which has significant implications in human health and phytoremediation. Although data regarding the CDFs/MTPs mechanism is gathering there is still little information with respect to metal selectivity determinants.
Key Words: cation diffusion facilitator, *Arabidopsis*, plant transporter, metals.

Rezumat. Ionii metalelor tranziţionale (în special mangan, fier, cobalt, nichel, cupru şi zinc) prezintă un spectru larg de roluri catalitice şi regulatorii în procesele de creştere şi dezvoltare ale tuturor organismelor vii. Cu toate acestea, excesul acestor ioni metalici este toxic pentru orice formă de viaţă, astfel că fiecare celulă şi implicit fiecare organism trebuie să menţină concentraţia lor în limite foarte stricte, proces denumit homeostazie. Ionii metalelor grele sunt absorbiţi intracelular prin intermediul unor transportori specifici. Odată acumulaţi însă intracelular, neputând fi degradaţi, menţinerea concentraţiei lor la nivel citoplasmatic se face printr-o serie de mecanisme care implică: chelatarea, sechestrarea în diverse subcompartimente celulare sau eliminarea lor din celule. Printre transportorii implicaţi în menţinerea homeostaziei metalice se numără familia proteinelor transportoare care facilitează difuzia cationilor metalici (Cation Diffusion Facilitators). Aceste proteine realizează transportul ionilor metalelor grele, dar prezintă o afinitate mai ridicată pentru ionii de zinc, fier şi mangan. La plante, CDF poartă denumirea de proteine care conferă toleranţă la metale (Metal Tolerance Proteins - MTPs). Acest grup de transportori vegetali a ridicat un interes deosebit deoarece conţinutul în metale a plantelor se reflectă prin lanţul trofic la nivelul animalelor şi implicit a omului. Astfel, cunoaşterea modului prin care homeostazia ionilor metalici este menţinută în cadrul celulor vegetale şi cunoaşterea modului de acţiune a MTP poate contribui la menţinerea sănătăţii umane şi mai mult, la procesul de fitoremediere. Deşi date privind modul de acţiune a CDF/MTP se acumulează, încă există puţine informaţii cu privire la determinaţii specificităţii pentru metale în cazul acestor transportori.
Cuvinte cheie: CDF, *Arabidopsis*, transportori vegetali, metale.

Introduction. Transition metals are indispensable nutrients for all living entities, playing important roles as part of numerous catalytic enzymes, regulatory and structural proteins. In plants, iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), nickel (Ni) are essential micronutrients, while molybdenum (Mo) and cobalt (Co) are beneficial for legumes for the fixation of atmospheric nitrogen (Marschner 1995). The principal mechanism by which the plants acquire these essential metals is through root uptake. However, the mechanism is not specific, and consequently plants are prone to accumulation of other, non-essential and even toxic heavy metals *e.g.* cadmium (Cd), lead (Pb), chromium (Cr), mercury (Hg), silver (Ag). Moreover, even the essential metals can, under certain conditions (*e.g.* contaminated soils and/or waters), accumulate in

plants in higher concentrations, posing a risk not only to plants, but also to animals and humans through the food chain. Metal homeostasis in plants is controlled by four main groups of transporters: **Z**inc/**I**ron **P**roteins (**ZIPs**), **N**atural **r**esistance **a**ssociated **m**acrophage **p**roteins (**Nramps**), **H**eavy **M**etals P_{1B}-type **A**TPases (**HMAs**) and **C**ation **D**iffusion **F**acilitators (**CDFs**) that are involved in the intake and export of heavy metals into and out from the cell and their transfer into cellular compartments. The current paper is centered upon knowledge up to date on plant CDFs known as Metal Tolerance Proteins (MTPs).

Cation Diffusion Facilitators. The ubiquitous family of CDFs (Cation Diffusion Facilitators) comprises of more than 400 members (Montanini et al 2007) recognized to play a major role in the efflux of transition metal cations from the cytoplasm to the outside of the cell or into subcellular compartments.

A recent phylogenetic analysis of CDFs from all kingdoms of life (Archaea, Bacteria and Eukaryota) has proposed their organization within three groups based on their specificity for the principal metal: zinc, manganese and iron/zinc (Montanini et al 2007). It is important to note that within each group, individual transporters can transport other transition metals with varying degrees of specificity. Thus, while some the members within the Zn-group are highly specific for Zn, others can also transport: Co, Cd or Ni. Members within the Mn group can sometimes transport Fe. Whilst mammalian CDF are mainly found within the Zn-group, the plant members are spread among the groups. Mammalian CDFs are named ZnT (Zinc Transporters), all the 10 members identified within the human genome being specific for Zn (Kambe et al 2004). *Arabidopsis thaliana* encompasses 12 members, spread through out all the metal specificity groups (AtMTP1-4, 5, 12 in Zn-group, AtMTP8-11 in Mn-group, AtMTP6 in Fe/Zn-group and AtMTP7 with no metal specificity annotation).

Since their discovery, in 1997 (Paulsen & Saier 1997), a lot of interest has been put into establishing the physiological role and unraveling the structure and functional mechanism of CDF transporters family. Most CDFs comprise of six putative transmembrane spans (TMD), a CDF signature sequence, N- and C-termini facing the cytosol and a characteristic C-terminus cation efflux domain (Pfam 01545).

There is evidence that CDFs assemble as homodimers (Blaudez et al 2003; Lu & Fu 2007; Cherezov et al 2008) and function as antiporters transporting metal ions against concentration gradients, using H^+ or K^+ to create an electrochemical gradient (Guffanti et al 2002 – *Bacillus subtillis* CzcD; Chao & Fu 2004 – *Escherichia coli* ZitB; Grass et al 2005; Kawachi et al 2008 – AtMTP1). The release of the crystal structure of *E. coli* YiiP, a member of the Fe/Zn-group of the CDF family, depicts the bacterial transporter as a homodimer adopting a Y-shape with the TMDs of the two monomers clearly separated and the carboxy-terminal cytoplasmic domains (CTDs) tightly associated, constituting the dimerization region (Lu & Fu 2007; Lu et al 2009). The *E. coli* CDF was crystallized at 3.8A resolution in the presence of Zn although *in vivo*, Fe represents its main substrate. In this Zn-bound state, four Zn ions per subunit were identified, the first within the groove created by the TMDs2 and 5, second found at the interface between the cytoplasm and the membrane and third and fourth located at the dimerization point between the CTDs of the 2 monomers. However, the physiological importance of the four Zn ions identified per subunit has not been recognized yet.

Both the structure of YiiP and the observations that mutagenesis of any of the conserved residues (H/DxxxH/D) in the TMD2 and 5 result in non-functional proteins (Blaudez et al 2003 and Montanini et al 2007 - *Populus trichocarpa* x *P. deltoides* PtdMTP1 D86A, H89A/K, D93A, H260D, D264A/E; Drager et al 2004 – *Arabidopsis halleri* AhMTP1-3 D90A; Desbrosses-Fonrouge et al 2005 - AtMTP1 D94A; Wei & Fu 2005 – *E. coli* YiiP D49A, D157A/C; Lin et al 2008 - *Saccharomyces cerevisiae* ScZRC1 D45A) suggest that the Z1 site is directly involved in metal transport. But binding of Zn at sites 2-4 within the CTD is thought to be important for stabilizing the dimmer. Moreover the Zn-bound crystal structures of the CTD alone (last 92 aa at the C-terminal) of *Thermus thermophilus* CzrB (cadmium-zinc resistance protein B; Cherezov et al 2008) showed four Zn^{2+} are associated with each of the carboxy soluble parts of the homodimer. In the

apo state, the two CTDs adopt a V-shape, whereas binding of Zn brings the two monomers closely together on their entire length. Thus the CTD dimer is stabilized, but Cherezov et al (2008) proposed that in this modified conformation, the angle between the CTDs and the TMDs would alter so that, a possible Zn-chaperone would be able to interact at the cytosol-membrane interface and pass the metal ion for its transport. However, this proposed mechanism has yet to be verified and a Zn-chaperone has still to be identified.

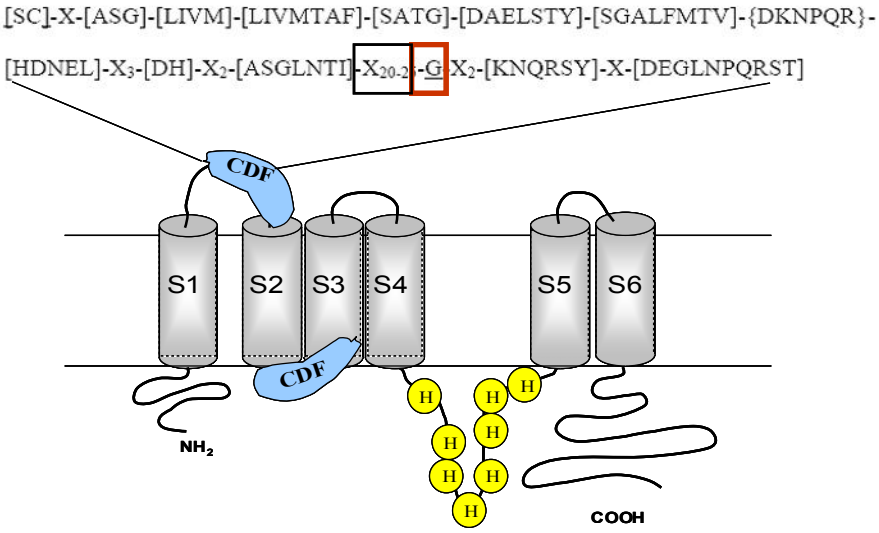

Figure 1. The general structure of Cation Diffusion Facilitator Transporters.

Nevertheless, knowledge up to date does not certify that the structure and the functional mechanism proposed for the bacterial CDFs (Lu & Fu 2007; Cherezov et al 2008; Lu et al 2009) can be extrapolated to other CDFs transporters within the Zn- group or within Mn-group. This is because members of the Zn-group of CDFs possess a long intracytoplasmatic histidine-rich loop (IL2) connecting TMDs 4 and 5 (Fig. 1), loop that is lacking in other metal-groups of this family transporters. In the bacterial YiiP (Fe/Zn-group) and CzrB (Zn-group) IL2 is represented solely by four residues, while in plant members of the Mn-group is also represented by 11 aa. The role of the His-rich loop has not been unraveled yet. Kawashi et al (2008) proposed that the His-rich loop functions as a sensor of the free metal ion concentration, and as a potential metal binding domain, which, only when saturated passes the ions to active transport site. Nevertheless, the role of His-rich loop is still to be determined. Also, although data regarding the mechanism of CDFs function is gathering, still little is known regarding the specific amino acids/sequences/domains within the protein that are involved in conferring metal specificity of CDFs.

The only characterized plant CDFs transporters are MTP1, MTP3 and MTP11, with MTP1 being by far the mostly described: *Arabidopsis thaliana* (Bloss et al 2002; Kobae et al 2004; Desbrosses-Fonrouge et al 2005), *Arabidopsis halleri* (Drager et al 2004), *Thlaspi caerulescens* and *T. geosingense* (Persans et al 2001; Kim et al 2004; Gustin et al 2009), Indian mustard – Brassica juncea (Muthukumar et al 2007), poplar - *Populus trichocarpa x deltoides* (Blaudez et al 2003) and *Nicotiana tabacum* and *N. glauca* (Shingu et al 2005). The only other plant member from the Zn-group that has been characterized is AtMTP3 a very close relative to AtMTP1 (Arrivault et al 2006). As for other plant CDFs, other than Zn-transporters, AtMTP11 is the only characterized so far.

At cellular level AtMTP1, similarly to other MTP1s, is localized at the vacuolar membrane and it is a highly specific transporter for Zn. Within plants, the AtMTP1 is not produced throughout the plant, but primarily in the subpopulation of dividing,

differentiating and expanding cells. RNA interference-mediated silencing of AtMTP1 causes Zn hypersensitivity and a reduction in Zn concentrations in vegetative plant tissues (Desbrosses-Fonrouge et al 2005).

Within plants, AtMTP11 belongs to a distinct subset of transporters (AtMTP8 – AtMTP11) whose main substrate is manganese. This group is closely related to the CDF ShMTP8 (formerly ShMTP1) identified in *Stylosanthes hamata* which has been implicated in vacuolar Mn^{2+} transport (Delhaize et al 2003; Delhaize et al 2007). Key characteristics which are common to this subset within the CDFs include the lack of the his-rich loop, common within the zinc group, and mostly possess five or six transmembrane domains. When expressed in the manganese sensitive *Saccharomyces cerevisiae* mutant *pmr1*Δ, lacking a Mn^{2+}/Ca^{2+} P-type ATPase transporter, tolerance to Mn^{2+} was conferred (Delhaize et al 2007; Peiter et al 2007). AtMTP11 fused with EYFP (Enhanced Yellow Fluorescent Protein) revealed a co-localization with the trans-Golgi marker sialyl transferase (Peiter et al 2007). *Atmtp11* mutants displayed manganese hypersensitivity whilst plants overexpressing AtMTP11 under the *CaMV35S* promoter demonstrated hypertolerance (Peiter et al 2007). The recent characterisation of AtMTP11 has suggested that it functions in a secretory pathway for Golgi-mediated detoxification of manganese.

The significance of metal tolerance proteins - bioremediation and biofortification. Research that focuses on heavy metal homeostatic transporters give insight into the molecular responses of a plant to metals. The principal process that triggers the metal homeostasis in plants is the cations uptake. However, plants are limited to an extent in their ability to control uptake, leading to deficiencies or excessive uptake. As a result, this leads to deficiencies or excess amounts further up the food chain to humans and animals. Thus plant metal homeostasis has significant implications in the nutrition of humans as well as plants. This is demonstrated by human mineral deficiencies, which are spread worldwide. The most common form of human micronutrient malnutrition is iron deficiency affecting more than two billion people, predominantly in countries where cereals are the staple food (Mandelbaum-Schmid - WHO 2004; WHO and FAO 2006). Through modern biotechnology edible crops such as rice, wheat and barley or vegetables can be made to contain more of the essential nutrients, like iron and zinc. This process is known as biofortification and can have a serious impact on improving nutrition throughout the developing world (*e.g*. golden rice) (Ye et al 2000). MTPs can potentially give rise to cereals with an increased nutritional value, by the production of transgenic seeds with an elevated micronutrient uptake. Moreover, engineering plants with increased tolerance to the accumulation of heavy metals has already enabled their successful use for bioremediation of soils and waters (*e.g.* Indian mustard - *Brassica juncea* for Pb and Cd – USA; yellow poplar – *Liriodendron tulipifera* for Hg (Rugh et al 1998). Thus, study of MTPs can also be used for the development of transgenic crops that are more tolerant of metal deficient soils, producing more resistant crops.

Understanding how a plant can select for and against different metals both in its external and internal environment is a key factor in harnessing the potential of these technologies. An in-depth knowledge of certain cellular structures and physiological processes such as; the network of processes that take up metals and transport them to above-ground/edible tissues and the homeostatic mechanism by which they are sequestered into the vacuole, is required before plants can be exploited for use in these technologies.

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