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CICLO XXI

IDENTIFICATION OF GENES INVOLVED IN HEAVY METALS TOLERANCE AND HYPERACCUMULATION IN *Arabidopsis halleri* AND CHARACTERISATION OF A bZIP TRANSCRIPTION FACTOR RESPONSIBLE FOR Cd UPTAKE AND TRANSLOCATION TO THE SHOOT IN *Arabidopsis thaliana*

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RIASSUNTO

Introduzione

Il cadmio (Cd) rappresenta uno dei principali fattori di rischio sia per la salute umana che per l'ambiente: a causa della sua elevata solubilità in acqua, si ha una vasta distribuzione nell'ecosistema, insieme ad un'immediata disponibilità all'assorbimento da parte delle piante e quindi l'ingresso nella catena alimentare. Un'alternativa ai tradizionali sistemi di risanamento ambientale da metalli pesanti, tra cui il Cd, è rappresentata dalla *phytoremediation*: una tecnica *in situ*, eco-compatibile ed a basso impatto economico che si pone come obiettivo la rimozione dall'ambiente di agenti inquinanti, sia di natura organica che inorganica, attraverso l'uso di organismi vegetali. In particolar modo, la fitoestrazione prevede l'utilizzo di piante in grado di rimuovere tali inquinanti da siti contaminati e accumularli nei propri tessuti epigei (Pilon-Smits, 2005). L'identificazione di numerose specie vegetali iperaccumulatrici in grado di tollerare, ma soprattutto di accumulare, elevate quantità di metalli pesanti nei propri tessuti, dimostra che le piante possiedono il potenziale genetico per tollerarli e rimuoverli da matrici contaminate. Un esempio è rappresentato da *Arabidopsis halleri* (appartenente alla famiglia delle *Brassicaceae*) in grado di tollerare elevate concentrazioni di Cd, Zn e Pb ed iperaccumulare Cd e Zn (van Rossum *et al*., 2004). Essendo vicina dal punto di vista filogenetico ad *Arabidopsis thaliana*, specie modello per studi di genetica molecolare in ambito vegetale, *A. halleri* riveste un particolare interesse per lo studio dei meccanismi responsabili dell'accumulo e tolleranza ai metalli pesanti. Negli ultimi anni, grazie a studi di genomica funzionale, sono stati identificati numerosi componenti molecolari responsabili della tolleranza e dell'accumulo, ma rimangono ancora da chiarire molti aspetti di fondamentale importanza alla base della regolazione dell'espressione genica, in cui i fattori di trascrizione (TFs), ad esempio, giocano un ruolo chiave. Inoltre, è necessario considerare il contributo apportato dai microorganismi della rizosfera della specie vegetale interessata. È noto infatti che tali microorganismi sono in grado di alterare la mobilità dei metalli presenti nell'ambiente, influenzandone di conseguenza il loro assorbimento da parte dell'apparato radicale della pianta (Lovley, 1995).

Presupposti ed obiettivi del progetto di ricerca

Una pianta ideale per il processo di *phytoremediation* dovrebbe possedere una serie di requisiti, primi fra tutti un'elevata biomassa e la capacità di tollerare/accumulare elevati livelli di sostanze inquinanti nei propri tessuti. Al fine di ottenere piante idonee al processo

di *phytoremediation*, lo scopo generale del progetto è l'identificazione e caratterizzazione in pianta di geni che sono alla base dei processi di tolleranza e accumulo di metalli pesanti, in particolar modo il cadmio. Inoltre, ci si è posti come obiettivo lo studio dell'interazione pianta-comunità microbica della rizosfera per valutare quali risposte si vengono ad attivare in pianta durante il processo di accumulo in presenza dei microorganismi stessi. A tal proposito si è considerato come modello di studio un ecotipo di *A. halleri* cresciuto in un sito contaminato da Cd, Zn e Pb (van Rossum *et al*., 2004).

Capitolo I: *characterisation of a B. juncea bZIP transcription factor responsible for Cd uptake and translocation to the shoot in transgenic plants*.

Il cDNA *full-length* del gene *BjCdR15* (*Brassica juncea Cd-regulated*) è stato isolato da *B. juncea* in seguito a trattamenti con Cd (Fusco *et al*., 2005) e presenta un'elevata similarità di sequenza nucleotidica e aminoacidica predetta con il gene *TGA3* di *A. thaliana*, il quale codifica per un TF di tipo bZIP. Tramite analisi dell'espressione genica, si è osservato che *BjCdR15* viene indotto precocemente in risposta al Cd in germogli e radici e da altri metalli pesanti (Pb e Ni) solo a livello radicale. Tramite ibridazione *in situ* si è potuto osservare un abbondante accumulo del trascritto sia nelle cellule dell'epidermide che nel sistema vascolare di foglie e radici di piante di *B. juncea* trattate con Cd. Per caratterizzare la funzione di BjCdR15 si è saggiata la sua sovraespressione in piante transgeniche di Arabidopsis e tabacco e si è eseguita una prova di complementazione utilizzando il mutante *tga3-2* di *Arabidopsis*. In presenza di Cd, le piante sovraesprimenti BjCdR15 (sia di *Arabidopsis* che di tabacco) presentavano, a livello dei germogli, un contenuto di Cd, un'area fogliare, un contenuto di clorofilla e un peso fresco superiore rispetto alle rispettive piante controllo. In particolar modo, i germogli delle piante *tga3-2* presentavano un contenuto di Cd più basso rispetto alle piante controllo e alle piante sovraesprimenti BjCdR15 (35S::*BjCdR15*): l'espressione ectopica di BjCdR15 in queste linee causava però un aumento significativo del contenuto in Cd a livello dei germogli rispetto alle altre prove. I risultati ottenuti avvaloravano quindi l'ipotesi che BjCR15 sia in effetti un ortologo di TGA3 poiché è in grado di complementare l'attività di TGA3 nelle linea mutante *tga3-2*. Si è poi analizzato il contenuto di AtPCS1 nelle diverse linee di *Arabidopsis* transgeniche prese in considerazione: i risultati ottenuti hanno indicato un putativo ruolo di BjCdR15 nella sintesi di AtPCS1 e quindi nella regolazione della sintesi di fitochelatine. Infine si è andati a osservare i livelli dei trascritti di alcuni trasportatori cellulari nelle radici delle diverse linee di *Arabidopsis* sovraesprimenti e non BjCdR15, al fine di verificare un coinvolgimento di tale gene nella loro regolazione in

seguito a trattamento con Cd. Questi geni hanno mostrato una normale induzione in seguito a trattamento con Cd nelle linee controllo, mentre, nel mutante *tga3-2*, la loro espressione non è influenzata dalla presenza del metallo nel mezzo di crescita. La sovraespressione di BjCdR15 in *tga3-2* causava una normale induzione dei geni in esame in presenza si Cd, indicando la complementazione dell'allele nullo TGA3 nel mutante.

Capitolo II: *identification of genes involved in heavy metals tolerance and hyperaccumulation in Arabidopsis halleri shoots in response to Cd and Zn and rhizosphere microorganisms.*

Con lo scopo di far luce sui meccanismi che stanno alla base della tolleranza e dell'iperaccumulo di metalli pesanti (in particolar modo il Cd) nella specie *A. halleri*, considerando l'interazione pianta-microorganismi, piante di *A. halleri* sono state trattate con metalli pesanti (Cd e Zn) in presenza o meno della comunità microbica stessa. Al termine dei seguenti trattamenti, a livello dei germogli si è effettuata un'analisi del contenuto totale delle clorofille e una misurazione del contenuto totale di entrambi i metalli. Dai risultati ottenuti si è potuto confermare che la presenza della totale comunità microbica proveniente dalla rizosfera autoctona di *A. halleri* cresciuta su terreno contaminato influenza positivamente l'accumulo sia di Cd che di Zn nella porzione vegetativa della pianta stessa. Al contrario, si è riscontrato che un inoculo con 8 ceppi in coltura pura, isolati dalla rizosfera di *A. halleri* cresciuta sul sito contaminato e in grado di tollerare elevate concentrazioni di Cd e Zn, non ha influenzato in modo analogo l'assorbimento dei metalli. Infatti, il contenuto di Cd e di Zn, riscontrato nella porzione vegetativa delle piante trattate con metalli e questi otto ceppi microbici, risultava minore rispetto alle altre prove. Sulla porzione epigea delle piante si è poi effettuata un'analisi molecolare delle proteine differenzialmente espresse, tramite 2-DE, in seguito ai diversi trattamenti. In totale sono stati individuati 78 spot proteici a diversa intensità, che sono stati analizzati mediante spettrometria di massa ESI-Q-TOF. In totale si sono identificate 57 proteine: la maggior parte di esse sono coinvolte in processi quali fotosintesi (43.6%), metabolismo cellulare (18%) e risposta a stress (33.3%). Si sono identificate, in percentuale minore (5.1%), anche proteine con funzione sconosciuta. Per alcune delle proteine identificate, tramite analisi western, si sono confermati i *pattern* d'espressione osservati tramite l'analisi proteomica bidimensionale, avvalorando ulteriormente tali risultati.

Prospettive future

Ci si pone come primo obiettivo l'espressione di *BjCdR15* nella linea mutante *tga3-2* sotto il controllo del promotore nativo del gene *TGA3*, al fine di comprendere meglio la loro correlazione funzionale e per confermare ulteriormente il fatto che i due geni in esame siano ortologhi.

In secondo luogo, ci si pone come obiettivo lo studio, a livello molecolare, di alcune proteine differenzialmente espresse nelle diverse prove, per cercare di comprendere meglio i meccanismi che sono alla base della tolleranza/accumulo in *A. halleri*.

Infine, si vuole continuare lo studio molecolare dei diversi ceppi isolati in coltura pura e poiché tali microorganismi rappresentano un potenziale *reservoir* di importanti geni coinvolti nella detossificazione dell'agente tossico, lo scopo finale del progetto è l'identificazione di alcuni di questi geni al fine di poterli trasferire in specie vegetali, ottenendo piante con un'alta efficienza di fitorimedio.

ABSTRACT

Introduction

Cadmium (Cd) is a main risk for human health and agriculture: this is due to its high solubility in water, which causes its rapid distribution into the environment, an immediate availability to plants and its access into the food-chain. An alternative strategy of environmental remediation from heavy metals, for example Cd, is the phytoremediation: a process applied *in situ*, ecologically safe (environment friendly) and inexpensive, which has as aim the strip of organic and inorganic contaminants from sites by plants. Particularly, the phytoextraction provide the use of plants able to remove heavy metals and accumulate them in the above-ground tissues (Pilon-Smits, 2005). The identification of numerous hyperaccumulator plants capable to tolerate and accumulate high heavy metal amounts in their tissues, demonstrates that plants own the genetic capacity to tolerate and remove heavy metals from contaminated sites. An example is *Arabidopsis halleri* (belonging to *Brassicaceae* family), a plant species tolerant to Cd, Zn and Pb and hyperaccumulator of Cd and Zn (van Rossum *et al*., 2004). Being phylogenetically related to *Arabidopsis thaliana*, *A. halleri* is considered an important model system in studies that concern phytoremediation. Much remains still unknown about the molecular components of the metal-induced signal transduction, and only recently, thanks to differential-expression analyses, it has been possible to identify several genes, for example transcription factors (TFs), involved in heavy metal stress response. Moreover it important to consider the role of microbial community of rhizosphere in heavy metal uptake and translocation to the shoot processes: rhizosphere microorganisms, in fact, are able to modify (alter), directly or indirectly, the heavy metal mobility in soil, influencing their absorption by plant roots (Lovley, 1995).

Aims of the work

With the purpose to obtain more information on the complex genetic network responsible for heavy metal accumulation and detoxification, the main aim of this work is the characterization of a transcription factor in response to heavy metal stress and to analyse the influence of microbial community of the rhizosphere on heavy metal uptake and translocation to the shoots. Therefore, to understand the mechanisms by which plants respond to heavy metal exposure is a primary goal of plant biotechnologysts, whose aim is to create plants able to recover high amounts of heavy metals, which can be used for phytoremediation.

Chapter I: *characterisation of a B. juncea bZIP transcription factor responsible for Cd uptake and translocation to the shoot in transgenic plants*.

The *BjCdR15* (*Brassica juncea Cd-regulated*) full-length cDNA was isolated from *B. juncea* after Cd-treatments (Fusco *et al*., 2005). *BjCdR15* sequence alignment showed an high sequence similarity with the *Arabidopsis TGA3* gene, coding a bZIP transcription factor. By expression analysis it was observed that *BjCdR15* is up-regulated in shoots and roots after Cd treatments and in roots after Pb/Ni treatments. Hybridisation *in situ* analysis showed an accumulation of *BjCdr15* transcripts mainly in epidermal cells and in vascular system of roots and shoots of Cd-treated *B. juncea* plants. *Arabidopsis* and tobacco WT plants and *tga Arabidopsis* mutant line were transformed with constructs for BjCdR15 overexpression to evaluate its influence in Cd tolerance and uptake and to verify whether it is able to complement the function of TGA3 in the mutant line. In presence of Cd, BjCdR15 overexpressing plants showed a higher Cd content in shoots. Furthermore, 35S::*BjCdR15* plants showed an increased leaf area, shoot fresh weight and chlorophyll content than control plants. Conversely, the *tga3*-2 mutant line showed a minor Cd content in shoot than control and overexpressing BjCdR15 plants. However, the ectopic expression of BjCdR15 in this mutant line caused an increased transport of Cd to the shoots. Indeed these plants showed the highest Cd content in shoots with respect to other lines. In addition, the content of AtPCS1 was analysed in shoots of the different transgenic *Arabidopsis* lines, to verify the role of BjCdR15/TGA3 in the phytochelatins synthesis. Finally, with the same purpose, the transcript levels of some metal transporters was determined in roots of transgenic *Arabidopsis* lines.

Chapter II*: identification of genes involved in heavy metals tolerance and hyperaccumulation in Arabidopsis halleri shoots in response to Cd and Zn and rhizosphere microorganisms.*

With the aim to identify and understand the molecular bases of heavy metal tolerance/hyperaccumulation (especially Cd) of *A. halleri* in presence of its rhizosphere microbial community, *A. halleri* plants were treated with heavy metals (Cd and Zn) in presence or not with rhizosphere microorganisms. After treatments the content of both metals was determined in shoots of plants differently treated. Moreover, the chlorophyll content was measured in shoots of all tests. The results obtained confirmed that the total microbial community of rhizosphere positively affect the uptake of both heavy metals in *A. halleri*. On the contrary, the *inoculum* with eight microbial strains, isolated from *A. halleri* rhizosphere and tolerant to high concentrations of Cd and Zn, does not positively

influence the uptake of these heavy metals: in fact, the heavy metal content in shoots of plants treated with metals and these eight microbial strains was less than other plants. Furthermore, on shoots of plants subjected to the different growth conditions, a molecular analysis (2-DE) was performed to identify the differentially expressed proteins. In total 78 protein spot were detected and analysed by ESI-Q-TOF mass spectrometry. After identification the 57 identified proteins were classified in their biological function: 43.6 % are involved in photosynthesis, 33.3% were grouped as stress-responsive proteins, 18% in cellular metabolism and only 5.1% correspond to unknown proteins. Finally, by western analysis, some expression patterns were confirmed.

Future perspectives

A main aim will be the expression of *BjCdR15* under the control of *TGA3* native promoter, with the purpose of understanding the functional correlation between both genes and to confirm that BjCdR15 is really an orthologous of TGA3.

Furthermore, an other important aspect of the research will be the molecular study of some of differentially expressed proteins in *A. halleri*, for a better understanding of the mechanisms of heavy metal tolerance and accumulation in this plant species.

Finally, the attention will be focused on the molecular study of the selected eight microbial strains tolerant to heavy metals. In particular, since these microorganisms can be considered a *reservoir* of important genes involved in detoxification of contaminants, the final goal of the project is the identification of some of these microbial genes, with the aim of transfer them into plants, obtaining thus plants with high phytoremediating efficiency.

LIST OF ABBREVIATIONS

2-DE:	Two Dimensional Electrophoresis
35SCaMV:	35S promoter of the Cauliflower Mosaic virus
a.m.u.:	Atomic mass unit
a.u.:	Arbitrary unit
ABA:	Abscissic acid
ACN:	Acetonitrile
AFLP:	Amplified Fragment Length Polymorphism
ATP:	Adenosine triphosphate
bp:	Basis pair
cDNA:	Complementary deoxyribonucleic acid
CFU:	Colony Formant Unit
CHAPS:	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Chl:	Chlorophyll
Da:	Dalton
DTT:	Dithiothreitol
DW:	Dry Weight
EDTA:	Ethylenediamine-tetraacetic acid
ESI:	Electrospray Ionization
ESI-Q-TOF MS:	Electrospray Ionization-Quadrupole-Time Of Flight-Mass Spectrometry
h:	Hours
HPLC:	High Performance Liquid Chromatography
IAA:	Indole-3-acetic acid
IEF:	IsoElectric Focusing
LAG:	Latency phase
LB:	Left Border
LC:	Liquid chromatography
LHC:	Light-Harvesting Complex
m/z:	Mass over charge
M:	Molarity
MIC:	Minimum Inhibitory Concentration
min:	Minute
mol:	Mole

Mr:	Relative Mass
MS/MS:	Tandem Mass Spectrometry
MS:	Mass Spectrometry
NL:	Non Linear
nt:	Nucleotide
OD:	Optical Density
OD_{600}	Optical Density at 600 nm
PAGE:	Polyacrylamide Gel Electrophoresis
PCR:	Polymerase Chain Reaction
PEG:	Polyethylene Glycol
pI:	Isoelectric point
ppm:	Part per million
PS:	Photosystem
RB:	Right Border
rev/min:	Revolutions per minute
RNA:	Ribonucleic acid
ROS:	Reactive Oxygen Species
RT-PCR:	Reverse Transcriptase-Polymerase Chain Reaction
SA:	Salicylic acid
SDS:	Sodium Dodecyl Sulphate
SE:	Standard error
SSP:	Standard Spot Protein
T-DNA:	Transfer-DNA
V/V:	Volume per volume
Vh:	Volt x hours
W/V:	Weight per volume
WT:	Wild-Type

GENERAL INTRODUCTION

I. SOIL AND WATER POLLUTION BY HEAVY METALS

Lands and waters are precious natural resources on which the sustainability of agriculture and the civilization of mankind rely. Nevertheless, they are subjected to massive exploitation and severely degraded and polluted by anthropogenic activities. Pollution derives from emissions, effluents and solid discharge from industries, vehicle exhaustion and metals from smelting and mining. Furthermore, the use of insecticides and pesticides, disposal of industrial and municipal wastes in agriculture and the excessive use of fertilizers represent sources for soil and water pollution (McGrath *et al*., 2001). Among environment contaminations, heavy metals are a serious alarm to plants and human beings: high concentrations of heavy metals in soil can negatively affect crop growth and, being introduced into the food-chain, can lead, in humans, to acute gastrointestinal and respiratory damages, acute heart, brain and kidney problems. More in detail, heavy metals may interfere with metabolic functions in plants, including physiological and biochemical processes. They inhibit photosynthesis and respiration processes, causing degeneration of cell organelles and eventually leading to plant death (Garbisu and Alkorta, 2001; Schmidt, 2003; Schwartz *et al*., 2003).

Conventionally, heavy metals are defined as elements with a density higher than 5 g/cm^3. According to this definition, 53 out of 90 natural elements are heavy metals (Weast, 1984). Based on their properties and solubility under physiological conditions, some of these may be available for living cells and have a physiological importance for organisms. For example, Fe, Mo, and Mn are important micronutrients. On the contrary, Zn, Ni, Cu, Co and Cr are toxic elements but essential for plant life as trace elements, while As, Hg, Ag, Cd and Pb have no known function as nutrients and seem to be toxic to plants and living organisms (Godbold and Huttermann, 1985; Breckle, 1991; Nies, 1999). The most common heavy metals identified in polluted environment include As, Cu, Cd, Pb, Cr, Ni, Hg and Zn and the presence of these metals may vary from site to site, depending from

the source of individual pollutant (Lone *et al*., 2008). These heavy metals have high stability and consequently persistence into the sites, and, for their chemical and physical properties, they cannot be destroyed biologically but can be only transformed from one oxidation state or organic complex to another (Garbisu and Alkorta, 2001; Gisbert *et al*., 2003).

II. CADMIUM IN THE ENVIRONMENT

In agricultural fields, Cd pollution is an increasing problem due to soil amendment and to the intense use of phosphate fertilizers that contain it as a contaminant (Schützendübel and Polle, 2002). Cd seems to be toxic to both eukaryotic organisms and microorganisms (Sanità di Toppi and Gabbrielli, 1999; Benavides *et al*., 2005): this is due to its high solubility in water, which causes its rapid distribution into the environment, an immediate availability to plants and its access into the food-chain. Cd finds important roles in several factory applications: it is used in processes of cadmium plating on metals (iron, steel and copper), for production of pigments, paints and as component for the synthesis of alloys with other metals (copper, zinc,...) in order to increase ductility and malleability. Furthermore, salts of Cd are often used as stabilizers in plastics (PVC production) and may be present as contaminants in phosphate fertilizers. In addition to industrial production, this heavy metal can be dispersed during coal burning and waste incineration (Lee, 1984; Alonso and Eva, 2001). In natural environments Cd is present at concentrations as 0.1-0.5 mg/kg (Schützendübel and Polle, 2002) but in contaminated sites its concentration reaches also the value of 150 mg/kg (Jackson and Alloway, 1991).

II.I Cd TOXICITY IN PLANTS

Symptoms induced by Cd toxicity in plants are easily identifiable: this metal is considered a non-nutrient element, since it has no known function in plant development and life, with

the exception of the Cd carbonic anhydrase of marine diatoms (Lane and Morel, 2000). In higher plants, Cd negatively affects both plant growth and development, resulting in growth stunting and eventually plant death. The critical tissue concentration, at which the metal causes injuries in plant physiology, is in the range of 8 to 12 mg/kg dry mass (Balsberg-Pahlsson, 1989). The base of Cd toxicity is still not completely understood, but it might due to its high affinity for sulphydryl groups (e.g. three-fold higher than Cu ions; Schützendübel and Polle, 2002). Cd, binding to sulphydryl groups of structural proteins and enzymes, leads to protein misfolding, inhibition of enzyme activity and/or interference with the redox enzyme regulation (Hall, 2002). Another important toxicity mechanism is due to the chemical similarity between Cd^{2+} and functional ions situated in active sites of enzymes and signalling components. Consequently, Cd^{2+} ions can interfere with homeostatic pathways for essential metal ions (Roth *et al*., 2006) and the displacing of divalent cations, such as Zn and Fe, from proteins could cause the release of “free” ions, which might trigger oxidative injuries via free Fe/Cu-catalyzed Fenton reactions (Polle and Schützendübel, 2002). However, *in vivo* Cd-related injuries on plants depend firstly on the plant species: for example, hyperaccumulators or tolerant plants may activate cellular mechanisms that weaken the impairment due to Cd stress. Moreover, the time of Cd exposure and its magnitude, together with external environmental conditions, contribute to modulate plant sensitivity to the metal (Sanità di Toppi and Gabbrielli, 1999).

- Photosynthesis and carbon assimilation

The most evident and studied effects of Cd toxicity are chlorosis on leaves together with necrotic maculature, water uptake imbalance and stomatal closure (Clemens, 2006).

Chlorosis, caused by Cd, might be due to changes in Fe:Zn ratio, as shown in corn leaves (Root *et al*., 1975) and to the negative effects on chlorophyll metabolism (Chaffei *et al*., 2004). In fact, at cellular level, Cd may damage the main components of photosynthetic apparatus, as the light harvesting complex II and the two photosystems (Figure I), and

cause a decrease in chlorophyll and carotenoid content, leading to higher non-photochemical quenching process (Sanità di Toppi and Gabbrielli, 1999).

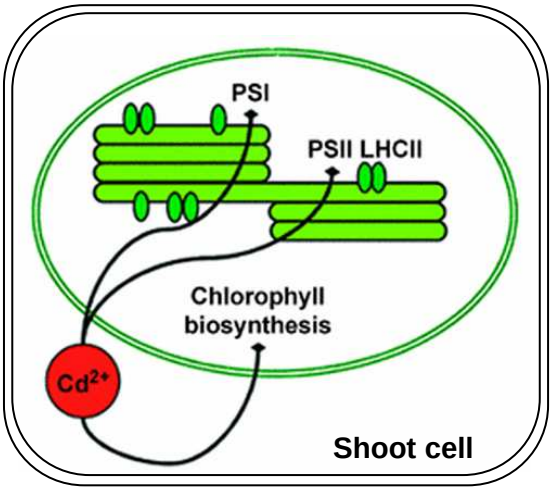


Figure I. Effects of Cd on photosynthesis and chlorophyll biosynthesis. PSI and II: photosystem I and II; LHCII: light harvesting complex II. (DalCorso *et al*., 2008).

Regarding stomatal closure, it has been shown that during Cd exposure stomata close independently to the water status. It is known that stomatal closure can be actively driven by ABA-induced Ca^{2+} accumulation in the cytosol of the guard cells: the increase in cytosolic free Ca^{2+} promotes the opening of plasma membrane anions and K^{+}_{out} channels. As more ions leave the cell, water follows and turgor is lost, with stomatal pore closure (MacRobbie and Kurup, 2007). Cd, being chemically similar to Ca ions, probably enters guard cells through voltage-dependent Ca^{2+} channels and once in the cytosol it mimics Ca^{2+} activity (Perfus-Barbeoch *et al*., 2002) (Figure II). Stomatal closure, damage to the photosynthetic machinery and interference with pigment synthesis are the main causes of a general decrease of the photosynthetic efficiency, lowering the effective quantum yield. Moreover, by inhibiting enzymes involved in CO_2 fixation, Cd decreases carbon assimilation (Perfus-Barbeoch *et al*., 2002).

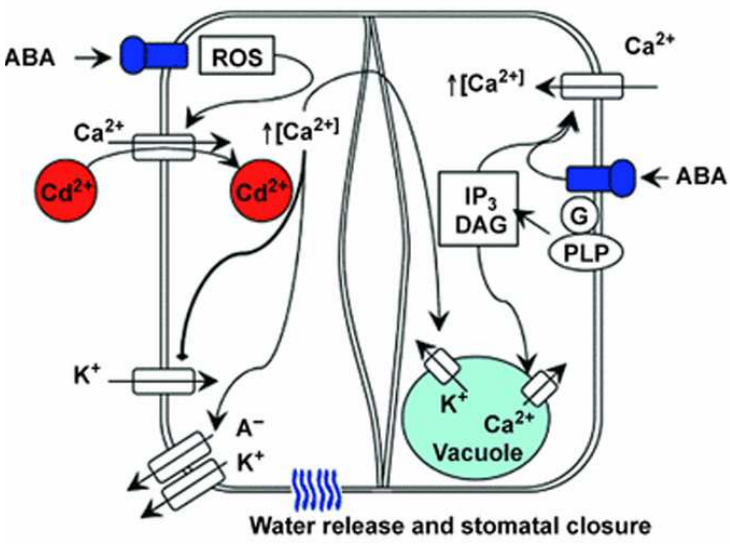


Figure II. Effects of Cd on guard cells: mimicking Ca^{2+} ions, Cd enters stomatal guard cell and activates opening of plasma membrane anion and K^{+}_{out} channels. IP3: inositol-3-phosphate; DAG: diacylglycerol; PLP: phospholipase protein, involved in DAG and IP3 mediated signalling. (DalCorso *et al*., 2008).

- Cd effects on cellular homeostasis and intracellular signalling

In different plant species cytotoxicity of Cd exposure is associated to chromosomal aberrations and inhibition of mitotic processes with consequent altered cell cycle and division (Benavides *et al*., 2005). Furthermore, in *Arabidopsis thaliana* Cd causes high mutation rates, floral anomalies, poor seed production and malformed embryos (Ernst *et al*., 2008). It also induces higher vacuolization and mitochondrial degeneration affecting cell metabolism and aerobic respiration (Silverberg, 1976). Moreover, Cd exposure causes oxidative injuries, such as lipid peroxidation, leading to alteration of membranes functionality and protein carbonylation (Romero-Puertas *et al*., 2002; Schützendübel *et al*., 2001), and converges into a general redox homeostasis impairment. Cd also imbalances the activity of antioxidative enzymes and affects catalase and super-oxide dismutase (SOD) activity, generating an over-accumulation of H_2O_2 and O^{2-}, the well known reactive oxygen species (ROS), even if it is still not clear whether the over-production of ROS

during Cd-treatment is the cause of redox cellular imbalance or if this is a specific stress-mechanism activated by the plant cell to cope with the heavy metal ions (Romero-Puertas *et al*., 2004). However, the accumulation of ROS in the different cell compartments causes the alteration of the signalling mediated by H_2O_2 and other oxygen species. In fact, hydrogen peroxide is considered a signal molecule which plays a significant role in triggering the induction of defence mechanisms against both abiotic stresses, such as temperature and ozone (Dat *et al*., 2000; Sharma *et al*., 1996), and pathogen attack as infections due to bacteria or powdery mildew fungi (Bestwick *et al*., 1998; Thordal-Christensen *et al*., 1997). It was then shown that Cd induces peroxisome senescence in leaves, activating the glyoxylate cycle enzymes, malate synthase and isocitrate lyase, as well as peroxisomal peptidases, the latter being involved as leaf senescence-associated factors (Chaffei *et al*., 2004).

Finally, Cd interferes with plasma membrane ion transporters and ATPase (Sanità di Toppi and Gabbrielli, 1999) disturbing ion and metabolite movement and accumulation. In addition, Cd exposure affects the normal cellular signalling interfering with ions such as Ca^{2+} and Zn^{2+} and inhibits the activity of metabolic enzymes such as glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, malic enzyme, isocitrate dehydrogenase, Rubisco and carbonic anhydrase (Sanità di Toppi and Gabbrielli, 1999). Moreover, being chemically very similar to ions, such as Zn^{2+} and Ca^{2+}, Cd^{2+} can hamper the activity of Zn-finger transcription factors, substituting Zn ions and interfering with transcription mechanisms (Sanità di Toppi and Gabbrielli, 1999), and replaces Ca^{2+} ions in calmodulin proteins, causing the perturbation of intracellular calcium level and altering calcium-dependent signalling (Ghelis *et al*., 2000; Perfus-Barbeoch *et al*., 2002).

- Effects on nutrient uptake and root physiology

In roots, Cd imbalances the water uptake and the nutrient metabolism (uptake, transport and use), interfering with the uptake of essential elements such as Ca, Mg, K, Fe and P

(Benavides *et al*., 2005). For example, in cucumber and sugar beet it was demonstrated that Cd leads to a Fe(II) deficiency caused by the inhibition of the root Fe(III) reductase (Alcantara *et al*., 1994). Moreover, both nitrate and nitrite reductase activity in roots and leaves are affected (Chaffei *et al*., 2004) as well as root-to-shoot nitrate transport (Sanità di Toppi and Gabbrielli, 1999), leading to a reduced nitrate assimilation by the whole plant (Figure III). In many plant species (e.g. tomato, maize, pea and barley), Cd alters the activity of enzymes involved in nitrogen metabolism (Nussbaum *et al*., 1988; Boussama *et al*., 1999). Also the activity of the enzymes responsible for the incorporation of ammonium molecules into the carbon skeleton (i.e. glutamine and glutamate synthetase) is compromised (Chaffei *et al*., 2004) (Figure III). In addition, the activity of the glutamate dehydrogenase (GDH) is enhanced during Cd stress (Boussama *et al*., 1999). Because high activity of GDH enzyme has been related with pathogen response and senescence induction (Osuji and Madu, 1996; Masclaux *et al*., 2000), and changes in nitrogen metabolism due to the Cd stress are similar to the ones induced during senescence, it has been hypothesized that Cd induces senescence-like symptoms, at least in tomato, leading to nitrogen mobilization and a storage strategy (Chaffei *et al*., 2004). Finally, during Cd-treatments, in nodules of soybean plants, it is evident that nitrogen fixation and primary ammonia assimilation decrease (Balestrasse *et al*., 2003).

Regarding sulphur metabolism, exposure to Cd induces a remarkable increase in the amount of thiol compounds, with a concomitant decrease in the activity of leaf ATP sulphurylase and O-acetylserine sulphurylase, enzymes involved in the sulphate assimilation pathway (Astolfi *et al*., 2004).

Phenotypically, at level of roots, Cd exposure inhibits root growth and lateral roots formation, while it induces differentiation of numerous root hairs both in *Arabidopsis* and tobacco plants (Farinati *et al*., unpublished). While in tomato plants, Cd-treated roots were thicker and stronger and the root biomass was less affected than the leaves (Chaffei *et al*., 2004).

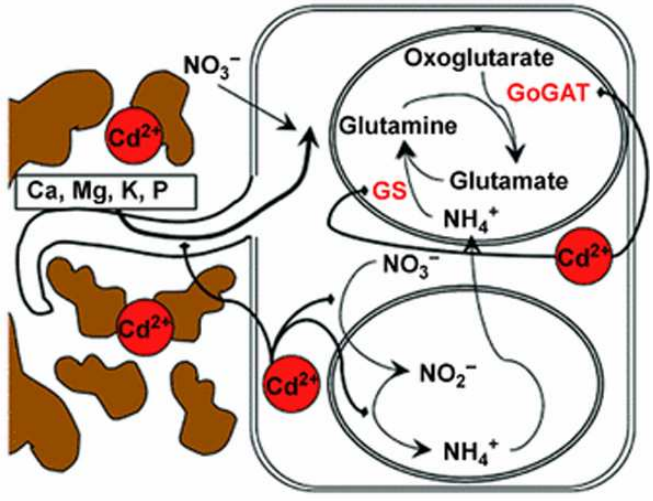


Figure III. Effects of Cd on root cells: Cd induces inhibition of root enzymes involved in nitrogen assimilation. Moreover, Cd interferes with activity of both GS and GoGAT enzymes, involved in ammonium assimilation. GS: glutamine synthase; GOGAT: glutamate synthase (DalCorso *et al*., 2008).

II.II PLANT RESPONSES TO Cd

Plants, as all other organisms, have evolved a complex network of homeostatic mechanisms to minimize the damages from exposure to nonessential metal ions, as Cd. To avoid Cd toxicity, land plants have developed different active and passive strategies of exclusion of the heavy metal ion from the cellular environment. For example, as first defence to Cd stress, plant organic acids, such as malate or citrate, can be exuded from roots: they bind metal ions to the soil matrix excluding them from root absorption (Delhaize and Ryan, 1995). Secondly, the cell wall (through pectic sites and hystidyl groups) and extracellular carbohydrates (callose, mucilage) can play a significant role in immobilizing toxic ions, preventing their uptake into the cytosol (Sanità di Toppi and Gabbrielli, 1999). To limit the concentration of toxic elements, active metabolism also takes charge producing chelating compounds (phytochelatins and metallothioneins) involved in the detoxification and compartmentalization of the heavy metals in specific cellular compartments. Moreover, as for other abiotic stresses, Cd resistance involves the synthesis of stress-related proteins and signal molecules (heat shock proteins, salicylic and abscissic acids, ethylene) (DalCorso *et al*., 2008).

- Modulation of signal transduction and gene expression by Cd

Responses to heavy metal stress relies on an intricate signal transduction pathway within the cell that begins with the sensing of the heavy metal (or heavy metal associated symptoms) and converges into transcription regulation of metal-responsive genes (Singh *et al*., 2002). This allows possible plant development and reproduction in adverse environmental conditions, by sharing common mechanisms for growth, development and adaptation to the environment.

Signal transduction begins with the activation of the receptor elements, that undergo to a conformational change after recognition of and binding to the ligand (e.g. a hormone). For example, ABA and ethylene are the major hormones associated with the stress responses and it has been shown that Cd exposure induces the biosynthesis of these molecules into roots (Sanità di Toppi and Gabbrielli, 1999). Some receptors are able to directly regulate gene expression following the binding to the ligand, but most require a wide range of intracellular mediators that activate each other, thus creating a cascade of reactions that lead to amplified signal (Alberts *et al*., 1995). Much remains still unknown about the molecular components of the metal-induced signal transduction, and only recently, thanks to differential-expression analyses, it has been possible to identify transcription factors (TFs) probably responsive to Cd stimulus (Fusco *et al*., 2005). As commonly found for other stress related TFs, also heavy metal responsive TFs share the same signal transduction pathway and might be activated by other abiotic stresses such as cold, dehydration, SA and H_2O_2 (Singh *et al*., 2002). Moreover, the modulation of TFs belonging to different families demonstrates the complexity of the response of plants to Cd stress, as other heavy metal stress, from the signal perception to the intracellular transduction cascade triggering the activation of Cd responsive genes.

- Role of phytochelatins

Phytochelatins (PCs) are small metal-peptide with the general structure (γ-Glu-Cys)*n*-X where *n* is a variable number from 2 to 11 and X an amino acid such as Gly, β-Ala, Ser, Glu or Gln (Cobbett and Goldsbrough, 2002). Due to the presence of the thiolic groups of Cys, PCs may chelate metal ions, in particular Cd, and form several complexes with molecular weight of about 2500 or 3600 Da, protecting the cytosol from free toxic ions (Cobbett, 2000). Their role in the protection from Cd toxicity is confirmed in both *Arabidopsis* and *Schizosaccharomyces pombe*, by the Cd-sensitive phenotype of *cad1* mutants (Ha *et al*., 1999). Glutathione is the building brick for PCs synthesis, which is catalyzed by the cytosolic PCs synthetase (PCS). It has been shown that PCS is constitutively expressed and post-translationally activated by heavy metals (Cobbett and Goldsbrough, 2002). After synthesis, PCs bind the heavy metal ions and facilitate their transport as complexes into the vacuole (Clemens, 2006) where they eventually form high-molecular weight (HMW) complexes (Figure IV). Several studies demonstrated that in *Arabidopsis* the transport of these HMW complexes across the tonoplast is mediated by ABC transporters (Cobbett and Goldsbrough, 2002). It was also reported that PCs play a role in Cd transport from root to shoot and it was demonstrated that a PCs dependent "overflow protection mechanism" would contribute to keep Cd accumulation low in roots, causing extra Cd transport to the shoot (Gong *et al.*, 2003). PCs play an important role in cellular homeostasis: because of their metal ion affinity they are involved in trafficking of essential nutrients such as Cu and Zn (Thumann *et al*., 1991). Anyway, an excessive amount of PCs does not confer, *per se*, any hyper-tolerance; indeed, while an enhanced PCs synthesis seems to increase heavy metals accumulation in transgenic plants (Pomponi *et al*., 2006), an excessive expression of *AtPCS* genes seems to determine an hypersensitivity to Cd stress (Lee *et al*., 2003).

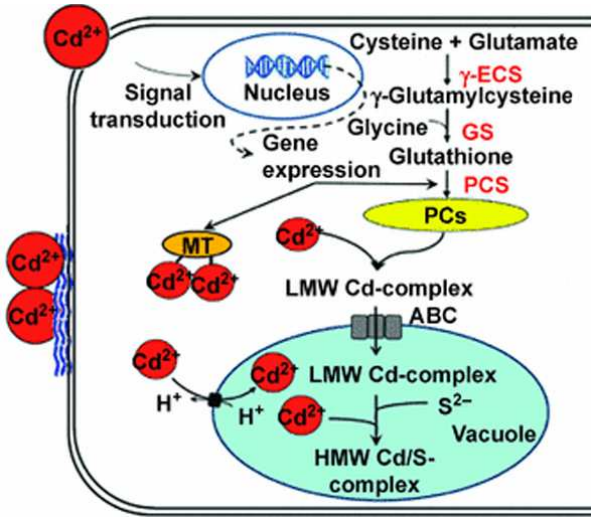


Figure IV. Role of phytochelatins: once Cd enters the cytosol, stimulates the synthesis of phytochelatins and probably metallothioneins. γ-ECS: γ-glutamylcysteine synthetase; MT: metallothioneins; GS: glutathione synthetase. (DalCorso *et al*., 2008).

- Role of metallothioneins

Like phytochelatins, also metallothioneins (MTs) are low molecular weight cysteine-rich peptides able to bind metal ions by means of mercaptide bonds. Differently from PCs, MTs are products of mRNA translation and induced in response to heavy metal stress (Cobbett and Goldsbrough, 2002) (Figure IV). In vertebrates MTs are characterized by a stretch of 20 Cys residues highly conserved, while plant and fungi isoforms do not contain this structure (Cherian and Chan, 1993). MT genes of wheat and rice can be induced by a variety of metal ions, such as Cu, Cd and Al, and abiotic stresses, such as high temperature and nutrients deficiency (Cobbett and Goldsbrough, 2002). Regarding their metal binding activity, it was shown that pea MTs (PsMTa) can bind Cd, Zn and Cu when expressed in *E. coli* (Tommey *et al*., 1991). Although the role of plant MTs in Cd tolerance is still largely unknown, overexpression of a mouse MT in tobacco plants enhances Cd tolerance *in vitro* (Pan *et al*., 1994), while *Brassica juncea* MT2 ectopically expressed in *Arabidopsis thaliana* confers increased tolerance to Cd and Cu (Zhigang *et al*., 2006). Moreover, there are some evidences that support their participation in Cu homeostasis (Cobbett and Goldsbrough, 2002). In fact, *Arabidopsis* MTs restore tolerance to copper in MT-deficient yeast strains (Zhou and Goldsbrough, 1994).

MT genes are expressed during various stages of plant development and in response to different environmental conditions (Rauser, 1999). In terms of transcript amount, many plant MT genes are expressed at very high levels in all plant tissues. *Arabidopsis* MT1a and MT2a seem to accumulate in trichomes, being involved in sequestration of heavy metal ions in these structures (Salt *et al*., 1995). Furthermore, since *Arabidopsis* MT expression has been detected in phloem elements, a role in metal ion transport has been postulated (Garcia-Hernandez *et al*., 1998). Finally a number of MT genes was isolated from ripening fruits (Rauser, 1999) and they also play a role in normal development processes.

- Metal ion transporters

An aspect of Cd tolerance in plant is correlated with its extrusion or intracellular compartmentalization mediated by specific transport processes. Thus metal transporters, situated in the tonoplast or in the plasma membrane, have a role in the maintenance of metal homeostasis within physiological limits. In plants there are different groups of metal transporters classified for their capacity to transport particular metal ions and for their ability to import or export metals into the cell or into different cellular compartments. Metal transporters appointed to ion import into the cytoplasm show low selectivity. An example is *At*IRT1 (Iron Response Transporter) (localized in the plasma-membrane of root cells): it is the primary root iron uptake system in *Arabidopsis* but can transport significant amounts of Cd (Korshunova *et al*.,1999). Conversely, intracellular transporters that export metal ions from the cytosol to both vacuoles and outside the cell, are highly selective. For instance, tonoplastic transporters *At*MTP1 and *At*MTP3 specifically export Zn into the vacuole (Krämer *et al*., 2007).

An important group of plant metal transporters is the ZIP family (for ZRT, IRT-like Protein). These plasma-membrane transport proteins are induced both in roots and shoots of *Arabidopsis* in response to Zn-limiting conditions (Krämer *et al*., 2007). ZIP members have

also been identified in several plant species, as well as in bacteria, fungi and animals and several results indicate that they are involved in divalent cations transport across membranes (Lòpez-Millàn *et al*., 2004). Members of ZIP family are thought to be implicated in Cd uptake from the soil into the root cell and in cadmium root-to-shoot transport, being involved in the xylem unloading process (Krämer *et al*., 2007). Enhanced root metal uptake mediated by ZIP transporters seems to be a factor necessary, but not sufficient, for hyperaccumulation in *Arabidopsis halleri* and *Thlaspi caerulescens* (Krämer *et al*., 2007) and accumulation capacity in these species varies with the expression of these proteins. For instance, in *A. halleri*, ZIP9 has a high expression level in roots already under Zn-sufficient conditions, while it is up-regulated in shoots in response to Zn deficiency (Krämer *et al*., 2007). *Vice versa* , in *A. thaliana*, ZIP9 is induced during Zn-deficiency in both root and shoot. Similarly, ZIP6 is highly expressed in under Zn-deficiency, while it is not induced in *A. thaliana* (Becher *et al*., 2004; Filatov *et al*., 2006).

The family of Natural Resistance-Associated Macrophage Protein (NRAMP) metal ion transporters represents another important group of transmembrane proteins involved in metal transport and homeostasis. These transporters are considered as “general metal ion transporters” because of their ability to transport Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} (Nevo and Nelson, 2006). As the ZIP transporters, also members of the NRAMP family share remarkable protein sequence identity among plants, yeast and mammalians (Nevo and Nelson, 2006). By cDNA microarray, it has been shown that the expression level of NRAMP genes is higher in hyperaccumulator species (Chiang *et al*., 2006), in which they are expressed in both roots and shoots and are implicated in transport of metal cations across the plasma-membrane into the cytosol or across the tonoplast (Krämer *et al*., 2007). In *A. thaliana* these metal transporters participate principally in Fe homeostasis: in heterologous systems, three members of the *Arabidopsis* NRAMP family, *At*NRAMP1, *At*NRAMP3 and *At*NRAMP4, can mediate uptake of Fe, Mn and Cd (Curie *et al*., 2000).

Interestingly, the overexpression of *AtNramp3* results in Cd hypersensitivity of *Arabidopsis* root growth and in an increased accumulation of Fe (Thomine e*t al*., 2000).

Concerning efflux systems, P_{1B}-ATPases metal transporters (HMA) are important to translocation of metal ions out of the cytoplasm (both outside the plasma membrane and into the vacuole) by ATP hydrolysis. As already mentioned, export metal transporters are more selective than import transporters: indeed, HMA members, as HMA2, HMA3 and HMA4, export exclusively Zn and Cd (Krämer *et al*., 2007). Recent works highlighted that members of this family, for example *Ah*HMA4, *Ah*HMA3 and *Tc*HMA4, deriving from hyperaccumulator species *A. halleri* and *T. caerulescens* respectively, are able to confer Cd or Zn tolerance when expressed in yeast (Papoyan and Kochian, 2004; Bernard *et al*., 2004). Therefore, it has been proposed that *Ah*HMA4, *Tc*HMA4, and probably the homolog in *A. thaliana At*HMA4, may contribute to Cd and Zn homeostasis extruding the metal ions from the cytosolic compartment (Krämer *et al*., 2007). Finally, their expression mainly in the vascular system of root and shoot suggests an implication of these transporters in metal root-to-shoot transport (Verret *et al*., 2004).

Recently, also ATP-Binding-Cassette (ABC) transporters have been shown to have a significant role in metal detoxification together with other processes like polar auxin transport, lipid catabolism, disease resistance and stomatal function (Rea, 2007; Kim *et al*., 2006). Important examples of the ABC family members involved in Cd response are the *Arabidopsis* mitochondria transporters *At*ATM (ABC Transporter of the Mitochondria). It has been found that *At*ATM3 is up-regulated in roots of plants treated with heavy metals such as Cd and Pb. Moreover, *At*ATM3 overexpressing plants were more tolerant to Cd, whereas *atm3* mutants showed increased sensitivity. Since the tonoplast transporter *At*ATM3 homolog in *Schizosaccharomyces pombe* (HMT1) is able to export Cd-phytochelatin complexes, it has been hypothesized that even *At*ATM3 could have a role in extruding Cd-GSH complexes formed in the mitochondria and that the sensitivity of the mutant is due to the oxidative damage of Cd accumulation in this organelle (Kim *et al*.,

2006). Another example of ABC transporter in *A. thaliana* involved in metal homeostasis is *At*PDR8 (Pleiotropic Drug Resistance): it was demonstrated that *At*PDR8 participates in both Cd tolerance and pathogen resistance (Kobae *et al*., 2006, Stein *et al*., 2006). *At*PDR8 is mainly localized in the membrane of root hair and epidermis after Cd exposure (Kim *et al*., 2007). Its overexpression decreases Cd accumulation in roots and shoots and it is proposed that *At*PDR8 might confer Cd tolerance by pumping it out of the plasma membrane to the apoplast (Kim *et al*., 2007).

Finally, members of the "Cation Diffusion Facilitator" (CDF) transporter group seem to mediate vacuolar sequestration, storage and transport of metal ions from the cytoplasm to the outer compartment (Krämer *et al*., 2007). CDF transporters have been characterized in both prokaryotes and eukaryotes and can transport across membranes divalent metal cations such as Zn, Cd, Co, Fe, Ni or Mn (Montanini *et al*., 2007).

III. PHYTOREMEDIATION: A NOVEL APPROACH TO CLEANING UP POLLUTED SOILS AND WATERS

Due to the acute toxicity of Cd and other heavy metals contaminants, there is an urgent need to develop low-cost, effective and sustainable methods to remove contaminants from the environment. In order to maintain good quality of soils and waters and to keep them free from contaminations, continuous efforts have been made to develop technologies that are easy to use, sustainable and economically feasible. Traditional physicochemical approaches have been widely used for remedying polluted soil and water, especially at a small scale. However, because of their high costs and side effects, these methods result to be onerous when applied in large scale. On the other hand, plant-based approaches for cleaning polluted soils and waters, such as phytoremediation, are relatively inexpensive since they are performed *in situ* and are solar-driven (Salt *et al*., 1995). For this reasons, in the last decade, these technologies have gained increasing

attention as emerging cheaper approaches. Phytoremediation is defined as the use of plants, and their associated microbes, for environment cleanup and can be applied to remediate soils, liquid and gaseous substrates (Pilon-Smits, 2005). This technology employs the naturally occurring processes by which plants and their rhizosphere microbial flora degrade and/or sequester organic and inorganic pollutants. Organic pollutants, toxic and often carcinogenic, are mostly produced by anthropogenic activities as military, industrial and agricultural activities. Depending on their chemical properties these contaminants may be degraded in the root zone of plants or taken up by the plant and subsequently degraded, sequestered or volatilized (Pilon-Smits, 2005). On the contrary, inorganic pollutants, as heavy metals, cannot be directly degraded (Nriagu, 1979) and therefore they can only be phytoremediated via stabilization or sequestration in harvestable plant tissues.

- Advantages and limitations of phytoremediation technology

The application of the phytoremediation technology is influenced by many aspects. Firstly, plants involved in phytoremediation processes must co-locate together with the pollutant. Secondly, phytoremediation is limited by the root depth, which should be high enough so that roots can reach the pollutant in the matrix. Aspects as the chemical/physical properties of soil, the toxicity level and also the climate are important for plant growth and consequently for remediation. Moreover, depending on the biological processes, phytoremediation may be slower than the more established remediation methods like excavation, incineration or pump-and-treat systems: the *flow-through* phytoremediation systems and plant degradation of pollutants are rather fast (days or months), but soil cleanup via plant accumulation often takes years, limiting the applicability of these *in situ* techniques. Finally, pollutant bioavailability might limit the phytoremediation process: if only a fraction of the pollutant is bioavailable but the regulatory standard cleanup requires

that all pollutants are removed, phytoremediation is not applicable by itself (Flechas and Latady, 2003).

Despite of these limitations, phytoremediation technology has gained popularity because of its low cost since biological processes are ultimately solar-driven. It has been estimated that phytoremediation is tenfold cheaper than engineering–based remediation methods, such as excavation, soil washing or burning and pump-and-treat systems (Glass, 1999). The low cost is also due to the fact that the phytoremediation is usually carried out *in situ*, reducing in addition the exposure of pollutants to humans. Ultimately, phytoremediation enjoys popularity with the public opinion as a "green clean" alternative to conventional remediation methods.

III.I PHYTOREMEDIATION TECHNOLOGIES AND THEIR USE

Plants and associated rhizosphere microorganisms can be extensively employed for phytoremediation processes. They can "work" as filters (rhizofiltration) in hydroponic setups (Raskin *et al*.,1997) or in wetlands (Horne, 2000). A second phytoremediating strategy is called phytostabilization: plants reduce, directly or indirectly, the mobility of pollutants in soils (Berti and Cunningham, 2000). This can occur either by preventing leaching, runoff and erosion of soil or by converting pollutants to less bioavailable forms, for example via precipitation in the rhizosphere (Arazi *et al*., 1999). Conversely, phytoextraction could be applied to extract and accumulate contaminants into the plant above-ground tissues (Blaylock and Huang, 2000). Successively the plant material can be used for non-food purposes, in the case of valuable metals, recycling the accumulated elements (phytomining) (Chaney *et al*., 2000). Microbes populating the rhizosphere also play an important role in decontamination of contaminated sites from organic pollutants: plant and bacteria can have a synergic effect on each other, in a phenomenon that is exploited for phytostimulation or rhizoremediation (McCutcheon and Schnoor, 2003). However if the degradation of contaminants occurs directly via plant enzymatic activity the

process is called phytodegradation (McCutcheon and Schnoor, 2003). Finally, with the term of phytovolatilization it is meant the process that occurs when, after uptake in plant tissues, certain pollutants can leave the plant in volatile form (Terry *et al*., 1995).

It is important to consider that these various phytoremediation technologies are not mutually exclusive. In fact, processes like accumulation, stabilization and volatilization can occur simultaneously in a wetland (Hansen *et al*.,1998) without human interference (Figure V).

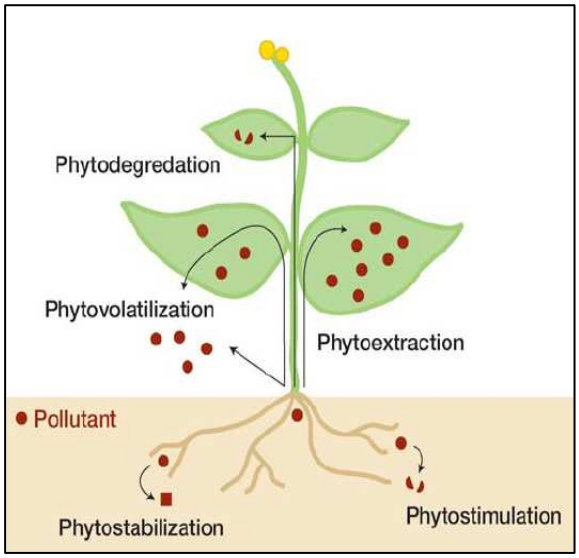


Figure V. Phytoremediation technologies: the pollutant (represented by red dots) can be stabilized or degraded in the rhizosphere, sequestered or degraded inside the plant tissue, or volatilized.(Pilon-Smits, 2005).

III.II PHYTOREMEDIATION OF HEAVY METALS

The different phytoremediation technologies above described are suitable for different classes of pollutants. For example, for toxic metals, such as Se, As, Cd and radionuclides, phytoextraction is mainly considered (Bãnuelos and Meek, 1990; Blaylock and Huang, 2000). Therefore, the use of plants with enhanced phytoextraction capacity for an array of metals could be an optimal phytoremediation strategy. Such plants would necessarily have a broad ability to take up and tolerate metals by means of specific protective mechanisms. Recent studies have shown that also genetic modifications of plants can

increase their phytoextraction efficiency (LeDuc *et al*., 2004; Ruiz *et al*., 2003). The identification of candidate genes involved into tolerance to heavy metals is a critical target of the nowadays research. A useful approach is the overexpression of enzymes that catalyze rate-limiting steps of detoxification pathways: for example appreciable results were obtained with the overexpression of ATP sulfurylase (APS) in Indian mustard (*Brassica juncea*) plants. Mature Indian mustard APS overexpressing plants successfully removed more Cd, Cr, Pb, Cu, Zn and Mn from polluted soils than WT plants, by their higher accumulation into the shoot (Wangeline *et al*., 2004). Moreover, transgenic plants that overproduce metal-chelators, such as citrate (De la Fuente *et al*., 1997), metallothioneins (Evans *et al*., 1992; Hasegawa *et al*., 1997) and ferritin (Goto *et al*., 1999), have been successfully developed for their increased ability to accumulate high levels of heavy metals. Finally, some strategies have focused on improving metal uptake by overexpressing metal transporters proteins (Arazi *et al*., 1999; Hirschi *et al*., 2000; Samuelsen *et al*., 1998; van der Zaal *et al*., 1999). However, it is important to consider that the mechanisms, developed to increase the efficiency of phytoextraction, have also practical limitations. For example, although the addition of metal-chelating agents, such as EDTA, can greatly increase metal uptake by plants (Blaylock *et al*., 1997), the increased metal bioavailability may lead to an enhanced metal leaching to the ground-water (Lombi *et al*., 2001).

IV. HYPERACCUMULATOR PLANTS: A NEW FRONTIER OF PLANT REMEDIATION

The different phytotechnologies employ numerous plant species and several plant properties. Favourable plant properties for phytoremediation have to be fast growth, high biomass, competitiveness and tolerance to pollution. In addition, high level of contaminant uptake, translocation and accumulation in harvestable tissues are important aspects of

phytoextraction of toxic compounds. An unique category of plants important for a phytoextraction process of heavy metals is that of the so-called hyperaccumulators. These species can accumulate high amounts of various heavy metals in their tissues (Reeves and Baker, 2000). In particular, they can accumulate one or more inorganic elements to levels 100-fold higher than other non-hyperaccumulator species grown under the same conditions (Brooks, 1998). Hyperaccumulation is an active process that depends on an endogenous hypertolerance mechanisms to resist the cytotoxic levels of the accumulated metals (Salt, 2006). To date, 400 metal hyperaccumulator species are known (Eapen and D'Souza, 2005). Most of them are Ni and/or Zn hyperaccumulators, while only few species hyperaccumulate Cd. The most common are *Thlaspi* species, such as *T. caerulescens* and *T. praecox*, *A. halleri* and *Sedum alfredii* (van de Mortel *et al*., 2008). *Thlaspi* species are polymetallic hyperaccumulators known to accumulate high amounts of Zn, Cd, Ni and Pb (Mari *et al*., 2006), whereas *A. halleri* is able to tolerate Zn, Cd and Pb and hyperaccumulates Zn and Cd (van Rossum *et al*., 2004). *S. alfredii* has been identified as a Zn hyperaccumulator, and only recently it has been confirmed to also hyperaccumulate Cd (Zhou and Qiu, 2005).

IV.I HYPERACCUMULATOR PLANTS AND GENETIC ENGENEERING

Hyperaccumulator plants can be directly employed as pollutant remover or can represent a sources of genes to improve non-hyperaccumulator plants. Nevertheless, natural hyperaccumulators have generally low biomass and slow growth rate. These restrictions may be overcome by transferring the genetic potential responsible for hyperaccumulation from hyperaccumulator species to plants characterised by fast growth, high biomass production and tolerance but also to plants lacking these properties. For example, Indian mustard is a suitable target specie, because of its large biomass production, a relatively high metal accumulation and the already well established transformation technology. Differently to non-hyperaccumulator plants, which normally accumulate heavy metals in

roots, hyperaccumulators are able to transport most of the absorbed metal to the shoots (Lasat *et al*, 1998). Thus, a metal translocation from root to shoot through the xylem is a determinant for the hyperaccumulation phenotype. In fact, it has been recently demonstrated that the metal transporter HMA4, expressed at higher level in the hyperaccumulator *A. halleri* if compared to the non-tolerant *A. thaliana*, is essential for the root-to-shoot transport (Hanikenne *et al*., 2008). At molecular level, even amino- and organic acids have been proposed to play a role in heavy metal hyperaccumulation and tolerance (Sharma and Dietz, 2006). Typically, chelation of the metal ion, transport of metal or its complexation and subsequent compartmentalization in vacuoles are the processes where biotechnology can play a part in the enhancing the phytoremediation capacity of plants. For example, transferring a single gene involved in metal transport, such as *HMA4*, from *A. halleri* to *A. thaliana* has increased the shoot metal up-loading in this non-accumulator specie (Hanikenne *et al*., 2008). Regarding metal-conjugates transport, plants overexpressing specific transport proteins (such as members of the CDF group,) might acquire higher detoxification and compartmentalization of GS-Cd conjugates into the vacuoles (Krämer *et al*., 2007). Transgenic *B. juncea* plants engineered to produce more glutathione and phytochelatins accumulated significantly more Cd than wild-type plants (Bennet *et al*., 2003), while *A. thaliana* and tobacco plants overexpressing MT genes showed more Cd tolerance and accumulation (Eapen and D'Souza, 2005). Furthermore, Cd tolerance and accumulation is also enhanced by overexpressing the γ-glutamylcysteine synthetase, an enzyme with important role in controlling glutathione synthesis and therefore metal chelation (Zhu *et al*., 1999). Another study revealed that the expression of the *AtPCS1* gene increased Cd and As tolerance and accumulation in *B. juncea* (Gasic and Korban, 2007) and in tobacco plants (Pomponi *et al*., 2006).

The comparison between hyperaccumulator with non-accumulator sister species (e.g. *A. halleri* with *A. thaliana*) suggests that the hyperaccumulating features could reside in sequence mutations, gene copy number and/or in different expression level of proteins

that contribute to metal tolerance (Hanikenne *et al*., 2008; Plaza *et al*., 2007). These findings highlight that probably part of the genetic potential for metal detoxification is already present in most plant species and that small sequence changes, which influence both metal sensing and activation of appropriate responses, make the difference.

AIMS OF THE WORK

The use of plants to strip heavy metals from soil is an emerging tool (Pilon-Smits, 2005). Therefore, to understand the mechanisms by which plants respond to heavy metal exposure is a primary goal of plant biotechnologysts, whose aim is to create plants able to recover high amounts of heavy metals, which can be used for phytoremediation. The identification of more than 400 plant species that can tolerate and accumulate high concentrations of heavy metals in their above-ground tissues suggests that the genetic potential for phytoextraction exists (Eapen and D'Souza, 2005). Progress towards this goal has been made and transgenic plants with enhanced tolerance and ability to accumulate heavy metals have been produced (Domínguez-Solis *et al*., 2001; Song *et al*., 2003; Gong *et al*., 2003). Nevertheless, the knowledge regarding the molecular mechanisms of plant metal response and hyperaccumulation remains limited and the investigation is still needed to unravel the complex genetic network responsible for heavy metal accumulation and detoxification.

It is well known that the presence of high concentrations of heavy metals in soil is perceived by plant cells as a stress signal that converges to the expression of different classes of proteins. This signal transduction pathway is controlled by an intricate interaction of genes among which transcription factors play crucial roles. With the intention of understanding the main molecular mechanisms activated in plants in response to heavy metal stress, in particular to Cd, the first aim of this PhD thesis was the characterization of a bZIP transcription factor (BjCdR15, *Brassica juncea Cd-Regulated*), isolated by cDNA-AFLP technique in *Brassica juncea* after Cd-treatment (Fusco *et al.*, 2005). The scope was to investigate whether and how this gene is involved in Cd response, to understand its putative involvement in Cd tolerance and accumulation in plant.

Another important aspect, in the phytoextraction, is the effect of the rhizosphere microorganisms on the plant metal uptake. In fact, metal bioavailability and plant metal uptake are greatly influenced by rhizosphere microbes (De Souza *et al*., 1999). Since the majority of molecular mechanisms regulating these plant-microbe interactions are still

unknown, the second aim of present study was to investigate these interactions considering an ecotype of the model hyperaccumulator species *Arabidopsis halleri*, adapted to growth and harvested in a heavy metal-contaminated soil (van Rossum *et al*., 2004). Moreover, although proteomics is crucial for understanding biological systems, there are few studies on stimulus-dependent change in plant proteome. Thus, to acquire more knowledge regarding the effects of plant-microbe interactions on metal uptake and accumulation, *A. halleri* plants were grown in presence of heavy metals with and without rhizosphere microbes. Afterwards, a proteome approach was applied to the shoot *A. halleri* plants, with the purpose to identify the genes involved in heavy metals tolerance and hyperaccumulation in this species.

Chapter I:

CHARACTERISATION OF A *B. juncea* bZIP TRANSCRIPTION FACTOR RESPONSIBLE FOR Cd UPTAKE AND TRANSLOCATION TO THE SHOOT IN TRANSGENIC PLANTS

1. INTRODUCTION

1.1 - Role of transcription factors in stress response

Plants have evolved numerous mechanisms to cope with various biotic and abiotic stress conditions, which cause adverse effects on growth and productivity (Gong *et al*., 2003). Consequently, plants trigger rapid defence responses by activating several signal transduction pathways, and the crucial step in the control of stress responses appears to be the modulation of gene expression (Kasuka *et al*., 1999). Stress is perceived and transduced through numerous signalling molecules, which may directly affect regulatory elements of stress-inducible genes to initiate the gene-product synthesis. Alternatively, these molecules might alter the activity of different classes of proteins including transcription factors (TFs), enzymes, molecular chaperons, ion channels and transporters (DalCorso *et al*., 2008) (Figure 1.1).

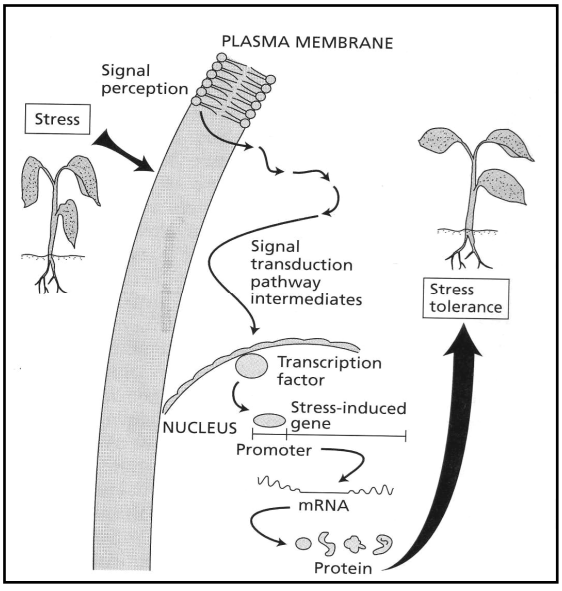


Figure 1.1 Signal transduction in response to abiotic stress. (*Plant Physiology*, Taiz and Zeiger, 1998).

Even the presence of high concentrations of heavy metals in soil is perceived by plant cells as a stress signal. Therefore, also heavy metal stress may induce a complicated

signal transduction pathway within the cell, that begins with the sensing of heavy metal (or heavy metal associated symptoms) and converges to transcription regulation of metal-responsive genes (Singh *et al*., 2002). In particular, TFs play an essential role since regulation of their expression may strongly influence plant stress responses (Uno *et al*., 2000). Thanks to the recent introduction of genomic technology, it has been possible to identify numerous genes, among which TFs, putatively involved in response to heavy metal stress: several genes induced in *B. juncea* after Cd-treatments were identified, for example, by cDNA-AFLP (Fusco *et al*., 2005) and changes in the transcriptome of *Arabidopsis* plants exposed to Cd and Pb were studied by Affymetrix DNA array (Kovalchuk *et al*., 2005).

1.2 - TFs in *Arabidopsis thaliana*

A. thaliana genome encodes more than 1500 TFs (Riechmann *et al*., 2000). Transcription factors belonging to the families of ERF (Ethylene Responsive Factor)/AP2 (APETALA2), bZIP (basic region/leucine zipper), Myb (Myeloblastosis), WRKY (Trp-Arg-Lys-Tyr domain) and several classes of zinc-finger proteins have been reported to play a role in plant stress responses regulating the expression of stress-responsive genes (Shinozaky and Yamaguchi-Shinozaky, 2000). The ability of TF proteins to influence the transcriptional regulation of effector genes is largely mediated through TFs-specific sequences. These may recognise and bind to *cis*-acting elements located in the promoter region of the corresponding gene (Miao *et al*., 1994).

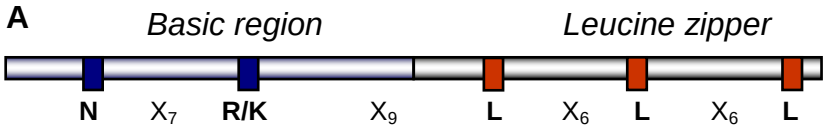
Numerous scientific works describe the involvement of TFs in response to heavy metal stress. For example, Cd affects the expression of ERF TFs belonging to the AP2/ethylene responsive-element-binding protein (EREBP) family. Indeed, it has been shown that *ERF1* and *ERF2* are induced after 2 h of Cd-treatment in roots of *A. thaliana* (Weber *et al*., 2006). Moreover, it has been also reported that DREB2A (Dehydration Responsive Element Binding 2A) is induced by Cd (Suzuki *et al*., 2001): DREB2A specifically interacts

with the DRE motif in the promoter region of the Rd29A (Desiccation Responsive gene) gene and regulates its transcription in Cd exposed plants. Rd29A is already known to be induced by cold, salt and dehydration stresses (Liu *et al*., 1998). OBF5 (Ocs-element Binding Factor) TF, a bZIP DNA binding protein, was shown to be induced by Cd: it binds to the promoter region of the glutathione S-transferase gene, an enzyme involved in ROS scavenging and xenobiotic detoxification (Suzuki *et al*., 2001). Moreover, it has been recently demonstrated that the expression of BjCdR15, a bZIP protein identified in *B. juncea*, is induced after short Cd-treatment (Fusco *et al*., 2005). WRKY53, a TF belonging to the WRKY family, was isolated as differentially expressed in Cd-treated *T. caerulescens* plants. This gene is also modulated by other environmental stresses such as salinity, drought, cold and salicylic acid and seems to participate in the stress related signal transduction pathway regulating the activity of other TFs rather than directly activating gene expression (Wei *et al*., 2008). Finally, MYB proteins, and in particular members of R2R3 MYB subgroup, also respond to heavy metal stress: in *A. thaliana* MYB4 is up-regulated after Cd- and Zn-treatment (van de Mortel *et al*., 2008), while *At*MYB43, *At*MYB48 and *At*MYB124 proteins are specifically induced by Cd in roots (Weber *et al*., 2006). In *T. caerulescens*, MYB28 is strongly expressed under Zn deficiency and high Cd concentrations and is involved in regulation of glucosinolate synthesis (van de Mortel *et al*., 2008).

1.2.1 - bZIP Transcription Factors in *Arabidopsis thaliana*

Among TF families, bZIP genes are well represented in the *Arabidopsis* genome and implicated in diverse biological processes, such as stress plant response (Jakoby *et al*., 2002). Structurally bZIP TFs have a basic region able to bind DNA and a leucine zipper motif responsible for dimerization. The basic region, about 16 amino-acid long, contains a nuclear localization signal followed by an invariant N-X_7R/K motif responsible for the contact to the DNA (Figure 1.2 A). The leucine zipper motif is characterised by a heptad

repeat of leucines, or other bulky hydrophobic amino acids (e.g. isoleucine, valine, phenylalanine or methionine), localized nine amino acids towards the C-terminus, and it folds originating an amphipathic helix (Hurst, 1995). The contact, via van der Waals interactions, between the hydrophobic sides of helices can induce a dimerization of bZIP TFs, creating a coiled-coil structure able to influence the binding to the DNA (Figure 1.2 B). The ability to generate homo- and hetero-dimers depends on the electrostatic attraction and repulsion of polar residues flanking the hydrophobic interaction surface of the helices (Hurst, 1995).



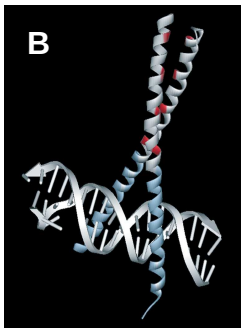


Figure 1.2 (**A**) Structure of the bZIP protein domain and (**B**) three-dimensional structure of the bZIP domain bound to DNA. (Jakoby *et al*., 2002).

Proteins with bZIP domains are present in all eukaryotes analysed to date. Some have been largely studied in animals and are important to understand the TF-DNA interactions, ternary complex formation, etc. However, *Arabidopsis* has about four times more bZIP genes (AtbZIP) than yeast, worm and human (Riechmann *et al*., 2000). The AtbZIP family can be subdivided into ten groups (A÷I and S groups) with a similar basic region and additional conserved motifs. Proteins belonging to the same groups have common features, as the size of leucine zipper, and it is possible that many of them recognize similar *cis*-elements (Jakoby *et al*., 2002). AtbZIP proteins, as well as all plant bZIP proteins, bind to DNA sequences with an ACGT core and the binding specificity is regulated by flanking nucleotides: generally, plant bZIPs preferentially bind to palindromic

sequence as A-box (TACGTA), C-box (GACGTC), G-box (CACGTG) but examples of binding to nonpalindromic sites also exist (Jakoby *et al*., 2002).

- D Group and TGA factors

AtbZIP TFs belonging to the D group participate mainly in two different processes: defence against pathogens and development (Jakoby *et al.*, 2002). For example, *Arabidopsis* TGA TFs (a plant conserved sub-family of bZIP TFs belong to group D) are transcription regulators mainly implicated in the expression of pathogenesis-related (PR) genes and in the induction of systemic acquired resistance (Zhang *et al*., 2003). It was shown that *Arabidopsis* plants lacking TGA factors (i.e. TGA2, TGA5 and TGA6) are severely compromised in PR-1 protein expression (Zhang *et al*., 2003). TGA factors are believed to regulate this systemic induction because early works indicated that these TFs bind to *as-1*-type elements present in promoters of PR genes. These regulatory elements are responsible for transcription in response to salicylic acid and xenobiotic stress (Liu and Lam, 1994; Qin *et al*., 1994). Even if a functional role for TGA factors in Cd-responsive gene expression has never been reported, it has been suggested that the *as*-1 element may confer Cd responsiveness (Kusaba *et al*., 1996). In particular, TGA factors, including TGA2 and TGA3, interact with NPR1 (Non-expressor of PR genes 1) which is necessary for SA dependent PR gene induction but does not bind to DNA by itself (Johnson *et al*., 2003). In addition, the presence of TGA4 TF able to interact with AtEBP (Ethylene Binding Protein) TF, responsible for the binding to ethylene responsive elements present in many PR gene promoters (Buttner and Singh, 1997), confirms that proteins belonging to the D group might be involved in integrating different systemic signals (salicylic acid and ethylene) in response to pathogen infection. Finally the member AtbZIP46 of the D group seems to be involved in developmental processes floral organ number in *Arabidopsis* (Jakoby *et al*., 2002).

2. MATERIALS AND METHODS

2.1 - Plant material and growth conditions

Plants of *Brassica juncea* (cv. Aurea), *Arabidopsis thaliana* (Col-0) and *Nicotiana tabacum* (cv. Petit Havana SR1) were cultured in hydroponic nutritive solution (Hoagland's solution; Hoagland and Arnon, 1938), treated with heavy metals and maintained in greenhouse, under controlled conditions as previously described (Fusco *et al*., 2005).

For *in vitro* tests, seeds of *B. juncea* (cv. Aurea), *A. thaliana* (Col-0) and *N. tabacum* (cv. Petit Havana SR1) were cultured *in vitro* on MS *medium* (Murashige and Skoog, 1962) under 16-h light/8-h dark regime at 22 ºC/18 ºC.

2.2 - Identification of the *Arabidopsis tga3-2* mutant line

Arabidopsis tga3-2 mutant line (SALK_088114) was identified in the SALK collection (http://signal.salk.edu/; Alonso *et al*., 2003). Seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). This mutant line was created by insertion of pROK2 T-DNA in *A. thaliana* (Col-0) WT plants.

Molecular analysis of the mutant line

Seeds were placed in Petri dishes on water wetted Whatman paper and incubated two days at 4℃ in the dark chamber to break dormancy. After germination, plants were grown on soil in greenhouse controlled conditions (16-h light/8-h dark regime). To identify homozygous *tga3-2* line, genomic DNA was isolated from shoot tissues of four weeks old plants. Tissues (100 mg) were ground in liquid nitrogen and solubilised in 500 µl of extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA, 1% v/v SDS, 10 mM β-mercaptoethanol) followed by isopropanol precipitation according to standard protocols (Sambrook and Russell, 2001).

T-DNA insertion site was recovered by PCR with the use of combinations of insertion-specific and gene-specific primers as described in (http://signal.salk.edu/tdnaprimers.2.html) and confirmed by means of the sequencing service. T-DNA specific primer **LBb1** for pROK2 was used in combination with the *TGA3* specific primer **1RP** (Table 2.1). Homozygosis was analyzed by PCR with the *TGA3* specific primer **1FP** designed in the *TGA3* promoter region, upstream the T-DNA insertion, in combination with *TGA3* 1RP primer (Table 2.1). The lack of the *TGA3* transcript was confirmed by RT-PCR analysis on cDNA obtained from homozygous plants using the ***TGA3* forward primer**, designed on the 5' UTR, and the ***TGA3* reverse primer** designed on the 3' UTR (Table 2.1). Actin cDNA was amplified as control using the primers **AtACT-F** and **AtACT-R** (Table 2.4).

Primer name	Primer sequence
LBb1	5'-GCGTGGACCGCTTGCTGCTGCAACT-3'
1RP	5'-GGACTCTCCCAACCAGATAACTG-3'
1FP	5'-TACCGGAATATTCCCCTCAAGCAT-3'
***TGA3* forward primer**	5'-TCGAGCTCCTCATGAATTTCTT-3'
***TGA3* reverse primer**	5'-GAAGATGATGCTCTCTAATGGA-3'

Table 2.1 Primers used for molecular analysis of *Arabidopsis tga3-2* mutant line.

2.3 - RNA isolation and cDNA synthesis

For Northern analysis, poly $(A)^+$ RNA was prepared from Cd-treated and untreated whole *B. juncea* plants by chromatography on oligo dT-cellulose (Bartels and Thompson, 1983). To determine gene expression by Real-time PCR, total RNA was extracted from whole plants and from shoots and roots, collected separately after the specific treatments, using the TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. In particular, total RNA was isolated from whole tobacco and *Arabidopsis*

plants, and from shoots and roots of *Arabidopsis* and *B. juncea* harvested separately. First-strand cDNA synthesis was performed using the SuperScript™ III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according the manufacturer's instructions.

2.4 - Rapid amplification of cDNA ends (RACE)

Total RNA (1 µg), isolated from leaves of 3-week-old *B. juncea* maintained in hydroponic culture and treated for 6 h with 10 µM $Cd(NO_3)_2$, was used for the synthesis of 5'-RACE and 3'-RACE cDNA (SMART^{TM} RACE cDNA Amplification kit, Clontech, Palo Alto, CA). Primers **RaceF** and **RaceR** (Table 2.2), designed on a 288 bp transcript derived fragment (accession n. DT317671) identified and isolated by a cDNA-AFLP approach in *B. juncea* (Fusco *et al*., 2005), were used for the 5'- and 3'-ends respectively. All steps were performed according to the manufacturer's protocol.

Primer name	Primer sequence
RaceF	5'-GAGAATCAATCACTCTACATC-3'
RaceR	5'-GCTCTGAGTTCTCTTTGGG-3'

Table 2.2 Primers used for RACE protocol.

2.5 - Cloning, generation of BjCdR15 overexpressing *Arabidopsis* and tobacco lines and complementation analyses of the *tga3-2* mutant

BjCdR15 cDNA full-length was amplified by PCR using the **BjCdR15 forward** and **BjCdR15 reverse** primers (Table 2.3). The amplified fragment was ligated by T4 ligase (Promega, Madison, USA) into the pBI121 binary vector (Clontech, Palo Alto, CA), under the control of the 35SCaMV promoter. All steps were performed according to the manufacturer's protocol. The construct was introduced by electroporation (2.5 KV) into competent *Agrobacterium tumefaciens* EHA105 strain (Hellens *et al*., 2000). *A. thaliana*

(Col-0) WT plants were transformed by *floral dipping* (Clough and Bent, 1998), while *N*. *tabacum* (cv. Petit Havana SR1) WT plants, grown *in vitro*, were transformed as described by Horsch *et al*., 1985. *Arabidopsis* and tobacco plants transformed with pBI121-empty vector were used as control.

To verify the complementation of BjCdR15 in *Arabidopsis tga3-2* mutant, *BjCdR15* cDNA full-length was inserted under the control of the 35SCaMV promoter, into the pCambia 1302 binary vector. The pCambia 35S*::BjCdR15* construct was introduced into *A. tumefaciens* EHA105 strain and used for transformation, by *floral dipping*, of *Arabidopsis tga3-2* mutant obtaining 35S::*BjCdR15-tga3-2* plants. Seeds of transgenic T_0 plants were collected separately and selfed, and T_1 lines were selected on the basis of their antibiotic resistance. The presence and the expression of the transgene were confirmed by PCR and RT-PCR. T_3 homozygous progenies obtained by self pollination and selection of transgenic tobacco and *Arabidopsis* lines were used for the following analyses.

Primer name	Primer sequence
BjCdR15 forward	5'-AGCTTCGTCCTTTCGATCTTC-3
BjCdR15 reverse	5'-CCTAGCTAACATCATTCGCG-3'

Table 2.3 Primers used for amplify BjCdR15 cDNA full-length.

2.6 - Quantification of transcription level by Real-time PCR

Real-time PCRs were performed in triplicate using the ABI Prism sequence detection system (Applied Biosystems, Foster City, CA) with the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Karlsruhe, Germany). Data were organized according to the comparative method described in the User Bulletin 2 (Applera).

The analysis was conducted separately on leaves and roots of 3-week-old *B. juncea* plants grown in hydroponic culture and treated with 10 μM $Cd(NO_3)_2$ or treated with either

10 μM $Pb(NO_3)_2$ or 10 μM $Ni(NO_3)_2$ (Fusco *et al*., 2005). Forward and reverse primers designed on *BjCdR15* cDNA sequence were **BjCdR15-F** and **BjCdR15-R**. In *B. juncea*, *BjCdR15* transcripts were quantified by normalization with **BjACT-F** and **BjACT-R** primers designed on β-actin cDNA sequence (Table 2.4).

To quantify the level of *TGA3* transcripts in leaves and roots of 3-week-old *Arabidopsis* plants grown in hydroponic culture and treated for 0, 2, 6 and 9 h with 10 μM $Cd(NO_3)_2$, forward and reverse primers, **TGA3-F** and **TGA3-R**, were designed on *TGA3* cDNA sequence. For *Arabidopsis* the quantifications of transcripts were normalized to a cDNA fragment of the β-actin cDNA sequence with the **AtACT-F** and **AtACT-R**, respectively forward and reverse primers (Table 2.4).

To examine the expression of *AtNramp3*, *AtHMA4*, *AtMRP3*, *AtPDR8* and *AtATM3* metal transporters in *Arabidopsis* roots, 3-week-old plants were treated *in vitro*, for 0, 24, and 120 h, on sterile Whatman 3MM paper saturated with liquid MS *medium* containing 20 μM $CdSO_4$. Before treatments, plants, grown on MS agar *medium*, were transferred for 2 days on sterile Whatman 3MM paper saturated with liquid MS *medium*. Real-time PCRs were performed using the following primers: **AtNramp3-F** and **AtNramp3-R**; **AtHMA4-F** and **AtHMA4-R**; **AtMRP3-F** and **AtMRP3-R**; **AtPDR8-F** and **AtPDR8-R**; **AtATM3-F** and **AtATM3-R** (Table 2.4). β-actin was amplified with the primers above reported.

The transcription levels of *BjCdR15* cDNA in overexpressing *Arabidopsis* and tobacco plants were measured with **BjCdR15-F** and **BjCdR15-R** primers and the normalization was performed using the **AtACT-F** and **AtACT-R** primers for *Arabidopsis* plants and **NtACT-F** and **NtACT-R** primers (designed on a fragment of tobacco actin 1 gene) for tobacco plants (Table 2.4).

Primer name	Primer sequence
BjCdR15-F	5'-TTTAGGAGAGTACTTCCACAG-3'
BjCdR15-R	5'-TTGACTCTTCTTCGTTGTGCTT-3'
BjACT-F	5'-TGTTCTGGACTCTGGTGATG-3'
BjACT-R	5'-AGGATCTTCATGAGGTAATCAG-3'
TGA3-F	5'-GCTCTCTCTCAAGGCTTAGAT-3'
TGA3-R	5'-TGCTTGAAGATTCTCCATGGC-3'
AtACT-F	5'-GAACTACGAGCTACCTGATG-3'
AtACT-R	5'-CTTCCATTCCGATGAGCGAT-3'
AtNramp3-F	5'-GTAAGCGTAGTAGCTATGTCT-3'
AtNramp3-R	5'-CAAAACCAGAGCTGCTTTATAA-3'
AtHMA4-F	5'-AAATGTTCCAGCAAAGGCAGT-3'
AtHMA4-R	5'-GGAATTGCAATACATAAAGTACT-3'
AtMRP3-F	5'-AGCCACAACACTAGCTGGAT-3'
AtMRP3-R	5'-GGGTTCAACAGTAGTAGAAGC-3'
AtPDR8-F	5'-TCTCTCAAACAACAAAGTCTCT-3'
AtPDR8-R	5'-ATGACACTACAATACATATTTAGA-3'
AtATM3-F	5'-TCTCATTGATTTTCTATGGAGC-3'
AtATM3-R	5'-CTCGTGTTAAAATTATATCATTCA-3'
NtACT-F	5'-ATCCCAGTTTGCTGAGAATAC-3'
NtACT-R	5'-GGCCCGCCATACTGGTGTGAT-3'

Table 2.4 Primers used for quantification of transcription level by Real-time PCR.

2.7 - Intracellular protein localization

The *BjCdR15* complete coding sequence was fused 5' to the coding sequence for the red fluorescent protein from the reef coral *Discosoma* (dsRED) in the pGJ1425 vector (Jach *et al*., 2001). The BjCdR15::dsRED construct and the pGJ1425-empty vector were used to transfect tobacco protoplasts. Mesophyll protoplasts were isolated from *in vitro* grown tobacco plants (cv Petit havana SR1) as described by Negrutiu *et al*., 1987. Freshly isolated protoplasts (1,5 x 10^6) were transfected with 10 μg of plasmid DNA by polyethylene glycol-mediated DNA uptake (Walden *et al*., 1994). Five hours prior the

analysis, Hoechst 33258 (Sigma-Aldrich, St. Louis, MO) was added (5 μg/ml) to the protoplast culture in order to stain the nuclear DNA.

2.8 - *In situ* hybridization

In situ hybridization experiments were carried out as described by Varotto *et al*., 2003. Roots and leaves of 3-week-old *B. juncea* plants, treated with $Cd(NO_3)_2$ for 6 h in hydroponic solution, were fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 16 h at 4 °C and embedded in Paraplast Plus (Sigma-Aldrich, St. Luis, MO). Sections (7-10 μm) were cut using a microtome RM 2135 (Leica, Nussloch, Germany) and collected on xylane-coated slides. Slides were deparaffinized, treated with 5 μg/ml proteinase K and hybridized with sense and antisense riboprobes in 50% formamide at 48 °C overnight. A 470 bp fragment of *BjCdR15* cDNA containing 110 bp of the 5' UTR and part of the translated region was cloned into the pBluescript vector (Stratagene, La Jolla, CA) restricted with *Xho*I and *Bam*HI for sense and antisense probes respectively. To obtain DIG-UTP (Roche Applied Science, Mannheim, Germany) labelled sense and antisense RNA probes i*n vitro* transcription was performed using T7 and T3 polymerases. DIG detection and signal visualization were done using NBT and BCIP stain following the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Images were acquired using the DC 300F camera (Leica).

2.9 - Measurement of Cd content

Arabidopsis and tobacco plants overexpressing BjCdR15 (35S::*BjCdR15* plants), *Arabidopsis tga3-2* mutant line, *Arabidopsis* 35S::*BjCdR15-tga3-2* plants and control plants (transformed with pBI121-empty vector) were grown in hydroponic culture and treated with 10 μM $Cd(NO_3)_2$ for 21 days in greenhouse controlled conditions. Shoots and roots were then harvested separately and rinsed with distilled water. Samples were oven-

dried at 60 °C for 36 h and DW was determined. Drie d samples were homogenized before analysis. Cd analysis was performed after microwave-assisted acid digestion (EPA 3052, 1996) by means of ICP-MS analysis (EPA 200.8).

2.10 - Assays of Cd sensitivity

Seeds of transgenic *Arabidopsis* and tobacco lines were sowed *in vitro* on solid MS *medium*. After three weeks, *Arabidopsis* and tobacco plantlets were transferred to the same *medium* supplemented with 200 or 400 μM $Cd(NO_3)_2$ respectively, to observe shoot development and leaf and root morphology. After 23 days for *Arabidopsis* and 31 days for tobacco plants, for each line, shoot fresh weight and chlorophyll content were measured.

2.11 - Determination of chlorophyll content

Leaf discs of plants grown in absence and in presence of Cd were weighed, frozen and ground to powder in liquid nitrogen. Pigments were extracted with 80% acetone saturated with Na_2CO_3 and absorbance values at 646.6 nm and 663.6 nm were measured. Chlorophyll concentration was determined according to classical equations (Porra, 2002).

2.12 - Western blot analysis

Total proteins were extracted from leaves of 3-week-old *Arabidopsis* plants treated with 20 μM $CdSO_4$ at different times (0, 24, 72 and 120 h) on sterile Whatman 3MM paper saturated liquid MS *medium*. Before treatments, plants, grown on MS agar *medium*, were transferred for 2 days on sterile Whatman 3MM paper saturated with liquid MS *medium*. Leaf tissues were grounded in liquid nitrogen and the powder was resuspended in extraction buffer (2% SDS w/v, 62.5 mM Tris-HCl pH 6.8, 10% glycerol v/v, 2.86 M β-mercaptoethanol). After centrifugation at 10000 g for 20', the supernatant was recovered and part of this was boiled for 10 min. Proteins (8 μg) were separated by 10% T SDS-PAGE electrophoresis and transferred to PVDF membranes (Hibond™-P,

Amersham Biosciences, Uppsala, Sweden) as described by Sambrook and Russell, 2001. After blocking with 5% w/v dry milk, membranes were probed with anti-AtPCS1 antibodies for over night at 4°. Detection was performed by me ans of chemiluminescence (ECL Plus Detection Kit, Amersham Biosciences, Uppsala, Sweden) and blot signals were visualized on X-ray film (Kodak). Membranes were subjected to a stripping in buffer (100 mM β-mercaptoethanol, 2% w/v SDS, 62.5 mM Tris-HCl pH 6.7) for 30 min at 60°. Re-probing with anti-AtActin antibodies at RT for 3 hours after stripping was needed to normalise the signal of anti-AtPCS1. The intensity of the chemiluminescence response was measured by scanning films and processing the image with the QuantityOne software Version 4.4.1 (BioRad).

2.13 - Hormone and stress treatments

Three-week-old *Arabidopsis* and *B. juncea* plants, grown on MS agar *medium*, were transferred for 2 days on sterile Whatman 3MM paper saturated with liquid MS *medium*. Plants were then subjected to several treatments as summarized in table 2.5 (untreated plants were used as a control). After treatments, shoots and roots were collected separately. Total RNA extraction, cDNAs synthesis and Real-time PCRs were performed to quantify *TGA3* and *BjCdR15* transcript levels in shoots .

Treatment type		Time of exposure
Abscissic acid (ABA)	0,1 mM	5 h
Salicylic acid (SA)	1,0 mM	5 h
Methyl-jasmonic acid (MeJa)	50 µM	5 h
High salinity (NaCl)	250 mM	5 h
High temperature	42 °C	5 h
Cold	4 °C	5 h
Drought	on dry Whatman 3 MM paper	5 h

Table 2.5 Experimental design for hormone and stress treatments.

2.14 - Microscopic analyses

Roots of *Arabidopsis* and tobacco plants, grown *in vitro*, were observed with an inverted Olympus IX70 microscope (St. Louis, MO) and images were acquired with a JVC KI-58 CCD camera. Transfected tobacco protoplasts were observed with a Leica TCS SP2 laser confocal microscope (Leica Microsystems, Heidelberg Germany). Images were collected frame by frame with the AOTF (acusto-optical tunable filter) using argon/krypton and hellion/neon laser.

2.15 - Statistical analysis

Data for leaf fresh weight, chlorophyll and Cd content in shoots and roots of *Arabidopsis* and tobacco transgenic plants were obtained in three replications. Data were analyzed by analysis of variance with a threshold P-value of 0.05, 0.01 or 0.001. Means were compared by the Student-Newman-Keuls method using the COSTAT software.

3. RESULTS

3.1 - Cloning and sequence analyses of *BjCdR15* from *B. juncea*

By cDNA-AFLP technique a transcription derived fragment of 288 bp (accession no. DT317671) was isolated from *B. juncea* after treatment for 6 h with 10 µM $Cd(NO_3)_2$ (Fusco *et al*., 2005). This fragment showed sequence similarity with the *TGA3* bZIP transcription factor of *A. thaliana* (At1g22070). By PCR-based methods, including 5'- and 3'-RACE, a 1511 bp cDNA derived fragment of *BjCdR15* (accession no. EU110098) was cloned. Sequence analysis revealed a predicted full-length cDNA with a presumed open reading frame (ORF) of 1161 bp (Figure 3.1), encoding a putative protein of 386 amino acids containing a bZIP signature (43.7 kDa; Figure 3.2). *BjCdR15* sequence alignments, by BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/), revealed high sequence similarity with the *Arabidopsis* TGA3 bZIP transcription factor (nucleotides identity of 89% and amino acids identity of 86%). High sequence similarity was also found with AtbZIP50 (At1g77920) of *A. thaliana* (80% and 81% for nucleotidic and amino acid sequences identity respectively). In addition, the phylogenetic tree, showing the relationship among several plant bZIP transcription factors, points BjCdR15 as a putative orthologous of TGA3 (Figure 3.3).

GACCCAAAAAAAAGCTTCGTCCTTTCGATCTTCTCTCCCAAAAACGGAATATACCCATTAACAAGAATGCGTTTCAATTGG
TGATTCACTCTTCATATCTCTTCTTCGTGATGAATTGTCA**ATG**GAGATGATGAGCTCTTCCTCTTCTTCTACTACACAAG
TTGTATCGTTCAGAGAGATGGGGATGTATGAACCGTTCCAACACTTGTCTGGTTGGGAAAATGCTTTCAACACTATAGGTA
GTAGTAATCAGAACAACAACAACAACAACAATCCGAGTTCATCCACAGTTGTTGAGGTGGATGCTGCTAGAGCAGAAGCAG
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ATCAAATCAATGATAAGATGAAACGGCGATTGGCTCAGAACCGAGAGGCTGCTCGCAAAAGCCGTTTGAGAAAGAAGGCAC
ATGTTCAGCAGTTAGAAGAAAGCAGGTTGAAGTTATCACAGCTCGAGCAAGAGTTTGCAAGAGCTAGGCAGCAGGGATTAT
GCGTACACAATTCATCAGATAATAGCTACCTTGGACCGGCTGGGACCATGAACACAGGAATCGCTGCATTTGAGATGGAAT
ACACACACTGGCTAGAAGAACAAAACAAGAGAGTGAGTGAGATTAGAACAGCTCTCCAAGCGCATATCAGCGACATTGAGC
TCAAAATGCTTGTTGACACTTGCTTAAACCACTACGCAAACCTCTTCCGCATGAAAGCTGATGCTGCAAAAGCTGATGTGT

TCTTCTTGATATCTGGTATGTGGCGAACTTCCACTGAACGTTTCTTCCAGTGGATTGGAGGTTTCCGCCCTTCCGAGCTCT
TAAATGTTGTAATGCCATACATTGAGCCATTGACAGATCAGCAACTGTTGGAGGTGAGAAACCTCCAACAGTCGTCTCAGC
AAGCAGAGGAAGCTCTTTCTCAAGGCTTGGATAAACTTCAGCAGGGTTTGGTCGAAAACATTGCAGTTGACATAAGAGTTG
TTAAGTCTGTGAGTCACGGGGCTCAAATGGCTTCAGCTATGGAGAATCTTCAAGCATTAGAGGGTTTTGTGAACCAGGCAG
ATCATCTGAGAAAGCAGACTCTGCAGCAAATGGGCAAGATCTTGACGACAAGGCAGGCTGCTCGAGGGTTGCTCGCTTTAG
GAGAGTACTTCCACAGACTTCGTGCTCTGAGTTCTCTTTGGGCAGCTCGTCCACGAGAACGCTCT**TAG**CAAGCTTTAGGA
GTCAAAACAAAGAGAAGAGAAGCACAACGAAGAAGAGTCAACCGCGAATGATGTTAGCTAGGGGCTTGTTTATCTGTAAGT
GAAAGTGTATGGAGATGTAGAGTGATTGATTCTCCATTAGAGCATATCGTCATCTTCCTCATTGATGTTTTTGGTTTTGCT
TAAATGAATTCGTCAGAGAAATGAATAAAGATGATGTAAATTTGGGTGGAAAACA

Figure 3.1 The *BjCdR15* nucleotide sequence (5'»3'). ATG initiation and TAG termination codons are shown in red bold. Polyadenilation signal is underlined.

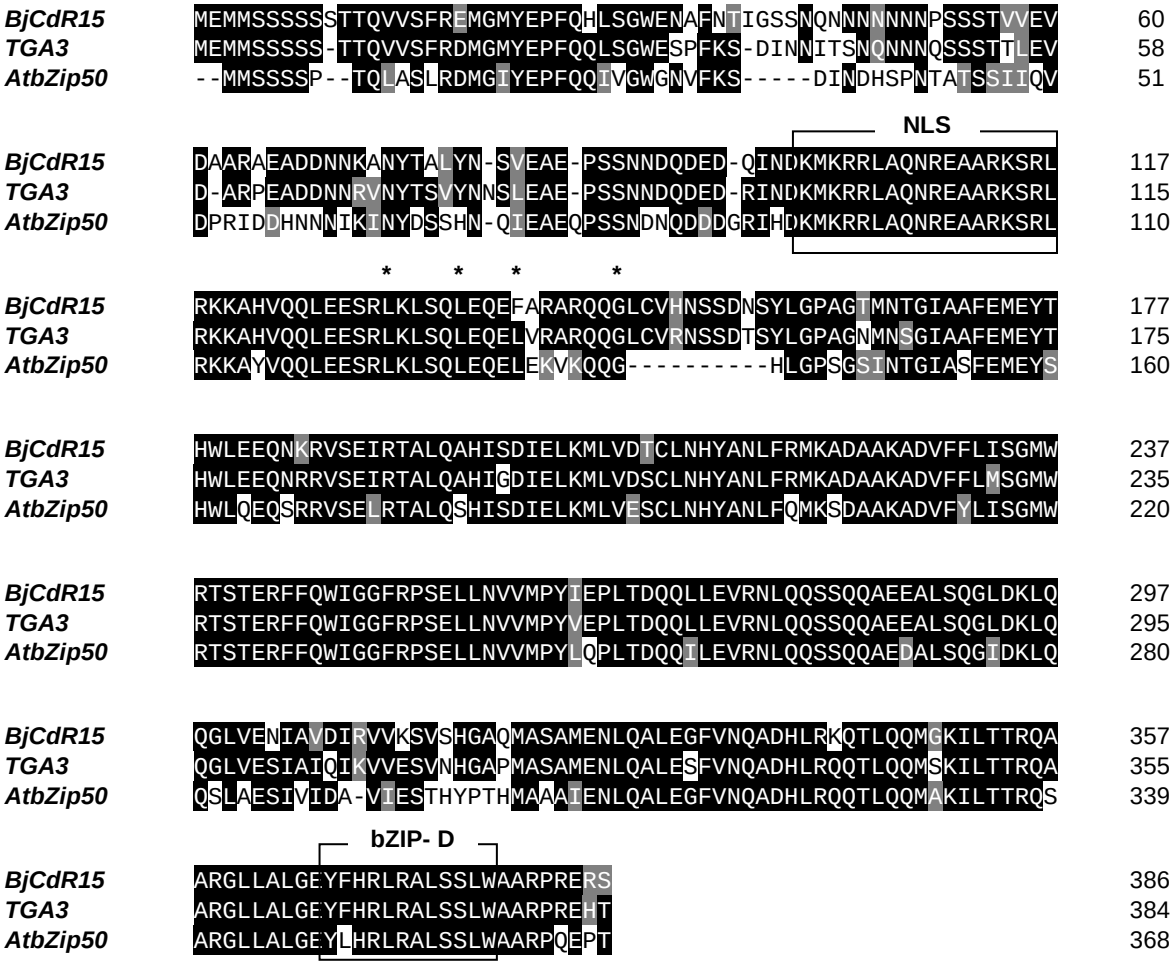


Figure 3.2 Amino acid sequence alignment of BjCdR15, TGA3 and AtbZIP50. The NLS box indicates the Nuclear Localization Signal and the bZIP-D box represents the signature domain of the class D bZIP transcription factors of *A. thaliana* (Jacoby *et al*., 2002). *amino acids of the leucin zipper domain.

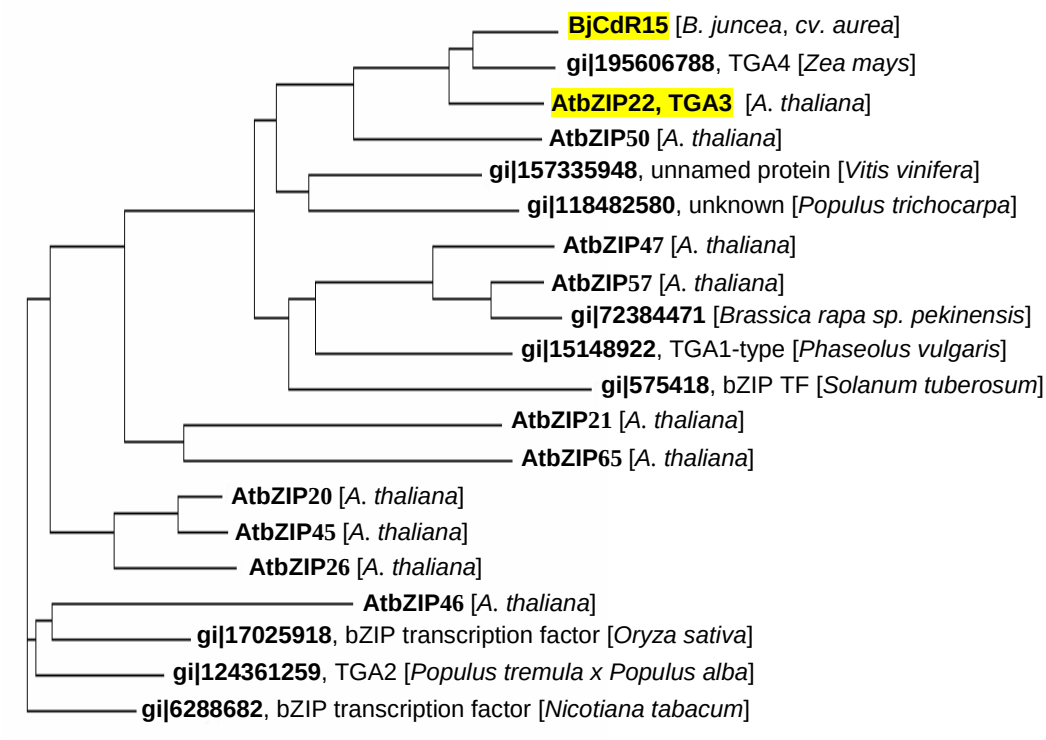


Figure 3.3 Phylogenetic tree (phylogram) showing the relationship among BjCdR15, class D bZIP transcription factors of *A. thaliana* (Jacoby *et al*., 2002) and other putative bZIP transcription factors of several species. Branch lengths are proportional to the amount of inferred evolutionary change. Protein sequences were aligned using the program CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and the phylogenetic tree was drawn with the PHILIP software and a clustering Neighbour-Joining (NJ) algorithm (Saitou and Nei, 1987).

3.2 - Identification of the *Arabidopsis tga3* mutant

An *Arabidopsis* mutant line (*tga3-2*), harbouring a T-DNA in *TGA3* sequence, was identified. The T-DNA insertion was mapped on the 5' UTR, 166 bp upstream of the start codon (Figure 3.4 A) (another *Arabidopsis tga3* mutant, *tga3-1*, was reported by Kesarwani *et al.*, 2007). Segregation analysis on selective *medium* and Southern blot experiments revealed the presence of a single insertion locus (not shown). No *TGA3* transcripts were detected by RT-PCR in homozygous *tga3-2* plants (Figure 3.4 B)

indicating a complete loss of gene function. Under standard growth conditions, mutant plants showed no morphological or developmental abnormalities if compared to WT plants, and T_3 generation was used for further analyses.

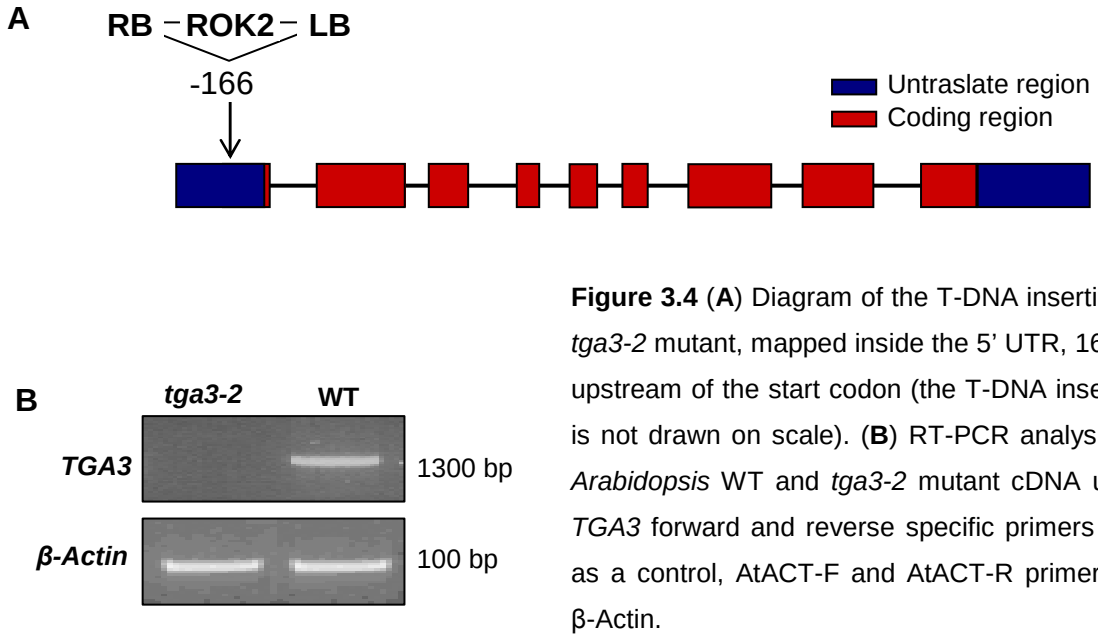


Figure 3.4 (**A**) Diagram of the T-DNA insertion in *tga3-2* mutant, mapped inside the 5' UTR, 166 bp upstream of the start codon (the T-DNA insertion is not drawn on scale). (**B**) RT-PCR analysis on *Arabidopsis* WT and *tga3-2* mutant cDNA using *TGA3* forward and reverse specific primers and, as a control, AtACT-F and AtACT-R primers for β-Actin.

3.3 - *BjCdR15* expression pattern

To confirm data obtained by the cDNA-AFLP technique, Northern-blot analysis was performed on RNA purified from whole *B. juncea* plants, using *BjCdR15* cDNA as probe. It was indeed confirmed that after 6 h of treatment with 10 µM $Cd(NO_3)_2$, *BjCdR15* was up-regulated and the level of transcription decreased with more prolonged Cd exposure times (Figure 3.5).

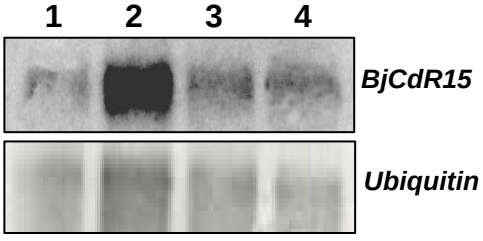


Figure 3.5 Expression pattern of *BjCdR15* by RNA blot analysis of poly $(A)^+$ RNA (2 µg per lane). Poly $(A)^+$ RNA was isolated from whole plants after Cd exposure for 0 h (**1**), 6 h (**2**), 24 h (**3**) and 6 weeks (**4**).

The expression pattern of *BjCdR15* was also monitored, by Real-time PCR, in shoots and roots of *B. juncea* at different times during the first 9 h of $Cd(NO_3)_2$ treatment. Transcript level increased in both leaves and roots after Cd addition and the highest *BjCdR15* mRNA amount was detected after 2 and 6 h in leaves and roots respectively. Up-regulation of *BjCdR15* was also observed in *B. juncea* roots after 6 h of Ni and Pb treatment (Figure 3.6 A). In addition, the *BjCdR15* expression pattern was compared with the *TGA3* mRNA amounts in roots and shoots of *Arabidopsis* plants grown in hydroponic culture and exposed to $Cd(NO_3)_2$ for the same times. Also the transcriptional level of *TGA3* increased in leaves and roots after 2 h of Cd-treatment and then decreased to the level of untreated plants after Cd prolonged exposure times (Figure 3.6 B).

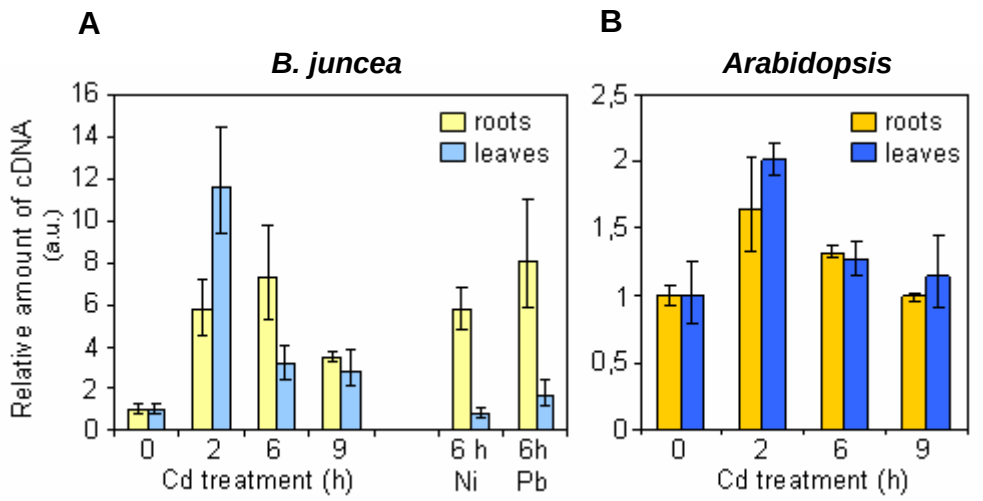


Figure 3.6 Analysis of the expression pattern of *BjCdR15* (**A**) and *TGA3* (**B**) in *B. juncea* and *A. thaliana* respectively at different times of Cd treatment by Real-time PCR. *BjCdR15* expression was also tested after treating plants for 6 h with Ni and Pb. Bars: SE.

To gain insights about the subcellular localization of the BjCdR15 protein, the complete *BjCdR15* ORF sequence was cloned at the 5' of the dsRED-sequence, under the control of the 35SCaMV promoter and transfected into tobacco protoplasts. Protoplasts transfected with the control dsRED protein showed red fluorescence throughout the entire

cytoplasm (Figure 3.7 A). On the contrary, protoplasts transfected with the construct encoding the BjCdR15::dsRED fusion protein exhibited red fluorescence in the nucleus, indicating that BjCdR15 product is a nuclear-localized protein (Figure 3.7 B, panel 1). This signal overlapped the fluorescence generated by a nuclear DNA dye, confirming that BjCdR15 product is a nuclear-localized protein (Figure 3.7 B, panel 2).

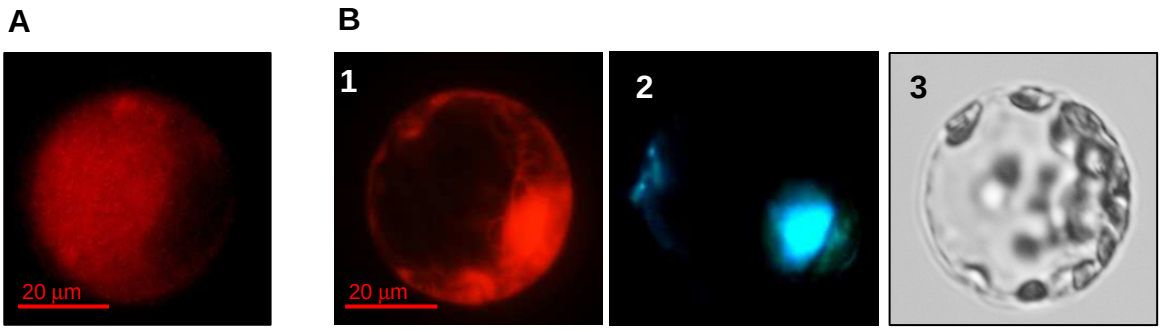


Figure 3.7 Fluorescence field of a mesophyll tobacco protoplast transfected with only dsRED protein (**A**) and BjCdR15-fusion protein showing the nuclear localization of BjCdR15 protein (**B**, panel 1). Fluorescence generated by the nuclear DNA dye Hoechst 33258 (Sigma-Aldrich) (**B**, panel 2). Bright field view (**B**, panel 3).

To determine the cellular and the tissutal expression of *BjCdR15*, *in situ* hybridization was performed in leaf and root tissues of *B. juncea* plants Cd-treated for 6 h using a fragment of *BjCdR15* antisense probe (470 bp). The analysis revealed a predominant accumulation of transcripts in epidermis and vascular system of both leaves and roots (Figure 3.8). In particular cross section of leaves showed *BjCdR15* mRNA in epidermis and internal and external phloem veins (Figure 3.8, panels 1-2), whereas in transverse section of mature roots, the hybridization signal was detected in the epidermis and in the vascular cylinder (Figure 3.8, panel 3). On the contrary, untreated plants showed a faint *BjCdR15* expression in leaf epidermis and vascular bundles whereas it was totally undetectable in roots (data not shown).

These results together suggest a putative role of *BjCdR15* gene in the Cd response. Moreover, its high expression in roots of plants treated with Ni and Pb might indicate a general function of BjCdR15 in response to heavy metals.

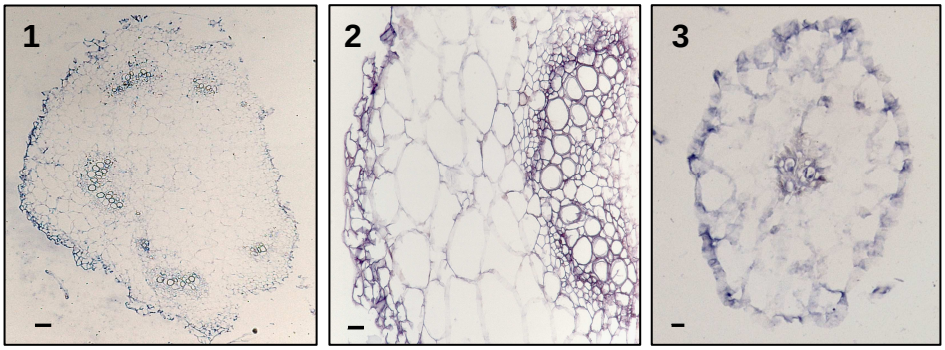


Figure 3.8 *In situ* hybridization of *BjCdR15* mRNA on leaf and root tissues of Cd-treated *B. juncea* plants. Cross sections of leaves (1 and 2) and roots (3). Blue staining represents the hybridization signal. Bars: 100 µm (1 and 3) and 50 µm (2).

3.4 - Cd content analysis

To investigate whether BjCdR15 can replace the function of TGA3 protein, *Arabidopsis tga3-2* homozygous mutant was transformed with the 35S::*BjCdR15* construct, obtaining 35S::*BjCdR15-tga3-2* plants. Moreover, to test the effect of BjCdR15 overexpression on Cd accumulation and tolerance, *Arabidopsis* and tobacco WT plants were chosen as heterologous systems and were transformed with 35S::*BjCdR15* constructs.

BjCdR15 transcription levels were determined by Real-time PCR in five 35S::*BjCdR15-tga3-2* lines and in four and seven transgenic lines of 35S::*BjCdR15 Arabidopsis* and tobacco respectively, to select the plant with the highest *BjCdR15* transcription level (data not shown). T_3 homozygous plants of the selected lines were tested for their ability to accumulate Cd and for further analyses. Cd content was determined in both roots and shoots of all different transgenic lines grown in hydroponic culture and treated for 3 weeks

with 10 μM $Cd(NO_3)_2$. 35S::*BjCdR15 Arabidopsis* and tobacco plants, generally, had significantly higher Cd ($p< 0.05$) in shoots than corresponding control plants (*Arabidopsis* and tobacco WT plants transformed with pBI121-empty vector) (Figure 3.9 A and B). In particular, in *Arabidopsis* plants the average Cd content in shoots was 13.2% higher in 35S::*BjCdR15* plants than in control plants (659 μg/g of shoot DW versus 582 μg/g). On the contrary, shoots of *Arabidopsis tga3-2* mutant plants had a significantly lower Cd content than control plants and 35S::*BjCdR15* plants ($p< 0.001$). Notably, the ectopic expression of BjCdR15 in *tga3-2* mutant (35S::*BjCdR15-tga3-2* plants) caused an increase in Cd accumulation, in shoots, significantly higher than all other assays ($p< 0.001$) (Figure 3.9 A). Notably, the accumulation of Cd in *Arabidopsis* roots was similar in all tested conditions except for the *tga3-2* mutant in which the Cd content was consistently higher ($p< 0.001$) (Figure 3.9 A). These observations suggest that *BjCdR15* gene might be an orthologous of the *Arabidopsis TGA3* since its product functionally replaces the TGA3 activity in *tga3-2* mutant plants in Cd uptake and translocation to the shoot.

Similar results were obtained with tobacco plants: shoots of 35S::*BjCdR15* plants had significantly higher Cd content ($p< 0.05$) than shoots of control plants; and there was no significant difference in Cd concentration in roots (Figure 3.9 B).

These results indicate that 35S::*BjCdR15 Arabidopsis* and tobacco plants transport more Cd to the shoot than control plants and suggest that this bZIP protein may be functionally involved in regulating the long-distance root-to-shoot transport. Remarkably, *Arabidopsis tga3-2* mutant plants accumulated about 1000 μg/g more Cd in roots if compared with all other tests. Moreover, in this mutant, the Cd transported to the shoot was only about 200 μg/g lower than the control line. This observation suggests that TGA3 is not only involved in Cd transport to the shoot but also keeps the Cd uptake under control in the roots.

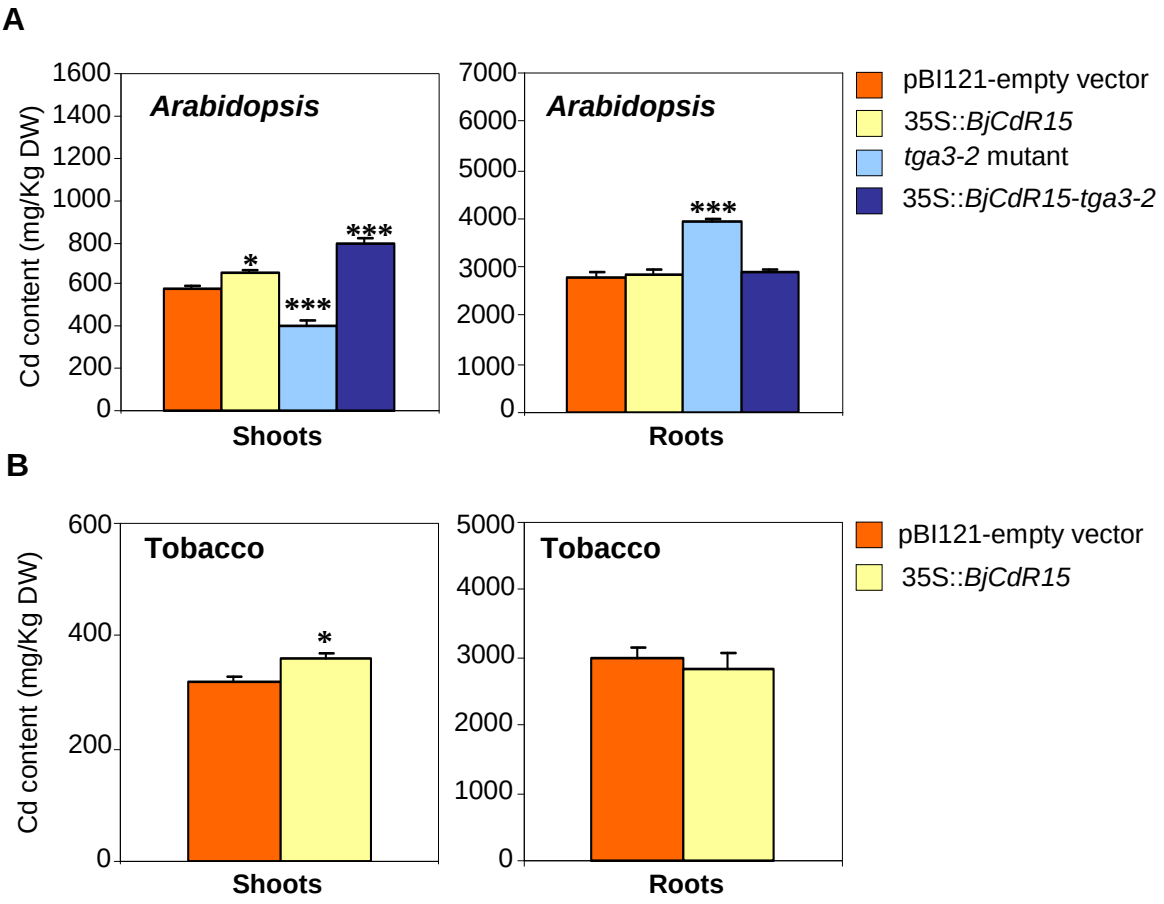


Figure 3.9 Cd content in *Arabidopsis* (**A**) and tobacco (**B**) plants. For *Arabidopsis*, values shown represent the average of 50 plants per three replicates; for tobacco, values are the average of 25 plants per three replicates). Bars: SE. Significant differences from control plants are indicated with * (P< 0.05); ** (P< 0.01) or ***(P< 0.001).

3.5 - *In vitro* phenotypic analysis and Cd tolerance

Seeds of all transformant lines were germinated and grown *in vitro* on MS *medium*. To evaluate their Cd tolerance, 3 weeks old plants were transferred on the same *medium* containing 200 and 400 µM $Cd(NO_3)_2$, for *Arabidopsis* and tobacco respectively. After treatment with Cd (lasted 23 days for *Arabidopsis* and 31 days for tobacco plants), *Arabidopsis* and tobacco treated and untreated plants were analysed.

In absence of Cd, the growth of *Arabidopsis* and tobacco BjCdR15 overexpressing plants (35S::*BjCdR15* plants) was similar to corresponding control plants (data not shown). In the

presence of Cd, phenotype differences were observed between BjCdR15 overexpressing and control plants both in the *Arabidopsis* and in the tobacco plants: 35S::*BjCdR15* *Arabidopsis* and tobacco lines grew better than control plants (Figure 3.10 A), their leaves were greener and broader and showed less chlorosis (Figure 3.10 B). On the contrary, after 3 weeks of Cd-treatment control plants were severely stressed and highly chlorotic (Figure 3.10 A and B). No differences were instead observed between *Arabidopsis* control plants and *tga3-2* mutant line after Cd-treatment, while *Arabidopsis* 35S*::BjCdR15-tga3-2* plants had a phenotype similar to *Arabidopsis 35S::BjCdR15* plants (data not shown). *In vivo* control plants also flowered early, probably induced by the stress conditions.

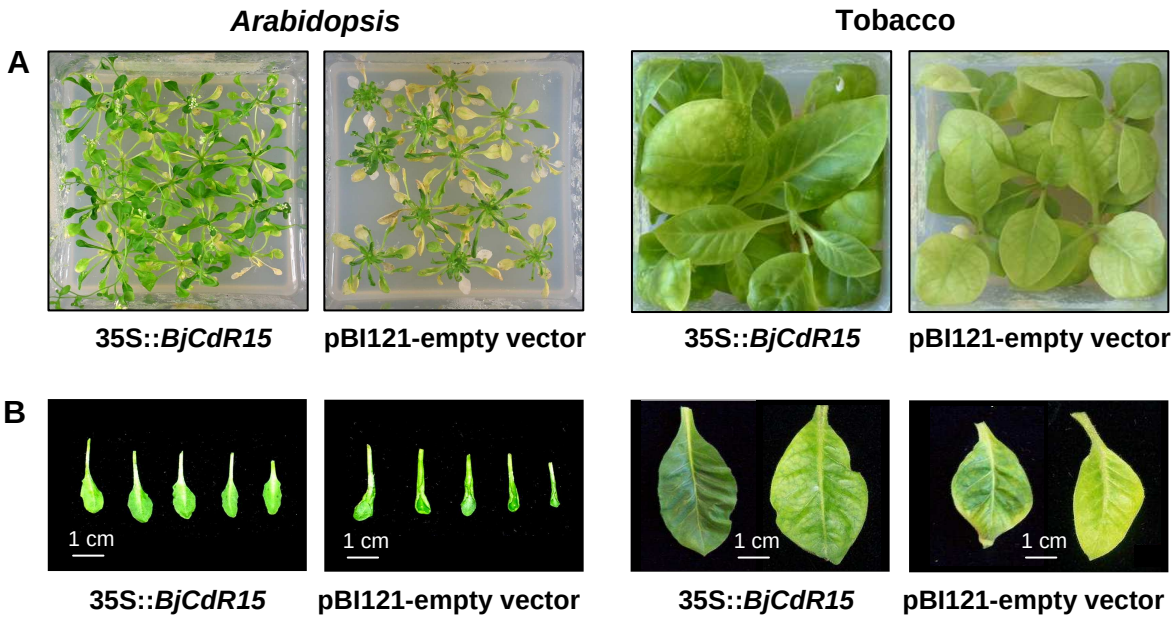


Figure 3.10 Phenotypes of plants grown *in vitro* on Cd-containing *medium*. (**A**) Comparison of *Arabidopsis* and tobacco BjCdR15 overexpressing plants with control plants. (**B**) Leaves of *Arabidopsis* or tobacco BjCdR15 overexpressing plants compared with leaves of control plants.

Considering the effects on roots, Cd-treated *Arabidopsis* control plants showed an abundance of root hairs accompanied by the presence of numerous lateral root primordia

that did not develop further (Figure 3.11 A). Similar phenotype was observed in roots of *tga3-2* mutant plants. In Cd-treated tobacco control plants, the induction of numerous root hairs was observed particularly in the region close to the root tip (Figure 3.11 B). *Arabidopsis* and tobacco plants overexpressing BjCdR15 showed instead a root morphology similar to that of the corresponding Cd-untreated plants (Figure 3.11 A and B).

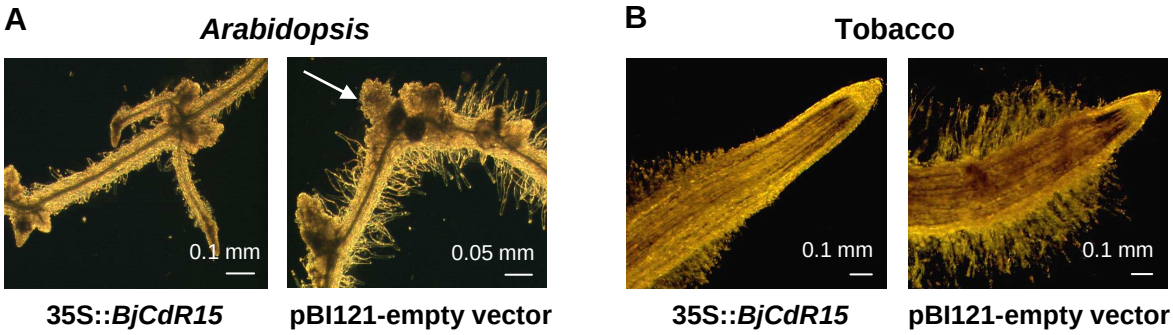


Figure 3.11 Light micrographs of roots of *Arabidopsis* (**A**) and tobacco (**B**) BjCdR15 overexpressing and control plants grown on Cd-containing *medium*.

3.6 - Chlorophyll content and biomass measure

Chlorophyll and biomass analyses were performed on *Arabidopsis* and tobacco plants grown *in vitro* for 3 weeks in *medium* without and with 200 and 400 µM $Cd(NO_3)_2$ for *Arabidopsis* and tobacco respectively.

In absence of Cd in the growth *medium* the total chlorophyll content was similar among the transgenic lines of *Arabidopsis* and tobacco, whereas, after Cd-treatment, a minor total chlorophyll content was observed in all treated plants if compared with the corresponding untreated plants (Figure 3.12 A and B). However, in presence of Cd, 35S::*BjCdR15* *Arabidopsis* plants showed a chlorophyll content 3.5 fold higher than *Arabidopsis* control plants ($p < 0.001$). The *tga3-2* mutant displayed a similar chlorophyll content to control plants, but the expression of BjCdR15 in this mutant line showed a

significant 2.3 fold increase (p< 0.001) in chlorophyll amount (Figure 3.12 A), probably due to BjCdR15 expression. A similar effect of BjCdR15 was also observed in tobacco plants (Figure 3.12 B) in which the chlorophyll level in 35S::*BjCdR15* tobacco line was 1.9 fold higher that tobacco control plants (p< 0.01).

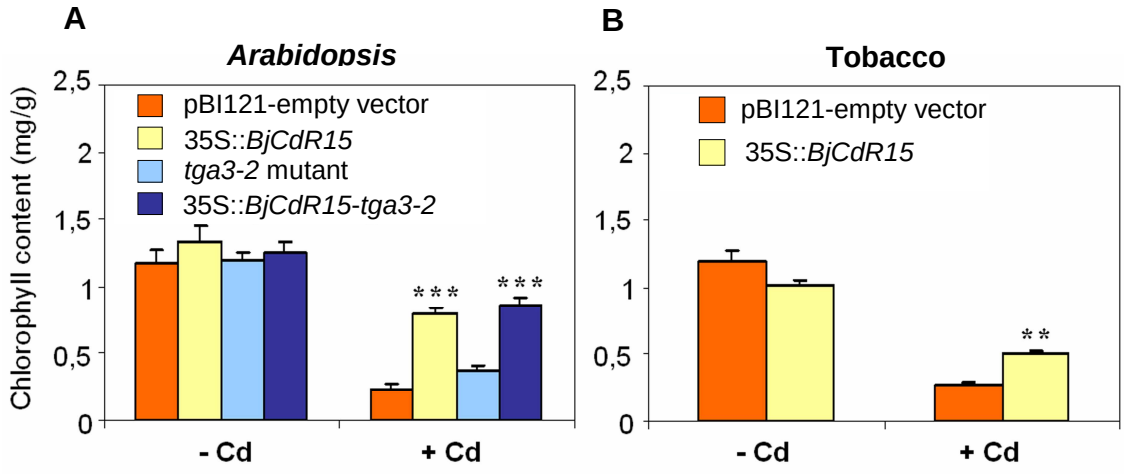


Figure 3.12 Chlorophyll content of control plants, 35::*BjCdR15* lines, *tga3* mutant and 35::*BjCdR15-tga3-2* line. For *Arabidopsis* (**A**), values showed are the average of four replicates from three plants each genotype grown with and without Cd; for tobacco (**B**), values reported are the average of three replicates from three plants each. Bars: SE.

Biomass accumulation was measured as shoot fresh weight of all tested plants. In the absence of Cd, the biomass accumulation was similar among the *Arabidopsis* tested lines and the tobacco lines, while significant differences between BjCdR15 expressing and controls plants were observed when Cd was added to the culture *medium* (Figure 3.13 A and B). In particular, in *Arabidopsis* 35S::*BjCdR15* and 35S::*BjCdR15-tga3-2* plants biomass were 1.2 fold higher (p< 0.001) than *Arabidopsis* control plants and *tga3-2* line respectively (Figure 3.13 A). 35S::*BjCdR15* tobacco shoot fresh weight was 1.4 fold higher (p< 0.05) that of control plants (Figure 3.13 B) in presence of Cd.

Both chlorophyll content and biomass accumulation analyses suggest that BjCdR15 transcription factor could be functionally implicated in Cd tolerance.

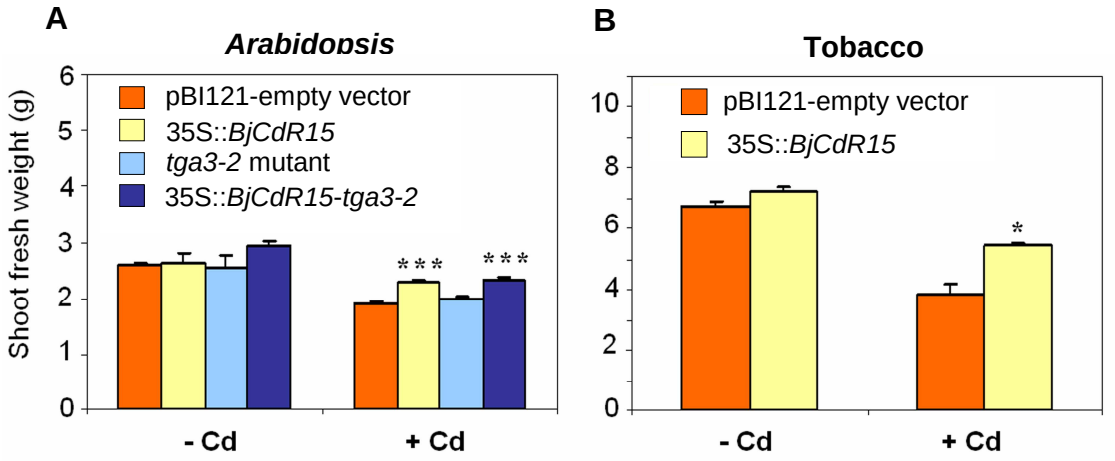


Figure 3.13 Shoot fresh weight of control plants, 35::*BjCdR15* lines, *tga3* mutant and 35::*BjCdR15-tga3-2* line. For *Arabidopsis* (**A**), values showed are the average of three replicates from ten plants each genotype grown with and without Cd; for tobacco (**B**), values reported are the average of three replicates from three plants each. Bars: SE.

3.7 - Role of BjCdR15/TGA3 on the expression of *Arabidopsis* PC synthase (AtPCS1)

Phytochelatins (PCs) are heavy metal binding peptides synthesized by the enzyme PC synthase (PCS) and play a key role in vacuolar sequestration and heavy metal detoxification. Because of the differences observed in Cd transport from root to shoot, Cd accumulation and Cd tolerance, among *Arabidopsis* control plants, *tga3-2* mutant and 35S::*BjCdR15* plants, the AtPCS1 expression level was measured by western blot. In absence of Cd in the growth *medium* (time 0 h) a basal expression level of AtPCS1 was found in leaves of all four lines tested of *Arabidopsis* (Figure 3.14 A and B). Interestingly, in the absence of Cd-treatment, 35S::*BjCdR15* plants showed an higher AtPCS1 constitutive level compared to control plants: this suggests that the ectopic expression of

BjCdR15 may positively regulate the expression of PCS. With the addition of Cd in the growth *medium* an increase of AtPCS1 by about 5-fold was detected in control plants (Figure 3.14 C). On the contrary, after Cd exposure, in 35S::*BjCdR15* plants a less pronounced increment (1.5 fold) was observed (Figure 3.14 C). Remarkably, accumulation of AtPCS1 in *tga3-2* mutant plants appeared to be Cd insensitive while the expression of BjCdR15 in *tga3-2* line doubles the production of this enzyme after 120 h of Cd-treatment (Figure 3.14 C). These data suggest that BjCdR15/TGA3 bZIP factors play a role in the regulation of PCS synthesis.

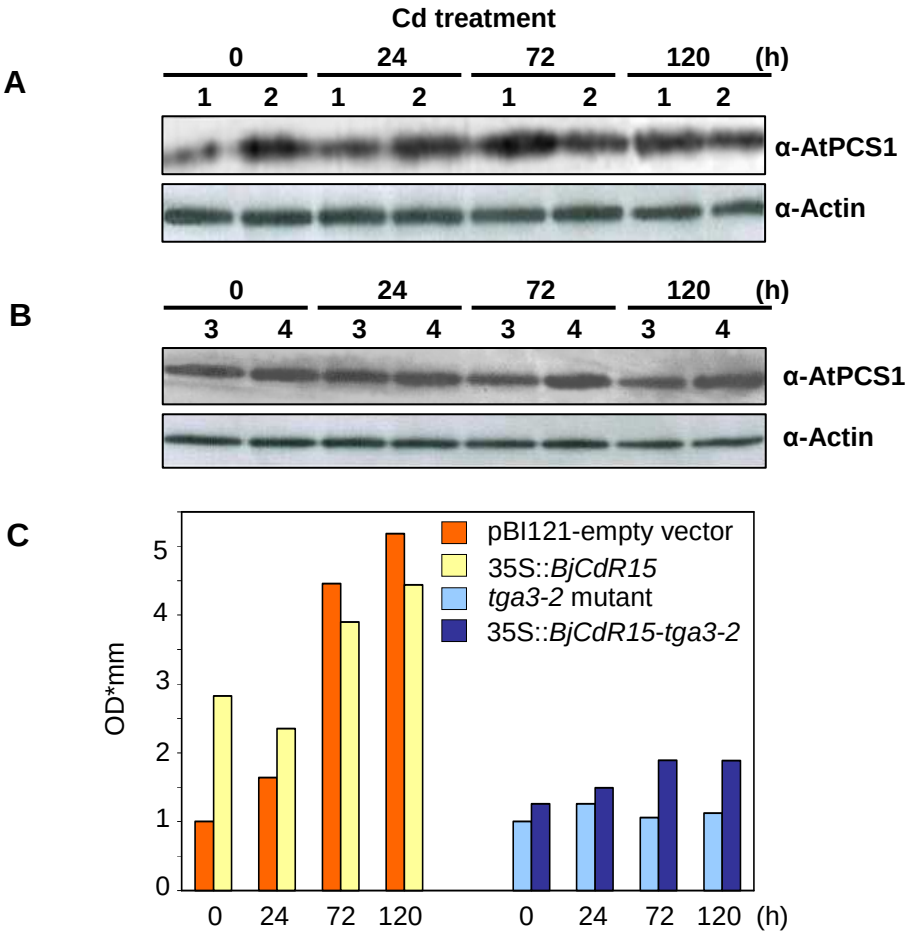


Figure 3.14 Analysis of AtPCS1 abundance. (**A**)-(**B**) Immunoblot analysis of total protein extracts from *Arabidopsis* control plants (1), 35S::*BjCdR15* line (2), *tga3-2* mutant (3) and 35S::*BjCdR15-tga3-2* line (4) at different times (0, 24, 72 and 120 h) of Cd-treatment. Antibody specific for AtPCS1 and α-Actin (as loading control) were used. (**C**) Densitometric analysis of immunoblot signals of lines tested in (**A**) and (**B**).

3.8 - Role of BjCdR15/TGA3 in the expression of heavy metals transporters

Since various types of cellular transporters have been shown to be involved in heavy metal detoxification mechanisms, transcripts of some of these genes were quantified by Real-time PCR in roots of *Arabidopsis* control and 35S::*BjCdR15* plants, *tga3-2* mutant and 35S::*BjCdR15-tga3-2* lines upon Cd-treatment. The attention was focused on *AtNramp3*, *AtHMA4*, *AtMRP3* (Multidrug-Resistance-related Protein), *AtPDR8* and *AtATM3* transcripts. *AtNramp3* transcripts in control and 35S::*BjCdR15* plants showed a normal induction after Cd-treatment, whereas its expression is Cd-insensitive in *tga3-2* mutant. Notably, when BjCdR15 is expressed in *tga3-2* mutant, *AtNramp3* was normally induced by Cd. BjCdR15 expression into *tga3-2* mutant restored *AtNramp3* transcription level similar to the level measured in control and 35S::*BjCdR15* lines. These expression patterns were analogous to those observed also for *AtHMA4* and *AtMRP3* genes in different lines after Cd treatment (Figure 3.15).

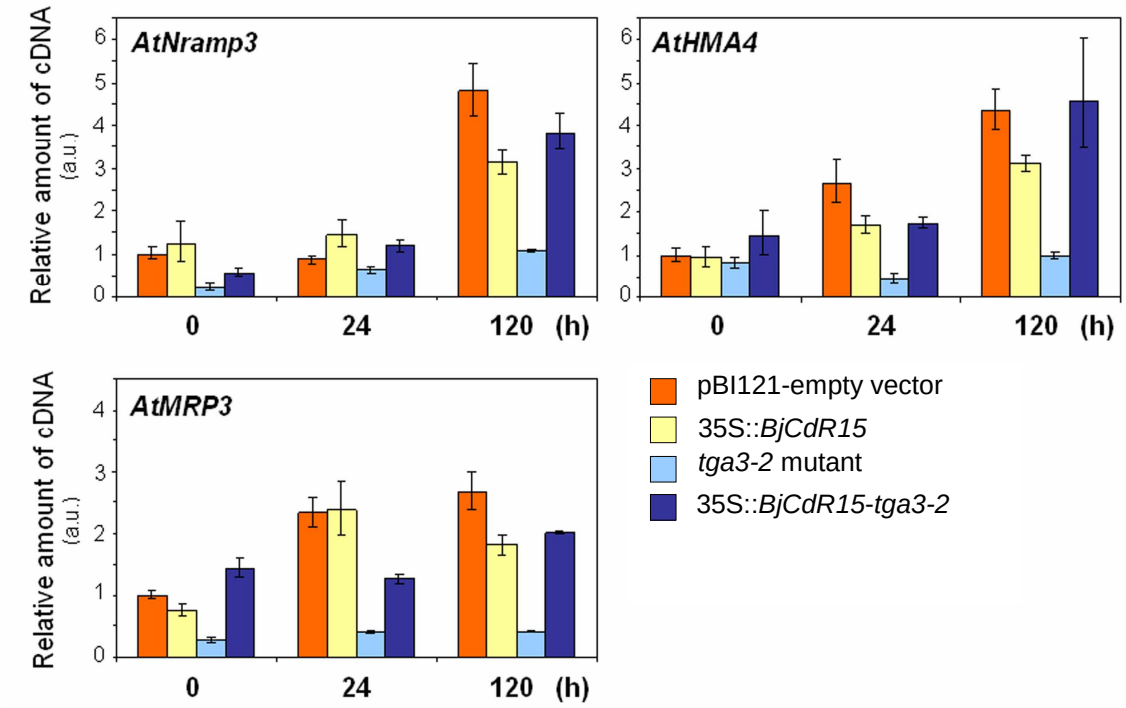


Figure 3.15 Transcript analysis of metal transporters *AtNramp3*, *AtHMA4*, *AtMRP3*, by Real-time PCR in roots of *Arabidopsis* plants at different times of Cd treatment (0, 24, 120 h). Bars: SE.

The expression of *AtPDR8*, is Cd-sensitive in control, 35S::*BjCdR15* and in 35S::*BjCdR15-tga3-2* plants and in *tga3-2* mutant after 120 h of treatment but, in absence of Cd, the level of transcript in the latter is not comparable to the level measured in control and 35S::*BjCdR15* lines (Figure 3.16).

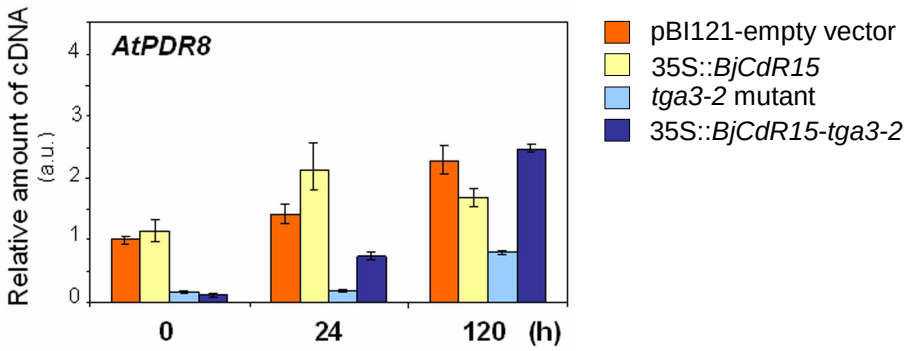


Figure 3.16 Transcript analysis by Real-time PCR of metal transporter *AtPDR8* in roots of *Arabidopsis* plants at different times of Cd treatment (0, 24, 120 h). Bars: SE.

Conversely, the expression of *AtATM3* gene is not affected by the lack of TGA3 protein, and a general slight increase in transcription level was observed in all lines (Figure 3.17). These results highlight a key role for BjCdR15/TGA3 transcription factors in the regulation of some of the main components of the metal transport system in plant.

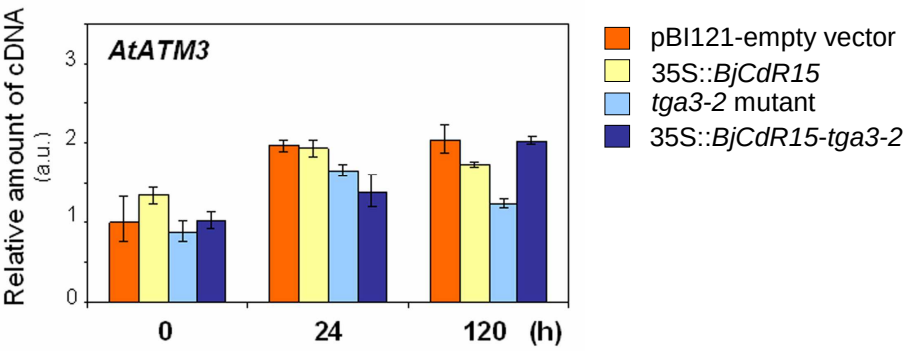


Figure 3.17 Transcript analysis by Real-time of metal transporter *AtATM3* in roots of *Arabidopsis* plants at different times of Cd treatment (0, 24, 120 h) PCR. Bars: SE.

3.9 - Expression of *BjCdR15* and *TGA3* mRNA in response to various treatment regimes

B. juncea and *Arabidopsis* plants were exposed to stress and hormone treatments to evaluate the expression of *BjCdR15* and *TGA3* in leaves in response to stress conditions. As shown in figure 3.18 A and B, both bZIP transcription factors are induced by cold (4 ºC) and almost unaffected by high temperature (42 ºC). In addition, these genes are induced by salt (NaCl) and drought treatments. ABA treatment revealed that both the genes are ABA responsive, although *TGA3* is more sensitive to ABA than *BjCdR15.* The transcription level of *BjCdR15* and *TGA3* were also measured after treatment with hormones such as methyl jasmonate and salicylic acid. Both genes are not induced by methyl jasmonate while only *TGA3* is up-regulated by salicylic acid.

The observed expression in diverse stress conditions suggest that these transcription factors could be involved in the activation of numerous stress responsive genes.

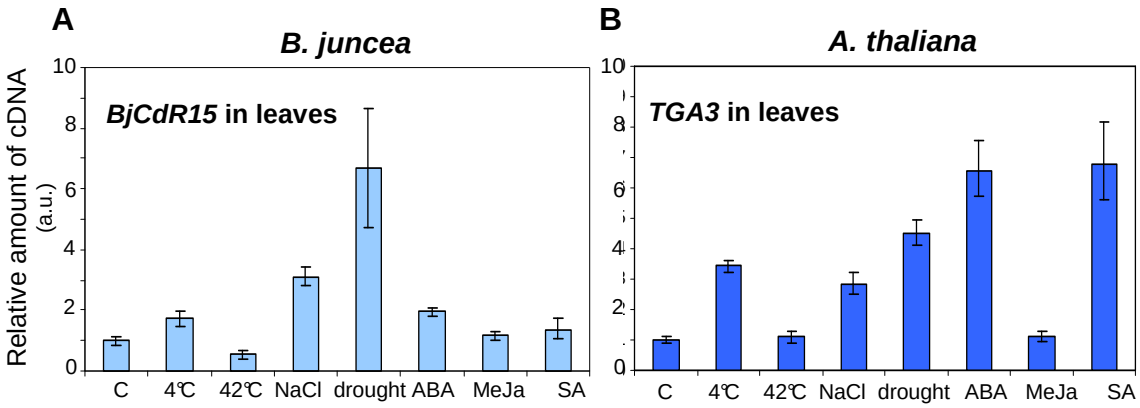


Figure 3.18 Expression analysis by Real-time PCR of *BjCdR15* and *TGA3* respectively in *B. juncea* and *Arabidopsis* plants following various treatments. C: untreated control plant. Bars: SE.

4. DISCUSSION

Molecular genetic studies of *Arabidopsis* bZIP TFs show that these proteins are involved in plant development as well as in stress signalling (Jakoby *et al*., 2002). It was demonstrated that TGA factors are required for basal defence and induction of PR genes (Kesarwani *et al*., 2007), and particularly, it is well known that TGA3 strongly interacts with NPR1 conferring disease resistance to bacterial and fungal pathogens (Zhou *et al*., 2000). In this study, a cDNA encoding a *B. juncea* bZIP transcription factor, BjCdR15, has been characterized. BjCdR15 is a putative orthologous of TGA3 and is up-regulated by heavy metals. Several evidences suggest a role of BjCdR15 in Cd tolerance, accumulation and translocation to the shoot. First of all, *BjCdR15* is induced by Cd and other heavy metals (Pb and Ni) in *B. juncea* plants and it is mainly expressed in the vascular system and the epidermal cells after Cd-treatment. Secondly, in presence of Cd, *Arabidopsis* and tobacco plants overexpressing BjCdR15 grew better and they were significantly more tolerant than control plants, showing higher chlorophyll content, shoot fresh weight and Cd content in the shoot than control plants. Moreover, BjCdR15 is able to complement the phenotype showed by the *tga3 Arabidopsis* mutant subjected to metal stress. This lets us to suppose that TGA3 and BjCdR15 share a similar function.

High sequence similarity between BjCdR15 and the *Arabidopsis* TGA3 TF and its nuclear localization confirmed that BjCdR15 is a transcription factor. Moreover, by means of northern and Real-time PCR analyses, it was shown that *BjCdR15* transcription increases shortly after Cd exposure and thereafter rapidly declines. This early induction in response to Cd exposure and its rapid shut off, point out to a key role for this TF in gene regulation in response to Cd. Furthermore, *in situ* localization of *BjCdR15* mainly in leaf and root epidermal cells and vascular tissues of Cd-treated *B. juncea* plants suggests a role of this bZIP factor in Cd absorption, transport and storage. In fact, root epidermis is the primary

contact site, between the plant and the soil, to which Cd gains access, while leaf epidermis is a major site of Cd storage in *B. juncea* (Salt *et al*., 1995).

Under Cd stress, lateral root formation was inhibited in control plants and promoted in *Arabidopsis* and tobacco overexpressing BjCdR15. The lack of TGA3 protein in the *tga3* mutant, seems not to affect the emission of lateral roots more severely than in control plants. Cd exposure also induced the formation of numerous root hairs in both *Arabidopsis* and tobacco control plants as well as in the *tga3* mutant line, while in *Arabidopsis* and tobacco overexpressing BjCdR15, the development of root hairs was comparable to that of Cd-untreated plants. Since Cd causes inhibitory effects on water and mineral nutrient uptake (Deckert, 2005; Meda *et al*., 2007), the normal development of roots in plants overexpressing BjCdR15 might represent an adaptive response to maximize the ability of the root system to uptake water and mineral nutrient in the presence of Cd and thus it can be considered as an aspect of Cd tolerance. On the contrary, changes in root structure, evident in control and mutant plants exposed to Cd, may instead be interpreted as symptom of Cd toxicity. However, the observation that Cd-treated *tga3* plants did not differ in root phenotype compared to control plants, does not allow to speculate about the role of TGA3 in lateral root and root hair development. Furthermore, during Cd exposure, the lack of TGA3 in knockout plants did not affect phenotype, biomass and chlorophyll content, more severely than in control plants. This result is not unexpected since the lack of TGA3 protein prevents, at least in part, the translocation and accumulation (see below) of Cd in shoots, and hence Cd toxicity symptoms are attenuated.

With regard to Cd accumulation, compared to control or BjCdR15 overexpressing plants (35S::*BjCdR15* plants), *tga3* mutant showed higher Cd accumulation in roots, but lower in shoots. These results remark that in *tga3* mutant both uptake and root-to-shoot long-distance transport of Cd are altered. On the contrary, when the function of TGA3 is restored by BjCdR15 protein, Cd transport to the shoot is reactivated: indeed the

overexpression of BjCdR15 in *tga3* mutant caused an increase of Cd content in shoots, while in roots it was similar to the control plants. The different effect of BjCdR15 overexpression has already been noted in WT or *tga3* backgrounds: in the latter BjCdR15 protein caused a higher Cd loading in shoots. All together, these data indicate that BjCdR15/TGA3 contribute to maintain a balanced Cd content in roots by acting on both Cd uptake and long distance root-to-shoot transport. This is consistent with the *in situ* BjCdR15 expression, predominantly localized in the vascular system of roots and leaves; moreover, it has recently been shown that the phloem could play a major role in the long distance "source to sink" transport of Cd (Mendoza-Cózatl *et al*., 2008). The apparent contradiction between increased Cd accumulation and tolerance has already been noted (Verret *et al*., 2004), and it has been observed that the major part of *Arabidopsis* biomass is formed by the shoot, which has large apoplastic and vacuolar compartments to enhance metal sequestration.

High Cd accumulation in roots was also reported for the *Arabidopsis* phytochelatins deficient mutant *cad1-3* (Gong *et al*., 2003). Phytochelatins have a role in heavy metal detoxification, chelating and sequestrating metal ions into the vacuoles (Salt and Rauser, 1995; Cobbett, 2000). It was proposed that a PCs dependent "overflow protection mechanism" would contribute to keep low Cd accumulation in roots by causing extra Cd transport to shoots (Gong *et al*., 2003). A possible role of BjCdR15 in cellular mechanisms of Cd detoxification (PCs synthesis and PC-Cd^{2+} complexation) and transport to the shoot, was therefore supposed, and the expression of AtPCS1 was measured in the different transgenic lines. In absence of Cd, AtPCS1 protein was less abundant in control plants than in 35S::*BjCdR15* plants. indicating that the ectopic expression of this bZIP TF is enough to influence AtPCS1 synthesis. Cd addition caused a further increase of AtPCS1 expression in both genotypes. Consistent with the Cd amount in roots, the level of AtPCS1 was not affected by Cd-treatment in *tga3* mutant, but it was increased in the

35S::*BjCdR15-tga3* plants. These findings suggest that BjCdR15/TGA3 might be involved in the regulation of PCS synthesis.

An other important aspect correlated to Cd tolerance is its extrusion or intracellular compartmentalization and hence the activation of specific transport processes. Indeed, although the transport pathways of Cd-complexes in plants have not yet been elucidated, several studies in *Arabidopsis* indicate that ABC transporters could be involved in Cd efflux, transport, sequestration and/or redistribution (Bovet *et al*., 2003; Bovet *et al*., 2005; Kim *et al*., 2007). Moreover, in *A. thaliana* and *A. halleri* HMA4 transporter contributes to Zn homeostasis and Cd detoxification (Mills *et al*., 2005; Verret *et al*., 2004, 2005; Courbot *et al*., 2007). In *T. caerulescens*, HMA4 may be involved in Cd transport and hyperaccumulation (Bernard *et al*., 2004; Papoyan and Kochian, 2004). It was proposed that AtNRAMP3 can transport heavy metal ions, modulating metal toxicity in plants (Thomine *et al*., 2003). The observations that the TGA3 mutation hampers Cd transport from root to shoot and strongly affects PCS expression and that BjCdR15/TGA3 positively regulate the expression of AtPCS1 and Cd translocation to the shoot, suggested to verify whether the expression of metal transporters is also regulated by these bZIP TFs. Moreover, the expression of several transporters mainly in vascular tissues of roots and leaves and in epidermis (Thomine *et al*., 2003, Verret *et al*., 2004; Kim *et al*., 2007), is consistent with BjCdR15 expression. All metal transporters tested in this work (*AtHMA4*, *AtMRP3*, *AtNramp3*, *AtPDR8* and *AtATM3)* resulted to be up-regulated by Cd-treatment in plants expressing TGA3 or BjCdR15 (see Figures 3.15, 3.16, and 3.17, section Results).

Regarding the expression of *AtHMA4*, it should be noted that a previous work reported the inhibition of this transporter after exposure to 100 µM Cd for 30 h (Mills *et al*., 2003). Oppositely, in this study the addition of 20 µM Cd for 24 and 120 h induced *AtHMA4* transcript. These conflicting observations may be due to different concentrations of the metal tested and/or to different times of Cd exposure.

Notably, *TcHMA4* transcription in *T. caerulescens* increased after Cd exposure (10 and 100 μM) for 20 days (Papoyan and Kochian 2004). Consistently, the absence of induction, under Cd supply, in the loss-of-function *tga3-2* mutant, and the restored upregulation of this gene in the 35S::*BjCdR15-tga3* plants demonstrate that BjCdR15/TGA3 regulate the expression of this transporter during Cd stress. Analogous results were obtained for *AtNramp3*, *AtMRP3* and *AtPDR8* transcripts. In particular BjCdR15 is able to complement the TGA3 function, restoring *AtPDR8* expression, only upon Cd-treatment. These observations led us to conclude that TGA3 and BjCdR15 might respond to Cd inducing redundant but distinct pathways. A further aspect worth of note is the evidence that BjCdR15/TGA3 affect the expression of genes that play multiple roles as transporters: both AtPDR8 and AtHMA4 contribute to Cd detoxification by functioning as Cd extrusion pumps (Mills *et al*., 2005; Kim *et al*., 2007); characterization of AtNRAMP3 has demonstrated its role in Fe and Cd transport and Cd sensitivity probably driving Cd efflux from the vacuole to the cytoplasm (Thomine *et al*., 2003). Experimental data showed that AtATM3 transporter has a mitochondrial localization, and contributes to heavy metal detoxification (Kim *et al*., 2006). Among the metal transporters considered in this study only *AtATM3* was not regulated by BjCdR15/TGA3.

It should be noted that *AtHMA4*, *AtMRP3*, *AtPDR8* and *AtATM3* genes possess, in their upstream promoter regions, at least one *as-1* binding motif (TGACG) sufficient to drive TGA3 recognition (Xiang *et al*., 1997), whereas *AtNramp3*, anyway induced by BjCdR15/TGA3, does not have the TGA binding sequence in its promoter. These findings suggest that several metal transporters could be directly activated by TGA3, while others may be indirectly regulated. Consistently, an early report indicated that the TGACG motif may confer Cd sensitive promoter activity, although straight relationship between Cd responsiveness and TGA transcription factors has yet been proved (Kusaba *et al*., 1996).

Finally, it is noteworthy that AtPDR8, a member of the pleiotropic drug resistance subfamily of the ABC transporters, is involved in both Cd resistance and pathogen

resistance (Kobae *et al*., 2006; Stein *et al*., 2006), even if no direct link between pathogen resistance and heavy metals has yet been found (Kim *et al*., 2007). It was suggested that AtPDR8 can recognize a very wide range of compounds, that might be toxic to the plant cell when accumulated at high concentration, and transport them out of the plasma-membrane (Kim *et al*., 2007). In this study, it is proposed that members of the TGA transcription factor family, TGA3 and BjCdR15, are able to induce the expression of genes responsible for the root-to-shoot Cd transport and are implicated in the extrusion of Cd into the apoplastic and/or vacuolar compartments. These results, together with the observation that TGA3 interacts with components that mediate diseases resistance (Kesarwani *et al*., 2007), point to a role of TGA3 in regulating the network of plant defence and other stress-induced signalling transduction pathways.

The results further indicate that regulator sequences of BjCdR15 and TGA3 might have, at least in part, a diverse response to different stress-associated signalling molecules. In fact, Real-time PCR results suggest that the promoter regions that control the transcription of *TGA3* in *Arabidopsis* contain salicylic acid inducible elements, whose role in the induction of systemic acquired resistance is well documented (Pontier *et al*., 2001; Kesarwani *et al*., 2007). Conversely, the transcription of *BjCdR15* in *B. juncea* is not modulated by salicylic acid nor by methyl jasmonate, suggesting that it is driven by inducible promoter elements mainly responsive to abiotic stresses. Thus, although TGA3 and BjCdR15 share high similarity, are both implicated in Cd transport and accumulation in the shoot and are both induced by abiotic stresses, they must differ in some promoter elements responsible for their activities in response to diverse stress *stimuli*. Such variability may reflect the divergent evolution of the related species *A. thaliana* and *B. juncea*.

In summary, the results reported show that BjCdR15/TGA3 regulate the expression of genes responsible for Cd tolerance and accumulation. The overexpression of BjCdR15 in heterologous systems, such as *Arabidopsis* and tobacco, enhances Cd translocation from

root to shoot. Eventually, this transcription factor might be considered as useful candidate for a biotechnological application in phytoextraction of Cd from polluted soils.

Chapter II:

IDENTIFICATION OF GENES INVOLVED IN HEAVY METALS TOLERANCE AND HYPERACCUMULATION IN *Arabidopsis halleri* SHOOTS IN RESPONSE TO Cd AND Zn AND RHIZOSPHERE MICROORGANISMS

1. INTRODUCTION

1.1 - The rhizosphere

The rhizosphere is defined as the interface between plant roots and soil and it extends approximately 1 mm around the roots (Pilon-Smith, 2005). As shown in Figure 1.1, in the rhizosphere, plants and microorganisms achieve mutual relationship by which both take advantage. These interactions are influenced by several factors such as characteristics and activity of plant and microorganisms, but also from soil chemical properties and climatic conditions. In particular, plant roots exude a variety of photosynthesis-derived compounds that serve as carbon sources for soil heterotrophic microorganisms (e.g. fungi and bacteria) (Olson *et al*., 2003). It has been estimated that about 20% of carbon fixed by plants is released from roots into the rhizosphere and consequently microbial density is 1-4 fold higher than in bulk soil (Salt *et al*., 1998). In turn, rhizosphere microbes support plant health enhancing water and mineral uptake (Kapulnik, 1996). For instance, rhizosphere bacteria are able to degrade organic compounds and modify the chemical state of inorganics, stimulating leaching of nutrients. Producing bacterio-toxins, microbes can also hamper the growth of pathogenic microorganisms thus promoting plant growth. Finally, fungi, associated via mycorrhiza to plant roots, may provide a larger nutrient-exchange area and mediate nutrient transport to the plant (Chopra *et al*., 2007).

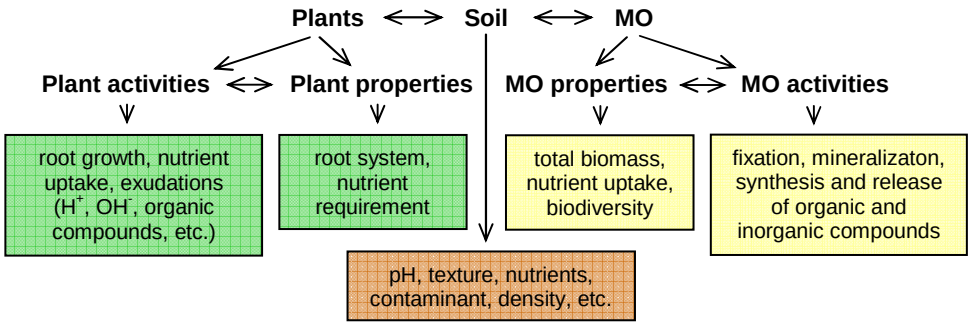


Figure 1.1 Scheme of interactions among plants, soil and microorganisms (MO) in the rhizosphere soil.

1.2 - Heavy metals phytoremediation assisted by rhizosphere microorganisms

Plants that might be employed in a phytoremediation process applied to heavy metals polluted sites should possess few but important features: rapid growth, high biomass production, heavy metal tolerance and accumulation (preferably, at high concentrations in shoots). Conversely, hyperaccumulator plants, as in the case of members belonging to the *Brassicaceae*, have exceptionally high accumulating capacity, but most of them are characterized by slow growth rate and limited biomass production (DalCorso *et al*., 2008). To overcome these unhelpful features of hyperaccumulators, some strategies have been defined as successful:

1) the use of plant species with a lower accumulating capacity but higher growth rate;

2) to supply the contaminated sites with complexing agents, such EDTA and synthetic aminopolycarboxylic acids (APCA), promoting an increasing uptake by plants (Pivetz, 2001; Doumett *et al*., 2008);

3) to amend the polluted sites with microorganisms naturally associated with the rhizosphere (Xiong *et al*., 2008): in fact, it has been observed that the use of these microbial communities in combination with plants, offers an efficient phytoremediating tool. Thus, a major aim of biotechnology researches is the investigation of mechanisms through which microorganisms influence plant heavy metal uptake.

Rhizosphere microbe cenosis are able to influence metal bioavailability altering heavy metal chemistry, for example by mechanisms that provide the use of these elements as terminal electron acceptors in anaerobic respiration: as result metal ion redox state is lowered, and this is enough to enhance plant uptake (Lovley, 1995). As a consequence of microbial metabolism and its impact on metal bioavailability, microbial populations of the rhizosphere may influence both the metal uptake and the plant growth. Soil rhizobacteria, producing compounds such as antibiotics and antifungals, organic acids, hormones (e.g. IAA), and metal-chelating agents, increase the bioavailability and facilitate the root absorption also of essential metals and nutrients, such as Fe, N and P (Crowley *et al*.,

1992; Xiong *et al*., 2008). For instance, microbial metal-chelators and Fe-siderophore, are exploited by plants as iron-delivery-system, and eventually as iron source (Arazi *et al*., 1999). Recent studies showed that high proportions of metal-resistant bacteria persist in the rhizosphere of hyperaccumulators such as *Thlaspi caerulescens* and *Alissum murale*, grown respectively under Zn and Ni contaminations and an increasing amount of these metals into the above ground plant tissues has been found under these conditions (Delorme *et al*., 2001; Abou-Shanab *et al*., 2003a). Moreover, enhancing root elongations, rhizosphere microbes significantly increase the uptake of Cd, Zn and Pb in *Sedum alfredii* (Xiong *et al*., 2008). De Souza and colleagues (1999) found that salt marsh bulrush plants, supplied with rhizosphere bacteria, accumulated 70-80% and 40-50% higher Se concentrations in their roots and shoots respectively, than plants grown under axenic conditions. In particular, rhizobacteria could enhance Se accumulation in plants by reducing selenate to organic Se, a chemical form taken up into roots faster than inorganic forms (Zayed *et al*., 1998). Chemolithotrophic bacteria have been shown to enhance environmental mobility of metal contaminants via soil acidification, or to decrease their solubility by precipitation as sulfides (Sharma *et al*., 2000). Delorme *et al*. (2001) found that soil acidification in the rhizosphere of *T. caerulescens* facilitates metal ion uptake by increasing metal ions mobility around the roots. Moreover, it has been also reported that the accumulation of Hg increases into plants when pH of culture solution is lowered (De Souza *et al*.,1999).

Concluding, defining the processes and the mechanisms that drive plant metal-uptake increase due to rhizosphere microbe population, is of primary importance to set-up efficient, low costly and environment friendly phytoremediation approaches.

2. MATERIALS AND METHODS

2.1 - Growth *media*

Nutrient *medium*	Nutrient broth (Oxoid, Garbagnate Milanese, Italy)	13 g/l
	Bacteriological agar (Oxoid)	10 g/l
	Deionised water	To volume
	pH	7.2

Malt *medium*	Malt broth (Micropoli, Milan, Italy)	20.0 g/l
	Yeast extract (Micropoli)	5.0 g/l
	Bacteriological agar (Oxoid)	10 g/l
	Deionised water	To volume
	pH	7.0

After sterilization, add riphampicin 15 mg/l.

Waksman *medium*	Glucose	10.0 g/l
	NaCl	5.0 g/l
	Bacteriological peptone (Micropoli)	5.0 g/l
	Beaf extract (Oxoid)	3.0 g/l
	Bacteriological agar (Oxoid)	10 g/l
	Deionised water	To volume
	pH	7.0

After sterilization, add amphotericin 4 mg/l, nystatin 50 mg/l and polymyxin 5 mg/l.

2.2 - Plant material and growth conditions

Seeds of *A. halleri* were collected from plants grown on a heavy metal contaminated site in 'Bois des Asturies' in the town of Auby in Northern France (van Rossum *et al*., 2004). They were surface sterilized in 70% ethanol for 1 min and in 1.5% v/v sodium hypochlorite solution for 20 min and then rinsed in sterile distilled water. Seeds were then germinated on MS *medium* (Murashige and Skoog, 1962) containing 0.7% agar and kept in growth chamber under 16-h light/8-h dark regime at 22 ºC/18 ºC. After 30 days plants were transplanted in perlite-filled 1 l polyethylene pots wetted with half strength Hoagland's solution (Hoagland and Arnon, 1938), up to substrate saturation and moved to the greenhouse.

2.3 - Estimation of rhizosphere microbial populations

Total counts of aerobic microbial communities (bacteria, actinomycetes and filamentous eumycetes) inhabiting the rhizosphere of *A. halleri* grown on the contaminated soil were evaluated. Two grams of rhizosphere soil, gently recovered from the surrounding of plant roots, were suspended in a 150-ml Erlenmeyer flask containing 18 ml of a 0.9% w/v NaCl solution. The flask was incubated at 28 ℃ in the dark for 2 h on an orbital shaker (250 rev/min). Serial dilutions of the suspension were then prepared in 0.9% w/v NaCl solution. Series of plates containing specific agarised *media* (Nutrient for bacteria, Waksman for actinomycetes and Malt for moulds) were streaked with 0.1 ml/plate of differently diluted suspensions. Plates were incubated for 5 days at 28 ℃. Colonies were counted for each microbial population and reported as "colony forming units per gram of rhizosphere soil" (CFU/g). All analyses were performed in triplicate.

2.4 - Isolation of Cd- and Zn-resistant microbial strains

Two grams of rhizosphere soil were suspended in a 150-ml Erlenmeyer flask containing 18 ml of a 0.9% w/v NaCl solution. The flask was incubated at 28 ℃ in the dark for 2 h on

an orbital shaker (250 rev/min). Serial dilutions of the suspension were then prepared and series of plates containing specific agarised Nutrient and Waksman *media* were streaked with 0.1 ml/plate of differently diluted suspensions. In order to isolate Cd- and Zn-resistant microbial strains, the *media* were previously supplied with 1 mM $CdSO_4$ (~ 100 ppm Cd) or 10 mM $ZnSO_4$ (~ 650 ppm Zn) and with 1 mM $CdSO_4$ plus 10 mM $ZnSO_4$. Plates were incubated for 5 days at 28 ℃. Afterwar ds, single morphologically different colonies were isolated and streaked on fresh agarised *media* containing $CdSO_4$, $ZnSO_4$ or both metal salts (at the concentration as mentioned above) as selective force, in order to obtain axenic cultures.

- Minimum inhibitory concentration (MIC) determination

MIC for Cd and Zn was determined on Nutrient agarised *medium*. Cd-and Zn-resistant microbial strains, previously isolated, were streaked on plates containing increasing concentrations of $CdSO_4$ and $ZnSO_4$ and checked for their growth ability after 5 days of incubation.

- Phylogenetic analysis

Genomic DNA of eight selected microbial strains was extracted with the NucleoSpin Tissue Kit (Clontech, Palo Alto, CA) following the manufacturer's instructions. Amplification of the 16S rRNA gene was performed with **F8** and **R11** primers (Table 2.1). The amplification product was sequenced on both strands and sequences were analysed by means of the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/).

Primer name	Primer sequence
F8	5'-GAGTTTGATCCTGGCTCAG-3'
R11	5'-ACGGCTACCTTGTTACGACT-3'

Table 2.1 Primers used for the amplification of 16S rRNA gene.

- Evaluation of microbial growth

Axenic culture for each of the eight microbial strains previously identified was obtained by inoculation of each strain in 250-ml Erlenmeyer flasks containing 100 ml Nutrient broth (initial OD_{600}: 0.01). Flasks were incubated on an orbital shaker at 200 rpm at 28 ℃ for 72 hours. At different times (0, 16, 24, 36, 48, 72 h), aliquots of the axenic cultures were streaked on agarised-Nutrient plates and microbial growth was estimate by CFU counting. All analyses were performed in triplicate.

2.5 - Microbial *inocula* for bioaugmentation tests

In order to understand the influence of rhizosphere microorganisms on metal-uptake in *A. halleri* plants, two different microbial *inocula* were tested:

1) an *inoculum* of the soil microbial community, derived from the rhizosphere of autochthonous *A. halleri*;

2) an *inoculum* with eight strains in axenic culture, isolated from the polluted soil and able to grow in *media* supplemented with both 1 mM $CdSO_4$ and 10 mM $ZnSO_4$.

Taking into account the actual size of the soil bacterial population as estimated by the serial dilution procedure, a proper aliquot of rhizosphere soil was suspended in 0.9% w/v NaCl solution, in order to obtain a final concentration of $1x10^7$ CFU rhizosphere bacteria per gram of perlite substrate. This suspension was filtered on Whatman paper and the filtrated fraction was uniformly distributed onto the perlite substrate supporting *A. halleri* plants (first *inoculum*). In this way, together with bacteria both autochthonous actinomycetes and filamentous fungi were added to the growth substrate.

For the second *inoculum*, axenic cultures were obtained by growing the eight different strains separately in 250-ml Erlenmeyer flasks containing 100 ml Nutrient broth. Flasks were incubated on an orbital shaker at 200 rpm at 28 ℃ for 42 hours. In order to obtain a final concentration of $1x10^7$ CFU of each strain per gram of perlite substrate, a proper aliquot of each bacterial culture, at the stationary growth phase, was collected and

centrifuged 4000 x g for 10 min, at 4°C. Pellets were resuspended together in 100 ml of 0.9% w/v NaCl solution. The microbial solution was uniformly distributed throughout the perlite bed.

2.6 - Experimental design

Ten plants of *A. halleri*, grown *in vitro*, were transferred to each pot, cultured in half strength Hoagland solution and subjected for a month to the following five different growth conditions:

1) control: untreated plants grown in the solely nutritive solution (**Ctr**);

2) only metals: addition of 1.0 mM $CdSO_4$ and 10 mM $ZnSO_4$ (**Mt**);

3) only microorganisms: addition of the native microbial community ($1x10^7$ CFU/g perlite) collected from the autochthonous rhizosphere of *A. halleri* (**Mc**);

4) metals plus microorganisms: addition of both 1.0 mM $CdSO_4$ and 10 mM $ZnSO_4$ together with the microbial community ($1x10^7$ CFU/g perlite) collected from the native rhizosphere of *A. halleri* (**Mt+Mc**);

5) metals plus eight microbial strains: addition of both 1.0 mM $CdSO_4$ and 10 mM $ZnSO_4$ together with the eight microbial strains (8MS) in axenic culture ($1x10^7$ CFU/g perlite each), isolated from the rhizosphere of *A. halleri* (**Mt+8MS**).

Plants of two pots for treatment were then harvested, roots were separated from shoots. Part of the sample were quickly frozen in liquid nitrogen and stored at – 80 ºC until use, while the rest was analysed for Cd and Zn contents.

2.7 - Measurement of Cd and Zn content

Cd and Zn concentrations were measured in samples belonging to the two soil layers (0-5 cm and 5-15 cm), collected from the contaminated site (town of Auby, Northern France). Moreover, Cd and Zn content was determined, in triplicate, in shoots of plants grown in the five experimental conditions and in shoots of plants collected in the native

contaminated soil. Samples were weighed and oven-dried at 85 ºC for 3 days. Dried samples were homogenized before analysis using a Wiley mill. Cd and Zn analyses were performed after microwave-assisted acid digestion (EPA 3052, 1996) by means of ICP-MS analysis (EPA 200.8).

2.8 - Determination of chlorophyll content

Plant leaves were frozen in liquid nitrogen, ground to power and weighed. Pigments were extracted with 80% acetone saturated with Na_2CO_3 and absorbance at 646.6 nm and 663.6 nm was measured. Chlorophyll concentration was calculated according to classical equations as previously described (Porra, 2002). Statistical analysis (Student's *t*-test) was performed to identify significant increase or decrease in chlorophyll content ($p< 0.05$).

2.9 - Protein extraction and solubilization

Total proteins were extracted from shoots of plants grown under the experimental conditions previously mentioned. Tissues were ground in liquid nitrogen and solubilised in 80 mM citric acid (pH 4), 1% w/v C7Bz0 detergent (Sigma-Aldrich, St. Louis, MO), 7 M urea, 2 M thiourea and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). After homogenisation, samples were centrifuged at 40,000 g for 20 min and supernatant proteins were precipitated by adding 8 volumes of acetone and 1 volume of methanol and incubated overnight at -20℃. Proteins were recovered by centrifugation at 20,000 g for 15 min and resuspended in solubilisation buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 40 mM Tris, supplemented with protease inhibitors). Protein concentration was estimated by Bradford assay (Sigma-Aldrich, St. Louis, MO), following the manufacturer's instructions.

2.10 - 2-DE and gel staining

Cysteine reduction and alkylation were performed by adding 5 mM fresh tributyl phosphine and 10 mM free acrylamide to 1.0 mg of total protein extracts. The reaction was stopped with 10 mM DTT. Afterward, 0.5% carrier ampholytes (pH 3 to 10) and traces of bromophenol blue were added to the samples. Each sample (2.2 mg/ml) was loaded by rehydration onto large-size 17 cm pH 3-10 NL IPG strips (BioRad, Hercules, CA) and five replica 2D-maps were performed. Isoelectric focusing (IEF), in first dimension run, was carried out with Protean IEF Cell (BioRad, Hercules, CA). Focusing was performed by applying a voltage program as follows: from 100 to 1000 V linearly for 5 h, 1000 V constants for 5 h, logarithmic voltage ramp from 1000 to 10000 V in 1 h, 10000 V constants until reaching a value of 75,000 Vh. At the end of the focusing phase each sample strip was reconditioned for 25 min with a solution containing 2% SDS, 6 M urea, 20% glycerol and 0.375 M Tris-HCl (pH 8.8). Second dimension electrophoresis (SDS-PAGE) was performed on polyacrylamide gradient gels (10 to 20% T) by using a PROTEAN® Plus Dodecacell (BioRad, Hercules, CA) in presence of glycine–Tris Buffer (pH 8.3) (192 mm glycine, 0.1% SDS, Tris to pH 8.3). The program set, for each gel, was: 40 mA for 3 min, 2 mA for 1 h and 10 mA overnight until the end of the run. All gels were then fixed with a solution containing 10% methanol and 7% acetic acid for 2 hours and subsequently stained with the Sypro Ruby fluorescent stain (BioRad, Hercules, CA).

2.11 - Analysis of differential protein patterns

Gels were scanned using the VersaDoc 1000 imaging system (BioRad). Spots analysis was performed by PDQuest software (version 7.3, BioRad). Each gel was analyzed for spot detection, background subtraction and OD-intensity quantification for each protein spot. Gel image showing the highest number of spots and the best protein pattern was chosen as a reference template and spots in reference gel were then matched across all gels. Values corresponding to spot quantities were normalised for each gel dividing the

raw quantity of each spot by the total quantity of all spots included in the reference gel. Gels were divided into separated groups (Ctr, Mt, Mc and Mt+Mc) and as previously indicated, for each protein spot, the average spot quantity value and its variance coefficient in each group were determined. Statistical analysis (Student's *t*-test) was performed to identify proteins that were significantly increased or decreased in the four sets of samples ($p < 0.05$). The following comparisons were performed:

- metal *vs* control (Mt *vs* Ctr);
- microorganisms *vs* control (Mc *vs* Ctr);
- metals plus microorganisms *vs* control (Mt+Mc *vs* Ctr);
- metals plus microorganisms *vs* metals (Mt+Mc *vs* Mt);
- metals plus microorganisms *vs* microorganisms (Mt+Mc *vs* Mc);

2.12 - In-gel digestion and MS/MS analysis

Spots of interest were excise from gels and subjected to in-gel trypsin digestion as described (Shevchenko *et al*., 1996). MS/MS analysis of the digested peptide was performed using QSTAR XL hybrid quadrupole-TOF instrument (Applied Biosystems, Foster City, CA, USA) coupled with LC Packings Ultimate nanoflow LC system (Dionex, Amsterdam, The Netherlands). The peptides samples, obtained from in gel trypsin digestion, were vacuum dried into a vacuum concentrator 5301 (Eppendorf, Hamburg, Germany) and stored at -20℃ until nanoHPLC ESI-Q-T OF MS analysis. The peptide pellets were resuspended immediately before analysis in 10 µl of solvent A (95% v/v water, 5% v/v ACN, 0.1% v/v formic acid). Five microliters of each sample were loaded and washed for 5 min onto the precolumn (300 µm i.d × 5 mm, C18 PepMap, 5 µm beads, 100 Å LC-Packings) using a flow rate of 30 µl/min solvent A via the LPG-3600 loading pump. Peptides were subsequently eluted at 300 nl/min from the precolumn over the analytical column (15 cm × 75 µm, C18 PepMap100, 3 µm beads, 100 Å LC-Packings) using a 35 min gradient from 5 to 60% solvent B (5% v/v water, 95% v/v ACN, 0.1% v/v

formic acid) delivered by the LPG-3600 micro pump and splitted at a ratio 1:1000 in the flow manager FLM-3100 (LC Packings). The total duration of the LC run was 65 min, including sample loading, column washing and equilibration.

Nanoscale ESI was performed using a Protana interface and nanoelectrospray needles set to 1.6 kV (New Objective,Woburn, MA). The QStar-XL operated in information-dependent acquisition (IDA) mode. In MS mode, ions were screened from 400 to 1800 m/z, and MS/MS data were acquired from 60-2000 m/z. Each acquisition cycle was comprised of a 1 sec MS and a 3 sec MS/MS. MS to MS/MS switch threshold was set to 10 counts per second (c.p.s.). All precursor ions subjected to MS/MS in the previous cycle were automatically excluded for 60 sec using a 3 a.m.u. window.

2.13 - Protein identification

The IDA settings were as following: default charge state was set to 2+, 3+, and 4+; MS centroid parameters were 50% height percentage and 0.05 a.m.u. merge distance; all MS/MS data were centroided, with a 50% height percentage and a merge distance of 0.05 a.m.u. The threshold peak intensity was set to 4 c.p.s. The MS/MS data from the protein sample was searched as a Mascot file against all entries in the public NCBI database (http://www.ncbi.nlm.nih.gov/) using the on-line Mascot search engine (www.matrixscience.com). Carbamidomethylation of cysteine residues, oxidation of methionine, deamidation of asparagine and glutamine were set as variable modifications for all Mascot searches. The score obtained from the analysis with Mascot software indicates the probability of a true positive identification; score value upon 60 was considered significant.

2.14 - Western blot analysis

Western analyses were performed according standard protocols (Sambrook and Russell, 2001). Total protein were extracted from shoots of tested plants, as mentioned in the

paragraph 2.9 section Material and Methods. Protein samples (8 µg) were loaded on 12% acrylamide SDS-PAGE-gels and transferred to PVDF membranes (Hibond^{TM}-P, Amersham Biosciences, Uppsala, Sweden). For hybridisation, specific antibodies against MDAR, PSBP1, PSBO2, Rieske and the δ-subunit of the plastidic ATPase (Agrisera, Vännäs, Sweden) were used. Detection was achieved employing the ECL–detection system (Amersham Biosciences, Uppsala, Sweden). The intensity of the chemiluminescence response was measured by scanning films and processing the image with the QuantityOne software Version 4.4.1 (BioRad).

3. RESULTS

3.1 - Determination of Cd and Zn content in contaminated soil

Measurements of Cd and Zn content were carried out in contaminated soil (van Rossum *et al*., 2004). In particular, metal concentrations were measured in a superficial layer (0-5 cm), where there were the majority of the *A. halleri* roots, and in a deeper layer (10-15 cm), characterised by a very compact soil structure. The upper zone showed a lower accumulation, of both Cd and Zn, than the deeper layer (Table 3.1), probably due to its physical structure, that permits heavy metal percolation.

Soil layer	mg/Kg Cd (ppm)	mg/Kg Zn (ppm)
0-5 cm	87	13475
10-15 cm	405	41755

Table 3.1 Cd and Zn contents that characterise the contaminated soil considered in this thesis.

3.2 - Microbiological analyses

3.2.1 - Microbial total counts

The aerobic microbial populations of *A. halleri* rhizosphere, grown in the contaminated area, was estimated by direct microbial *inoculum* on three agarised growth *media*: Nutrient, Waksman and Malt *medium*, for bacteria, actinomycetes and filamentous eumycetes respectively (see paragraph 2.3 section Materials and Methods). Average values of total microbial counts, for each microbial group, are reported in Table 3.2.

Growth *medium*	Microbial counts (Log CFU/g)
Nutrient	8.0 ± 0.24
Waksman	7.7 ± 0.15
Malt	7.2 ± 0.08

Table 3.2 Estimation of aerobic microbial populations of the *A. halleri* rhizosphere.

3.2.2 - Isolation of Cd- and Zn-resistant microbial strains from *A. halleri* rhizosphere

Cd- and Zn-resistant microbial strains were isolated as axenic cultures from the rhizosphere of *A. halleri* grown on the Cd and Zn (and Pb) polluted soil as indicated in the paragraph 3.1 (this section). Strains were selected on agarised Nutrient and Waksman *media* supplied with only $CdSO_4$ (~ 100 ppm Cd), only 10 mM $ZnSO_4$ (~ 650 ppm Zn) and $CdSO_4$ plus $ZnSO_4$. 63 microbial strains were tolerant to 1 mM Cd, 50 microbial strains to 10 mM Zn and 8 microbial strains were able to grow in the *media* supplemented with both 1 mM $CdSO_4$ and 10 mM $ZnSO_4$. In further analyses, the attention was focused on these eight microbial strains characterised by a MIC for $CdSO_4$ corresponding to 1 mM and for $ZnSO_4$ to 10 mM. Microbial genomic DNA was extracted and amplification of the 16S rRNA gene was performed. Sequence alignments, by BLAST algorithm, allowed the taxonomic identification of the strains, revealing high sequence similarity with strains listed in Table 3.3.

Microoganism	Sequence similarity
Pseudomonas putida	100%
Chryseobacterium joostei LMG18208	98%
Chryseobacterium sp. BW2	100%
Chryseobacterium sp. R139	98%
Tsukamurella strandjordii	99%
uncultured Escherichia sp.	99%
Novosphingobium sp. FND	99%
Curtobacterium sp.	99%

Table 3.3 Taxonomic characterisation of Cd- and Zn-tolerant microbial strains isolated as axenic culture from *A. halleri* rhizosphere.

For each axenic culture, the microbial growth in liquid *medium* was evaluated, counting CFUs on Nutrient broth agarised plates. These data were organized in the growth-phase diagram showed in Figure 3.1.

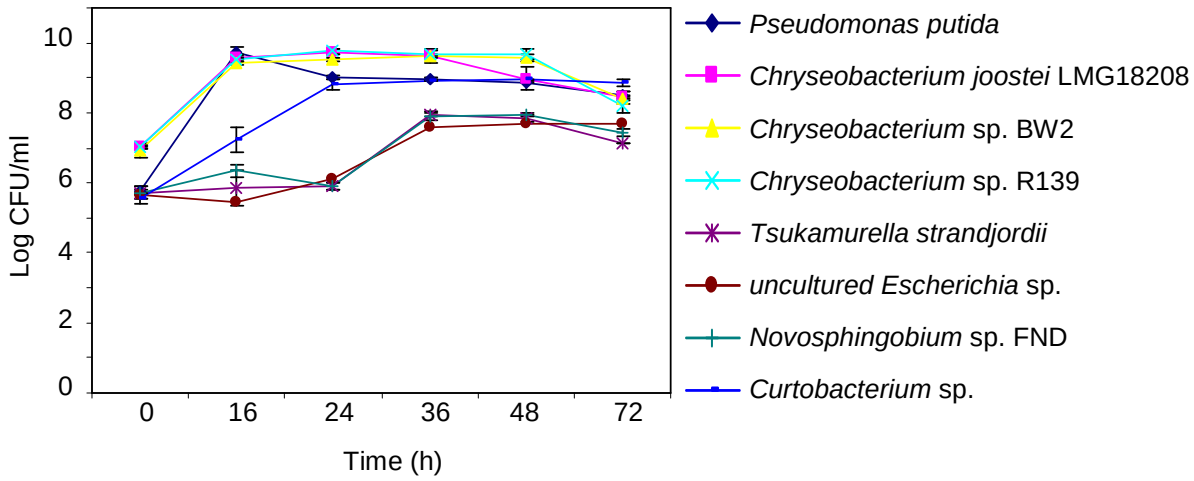


Figure 3.1 Growth trend of the eight strains resistant to both Cd and Zn. In the diagram, typical growth phases can be seen (the LAG phase, the early EXPONENTIAL phase, the late EXPONENTIAL phase and the STATIONARY phase).

As reported in Figure 3.1, all microbial strains reach their stationary growth in about 42 h after *inoculum* in axenic culture. Consequently, this time frame was employed during the bioaugmentation test (Mt+8MS, see paragraph 2.5 section Materials and Methods).

3.3 - Plant growth and metals accumulation

A. halleri plants grown in the greenhouse were subjected for one month to different treatments with heavy metals, rhizosphere microbial total community and with the axenic culture of eight microbial strains added to the nutrient solution (see paragraph 2.6 section Materials and Methods). This apparently long experimental time to identify differentially expressed proteins in *A. halleri* shoots was chosen to allow the establishment of plant-microbe interactions (Figure 3.2).

Figure 3.2 Experimental design of *A. halleri* treatments in greenhouse. Ctr: control; Mt: only metals; Mc: only microorganisms; Mt+Mc: metals plus microorganisms; Mt+8MS: metals plus the eight microbial strains resistant to both Cd and Zn.

At the end of the experiment, plants kept under the different treatment conditions exhibited the same phenotype of control plants maintained in the solely nutritive solution. They didn't show any chlorosis or toxicity symptoms (Figure 3.3 A). Furthermore, analysis of the total chlorophyll content in shoots, indicated that plants treated with only heavy metals or with heavy metals plus the rhizosphere microorganisms had a significant higher chlorophyll content than control plants, plants treated solely with rhizosphere microorganisms and than plants treated with heavy metals plus the eight microbial strains (Figure 3.3 B).

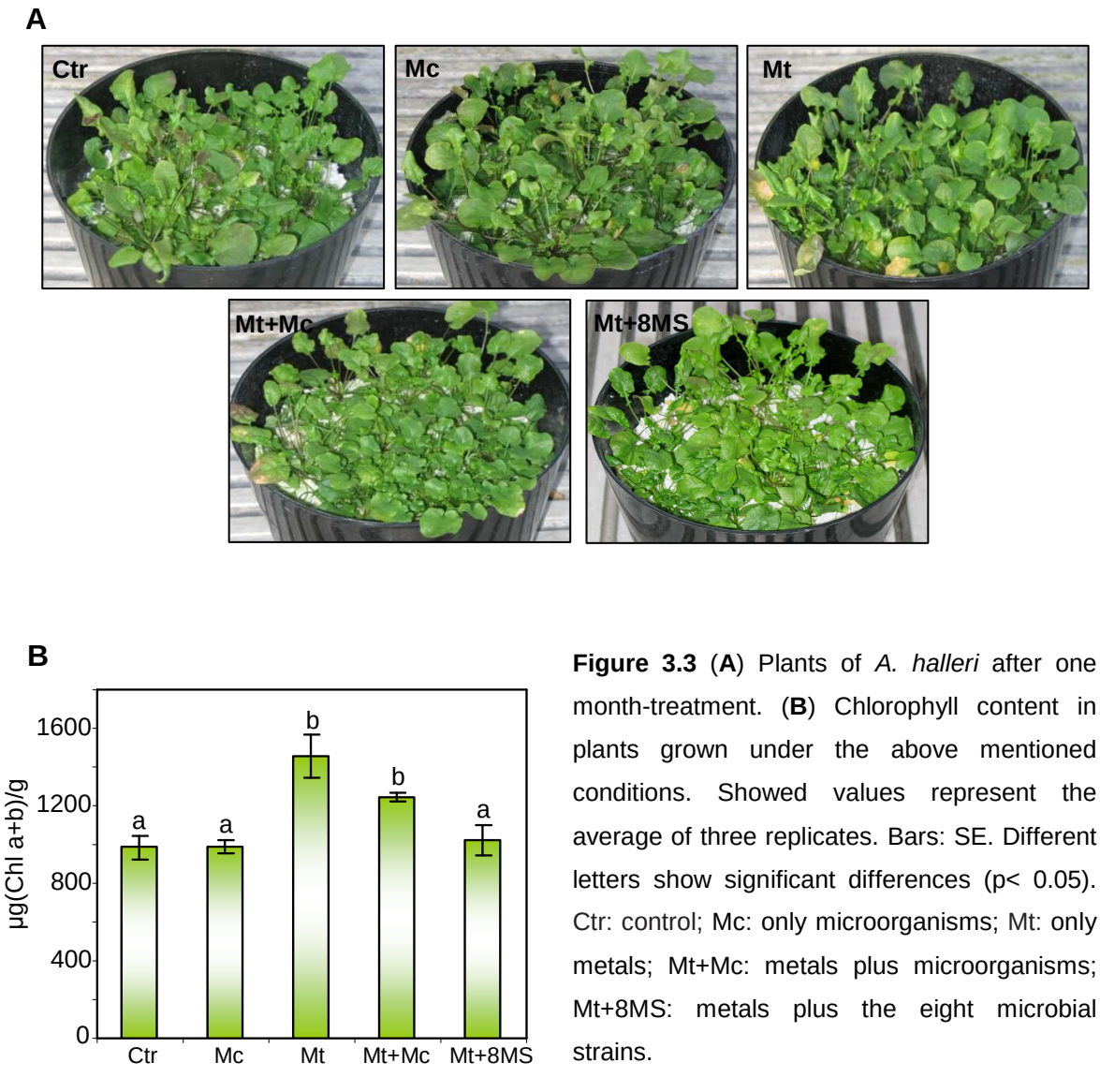


Figure 3.3 (**A**) Plants of *A. halleri* after one month-treatment. (**B**) Chlorophyll content in plants grown under the above mentioned conditions. Showed values represent the average of three replicates. Bars: SE. Different letters show significant differences ($p < 0.05$). Ctr: control; Mc: only microorganisms; Mt: only metals; Mt+Mc: metals plus microorganisms; Mt+8MS: metals plus the eight microbial strains.

Plants were harvested, roots were separated from shoots and the latter, content of Cd and Zn was measured. In shoots of control plants and of plants treated with only the rhizosphere microbial community, Cd was undetectable (Figure 3.4 A), whereas the concentration of Zn increased in dry biomass in plants treated with only the microbial population of *A. halleri* rhizosphere (Figure 3.4 B). As expected, in shoot dry biomass both Cd and Zn contents increased after exposure to 1.0 mM $CdSO_4$ and 10 mM $ZnSO_4$. Likewise, a further substantial metal accumulation increment was observed in plants treated with 1.0 mM $CdSO_4$ and 10 mM $ZnSO_4$ together with the *A. halleri* rhizosphere microbial community (Figure 3.4 A and B). On the contrary, plants treated with heavy metals plus the eight microbial strains accumulated in shoot a lower amount of Cd and Zn than plants treated with only heavy metals and plants treated with heavy metals plus the rhizospheric cenosis (Figure 3.4 A and B).

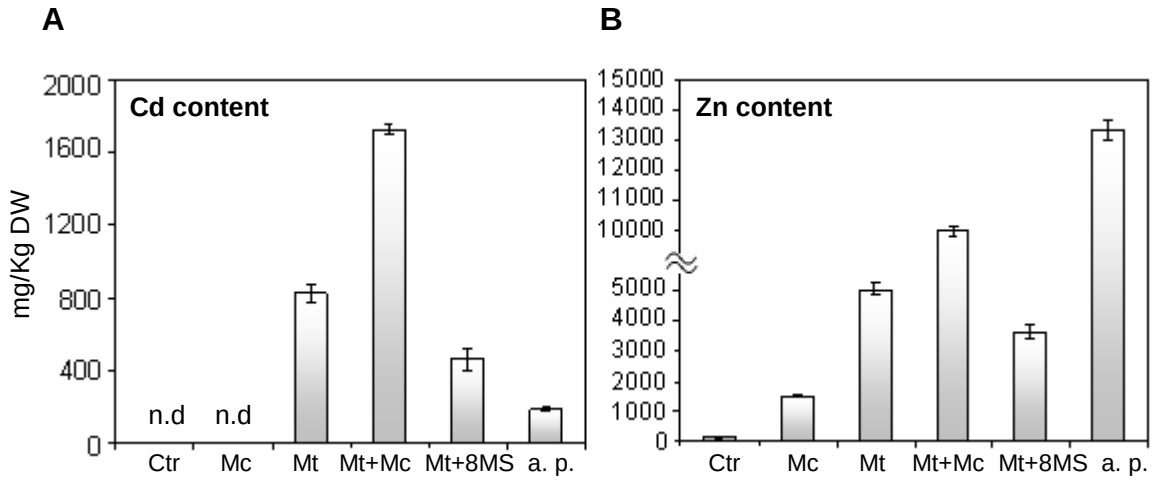
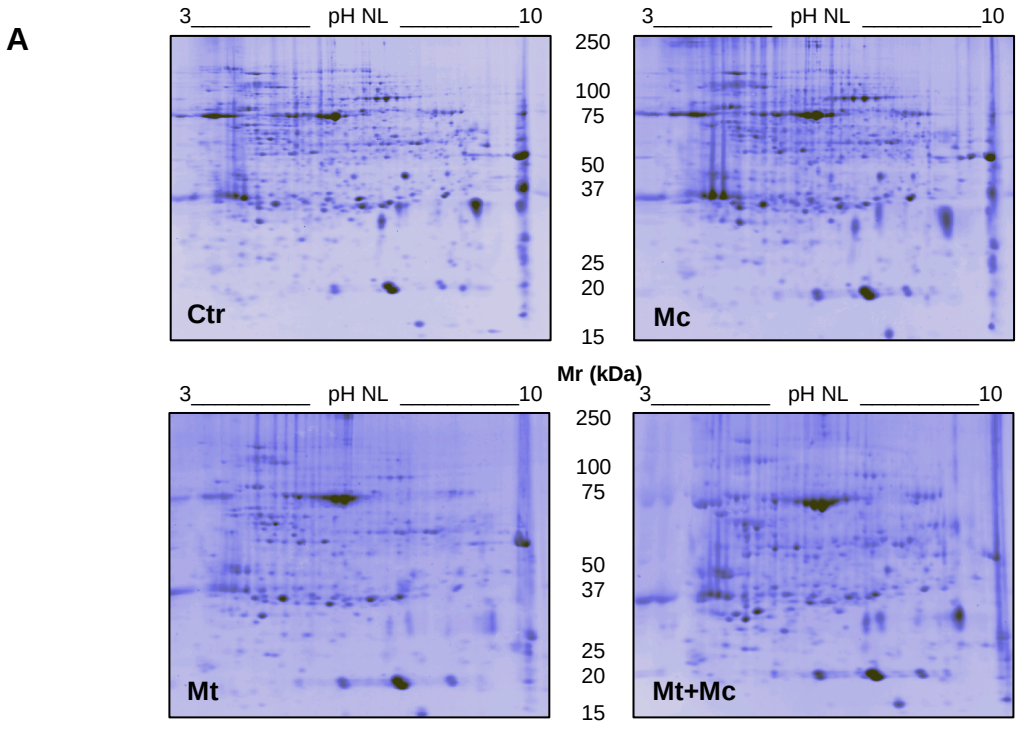


Figure 3.4 (**A**) Cd concentration and (**B**) Zn concentration in shoots of *A. halleri* plants after different treatments. Ctr: control; Mc: only microorganisms; Mt: only metals; Mt+Mc: metals plus microorganisms; Mt+8MS: metals plus the eight microbial strains. a.p.: autochthonous *A. halleri* population. n.d.: not detectable. Showed values represent the average of ten plants per three replicates. Bars: SE.

3.4 - Proteomic analysis of differentially expressed proteins

Although roots are the first site of the interaction with rhizosphere microorganisms as well as of the exposure to toxic metals, proteomic analysis was carried out on shoot tissues. This finds its rationale considering the biotechnological application of the molecular mechanisms that govern heavy metals accumulation in the above ground part in *A. halleri*, and their adaptation to rhizosphere microbe cenosis. It is important to underline that in this proteomic analysis it was focused the attention on the influence of the total microbial community of *A. halleri* rhizosphere on metal uptake and translocation to the shoots in this species.

In order to maximize the number of identifiable significant changes in the shoot proteome under the chosen conditions (Ctr, Mt, Mc, Mt+Mc), 2D-gels were performed using a large gel format with a 3-to-10 pH gradient for the first dimension. Separated protein spots were visualized on 2D-gels by Sypro Ruby staining, which allows good reproducibility and protein spot quantification for comparison among samples (see paragraph 2.10 section Materials and Methods). To highlight the effects of different treatments, representative gels for each sample were performed in five replicates and variations on spot intensity were confirmed by statistical analysis. 2D-maps for the four conditions together with the master map obtained by PDQuest analysis and corresponding to the sum of the spots visualized in the different gels, are reported in Figure 3.5 A and B. The average number of spots detected was 573 (± 10), 444 (± 70), 384 (± 9), and 387 (± 29), for 2D-maps of Ctr, Mc, Mt, and Mt+Mc respectively. A total of 78 different spots (matched across all the 2D-replica maps) were excised from gels and analyzed by MS/MS. Consequently, 57 protein spots (marked in Figure 3.5 B) were unambiguously identified as up- or down-regulated ($p < 0.05$) during the different treatments and they have been assigned to 39 proteins (Table 3.4).



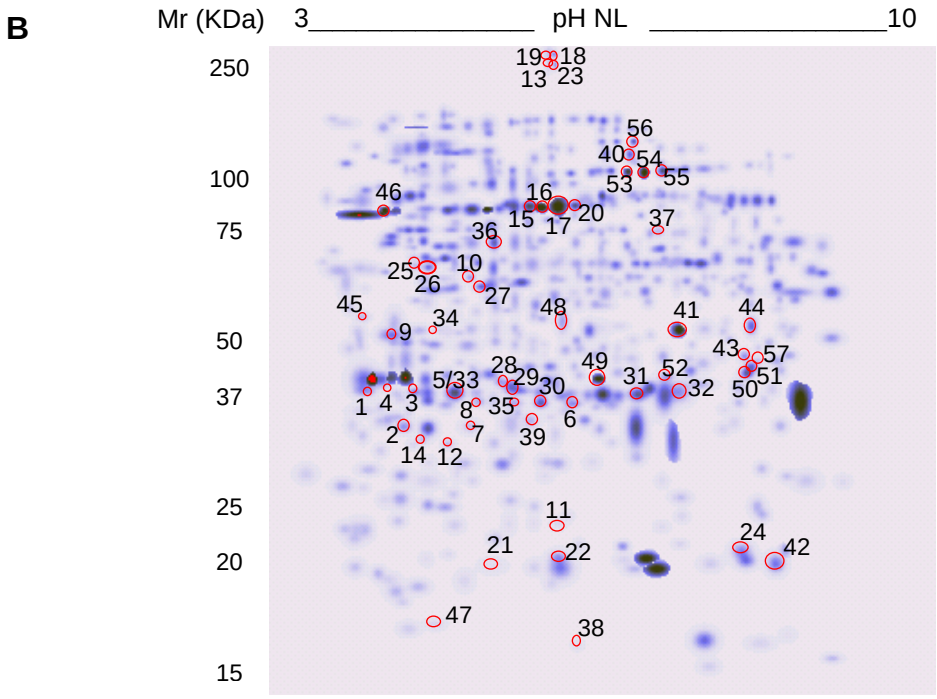


Figure 3.5 (**A**) 2D-maps of proteins extracted from shoots of *A. halleri* plants grown at the different growth conditions. For each condition, five replicates were performed. Ctr: control; Mc: only microorganisms; Mt: only metals; Mt+Mc: metals plus microorganisms. (**B**) Master map of the 2D-gels showing the protein spots revealing statistically significant changes in density between tested conditions.

3.5 - Bioinformatic analysis of the identified proteins

Functional annotation of the identified and differential expressed proteins was carried out subdividing proteins in groups based on functional categories. Of the 39 identified proteins, 43.6% are involved in photosynthesis, 33.3% were grouped as stress-responsive proteins, 18% in cellular metabolism and only 5.1% corresponded to unknown proteins (Table 3.4).

No	SSP	Protein Name	AC number (gi NCBI) and reference organism	MW (kDa)/pI Theoretical	Peptide count	Mascot score	Fold variations[3]				
							Mt *vs* Ctr	Mc *vs* Ctr	Mt+Mc *vs* Ctr	Mt+Mc *vs* Mt	Mt+Mc *vs* Mc
Photosynthesis – Light Dependent Reactions											
1	0206	Light harvesting chlorophyll a/b binding protein	gi|53627228 *Picea sitchensis*	26707/5,37	5	189			↑ 3.4		
2	1105	LHCA1	At3g54890 *A. thaliana*	25966/6,52	4	121			↑ 2.1		
3	1204	PSBP1*	At1g06680 *A. thaliana*	28078/6,90	11	445	↑ 3.9		↑ 3.0		
4	1209	PSBP1	At1g06680 *A. thaliana*	28078/6,90	6	149	↑ 2.3			Nd[1]	
5	2204 b	PSBP1	At1g06680 *A. thaliana*	20078/6,90	10	369	↑ 2.1				
6	5202	PSBP1	At1g06680 *A. thaliana*	28078/6,90	4	83			↑ 1.6		
7	2105	PPD1 (PSBP1-like)	At4g15510 *A. thaliana*	32245/8,92	9	355			↑ 2.0		
8	3203	PPD1 (PSBP1-like)	At4g15510 *A. thaliana*	32245/8,92	6	188	↑ 2.4			↓ 2.5	
9	1302	PSBO2 *	At3g50820 *A. thaliana*	34998/5,92	9	388		↑ 2.2	↑ 2.6		
10	2407	HCF136	At5g23120 *A. thaliana*	44076/6,79	9	417	↑ 2.3				
11	4006	Rieske Protein *	At4g03280 *A. thaliana*	24334/8,80	2	88				↑>10	
12	2103	δ-subunit plastidic ATPase *	At4g09650 *A. thaliana*	25653/9,04	7	346			↑ 2.0		
Photosynthesis – Light Independent Reactions											
13	4910	Rubisco - Large Subunit	AtCg00490 *A. thaliana*	52923/5,88	5	203	↑ 8.0				
14	1106	Rubisco - Large Subunit	AtCg00490 *A. thaliana*	52922/5,88	5	148	↑ 4.7				↑ 4.2
15	4603	Rubisco - Large Subunit	ArhiCp028 *Arabis hirsuta*	52933/5,96	15	549			↑ 2.3		↑ 2.3
16	4611	Rubisco - Large Subunit	ArhiCp028 *Arabis hirsuta*	52933/5,96	15	614	↑ 3.6	↑ 2.0	↑ 5.1		↑ 2.5
17	4612	Rubisco - Large Subunit	ArhiCp028 *Arabis hirsuta*	52933/5,96	13	591			↑ 2.7		
18	4907	Rubisco - Large Subunit	ArhiCp028 *Arabis hirsuta*	52933/5,96	11	359	↑ 6.1		↑>10	↑ 4.6	↑>10

19	4908	Rubisco - Large Subunit	ArhiCp028 *Arabis hirsuta*	52933/5,96	9	325	↑ 5.0				↑>10
20	5704	Rubisco - Large Subunit	ArhiCp028 *Arabis hirsuta*	52933/5,96	19	761	↑ 3.2		↑ 3.4		
21	3008	Rubisco - Small Subunit	At5g38430 *A. thaliana*	20273/7,59	4	160		↑ 3.2			
22	4001	Rubisco - Small Subunit	gi|406727 *Brassica napus*	20178/8,24	4	95	↑ 2.3	↑ 2.3	↑ 3.2		
23	4904	Rubisco - Small Subunit	gi|336456 *Batis maritima*	53510/6,09	5	198	↑ 8.5				
24	8001	Rubisco - Small Subunit	At1g67090 *A. thaliana*	20448/7,59	6	253	↑ 2.7		↑ 2.1		
25	1508	Phospho-Ribulo Kinase	At1g32060 *A. thaliana*	44436/5,71	18	560			↑ 2.2		
26	2501	Phospho-Ribulo Kinase	At1g32060 *A. thaliana*	44436/5,71	17	678			↑ 2.3	↑ 2.3	↑ 3.9
27	3401	Fructose-bisphosphate aldolase	At4g38970 *A. thaliana*	42961/6,78	6	215			↑ 1.9		
				Stress Response							
28	3205	AtGSTF10	At2g30870 *A. thaliana*	24215/5,49	8	363	↑ 2.8				
29	3206	AtGSTF10	At2g30870 *A. thaliana*	24215/5,49	8	302					↑ 2.6
30	4202	AtGSTF10	At2g30870 *A. thaliana*	24215/5,49	9	317		↓ 4.1			
31	6204	AtGSTF2	At4g02520 *A. thaliana*	24036/6,08	4	123		↓ 2.1			
32	7203	AtGSTF2	At4g02520 *A. thaliana*	24036/6,08	4	102	↓ 3.3	↓<10	↓<10		
33	2204 a	Chaperonin 20	At5g20720 *A. thaliana*	26785/8,86	11	572	↑ 2.1				
34	2308	Glyoxalase I	At1g67280 *A. thaliana*	29404/5,14	6	210	↑ 2.7				
35	3207	Fe-Superoxide Dismutase (FSD1)	At4g25100 *A. thaliana*	25409/6,30	1	66	↑ 2.2				
36	3504	Monodehydroascorbate reductase*	At5g03630 *A. thaliana*	47450/5,24	16	496	↓ 2.1				
37	7601	Hydroxypyruvate reductase	At1g68010 *A. thaliana*	42221/6,68	6	240			↑ 3.1		
38	5003	Malate dehydrogenase	At1g04410 *A. thaliana*	35548/6,11	3	97		↑ 3.5	↑ 2.7	↓ 1.7	
39	4101	Allene Oxide Cyclase	At3g25770 *A. thaliana*	21199/5,65	6	247					↓ 3.7
40	6804	TGG2	At5g25980 *A. thaliana*	61314/7,11	7	314	↓<10				

41	7307	Endochitinase (Class I)	At3g12500 *A. thaliana*	32004/5,93	5	156	↓ 4.7		↓ 2.9		
42	8005	Endochitinase (Class I)	At3g12500 *A. thaliana*	32004/5,93	3	133		↓ 3.2	↓ 4.2		
43	8301	Endochitinase (Class I)	At3g12500 *A. thaliana*	36091/8,05	3	181	Nd				
44	8302	Porin	At3g01280 *A. thaliana*	29407/8,77	2	125				↓ 2.1	
			Cellular Metabolism and Unknown Proteins								
45	0302	Fibrillin	At4g04020 *A. thaliana*	34927/5,45	11	556	↑ 2.0				
46	1711	Tubulin 5	At1g20010 *A. thaliana*	50310/4,66	7	451			↓ 2.4		
47	2005	Unknown Protein	At5g22580 *A. thaliana*	12341/5,42	2	97				↓3.3	↓ 2.0
48	5301	Unknown Protein (belonging to the Cyclase Family)	At4g34180 *A. thaliana*	28367/5,99	4	105					↓ 2.4
49	5209	VSP2	At5g24770 *A. thaliana*	29806/6,17	6	239	↓<10		↓ 7.7		↓ 4.5
50	8202	VSP2	At5g24770 *A. thaliana*	29806/6,17	6	281		↑ 2.8			↓ 2.9
51	8210	VSP2	At5g24770 *A. thaliana*	29206/6,17	6	209					↓ 2.1
52	7202	VSP1	At5g24780 *A. thaliana*	30026/5,47	2	96		↑ 2.0		Ni[2]	
53	6712	β -glucosidase 1	At1g52400 *A. thaliana*	60462/6,89	11	383	↓ 2.6		↓ 2.5		↓ 2.8
54	6713	β -glucosidase 1	At1g52400 *A. thaliana*	60462/6,89	13	434	↓ 3.6		↓ 3.4		↓ 3.7
55	7702	β -glucosidase 1	At1g52400 *A. thaliana*	60462/6,89	7	228			↓ 3.2		
56	6806	Cobalamine Independer Methionine Synthase	At5g17920 *A. thaliana*	83941/6,02	13	776		↓ 2.0			
57	8310	NADH-cytochrome b5 reductase	At5g20080 *A. thaliana*	35964/8,76	7	131		Nd			

Table 3.4 [1]Not detected; [2]Newly induced; [3]Fold variations in spot abundance between the different treatments and the corresponding controls; * Proteins whose expression pattern have been confirmed by Western analyses.

The number of differentially expressed spots associated to photosynthesis-related proteins was clearly higher than the number of spots belonging to other categories (Table 3.4). A consistent up-regulation of proteins involved in photosynthetic processes was observed. This finding is in disagreement with what is usually observed in plants after heavy metal treatment: in fact, the first visible symptom of heavy metal toxicity is generally leaf chlorosis followed from a growth inhibition. On the contrary, in this work, members of the light harvesting complex (LHC, chlorophyll a/b binding protein) were up-regulated in plants treated with metals plus microorganisms. In addition, PSBP1 (PSII binding protein) and PPD1 (PSBP1-like), two PSBP subunits of photosystem II, resulted to be induced by Cd and Zn treatment and by these metals plus microorganisms. In the presence of only Cd and Zn also HCF136 protein, essential for assembly of the photosistem II reaction centre (Plücken *et al*., 2002), is up-regulated. A synergic effect of heavy metals plus rhizosphere microorganisms was found for the induction of the δ-subunit of plastidic ATPase and a clear effect of the rhizosphere microorganisms was observed for the up-regulation of PSBO2 (PSII subunit O-2) and Rieske proteins. Eight protein spots corresponding to the Rubisco large subunit showed a statistically significant up-regulation, particularly marked by the treatment with heavy metals. Similarly, Rubisco small subunit, identified in four spots, was induced by Cd and Zn treatment and by the addition of rhizosphere microorganisms. Finally, upon the treatment with metals and microorganisms, a phosphoribulokinase (PRK) and a fructose-bisphosphate aldolase, two enzymes of the Calvin cycle, resulted overexpressed.

Numerous proteins were included in the functional category of stress response (Table 3.4). For example, glutathione S-transferase (GTS) is generally considered to play a role in detoxification of endogenous and xenobiotic compounds. Among the members of the GST family, identified in the *A. thaliana* genome (Wagner *et al*., 2002), in this work the modulation of two GST proteins belonging to the phi class is reported. In particular, GSTF10 is up-regulated by heavy metals and is down-regulated by the treatment with

only rhizosphere microorganisms, whereas the expression of GSTF2 is repressed by both heavy metals and microorganisms. A protein identified as chaperonin 20 has also been detected, and its expression is increased by the addition of Cd and Zn to the nutrient solution. Treatment with these heavy metals caused an increment of glyoaxalase I and Fe-superoxide dismutase and a reduction of monodehydroascorbate reductase (MDAR). Moreover, it was found that the hydroxypyruvate reductase (HPR) enzyme increased in plant cultivated in the presence of heavy metals plus microorganisms. The allene oxide cyclase, an enzyme belonging to the jasmonate biosynthetic pathway, was down-regulated in plants treated with Cd and Zn plus rhizosphere microorganisms if compared to plants treated with only microorganisms. A malate dehydrogenase (MDH) showed to be induced mainly by rhizosphere microorganisms even if the reduction of its expression was observed in presence of heavy metals plus rhizosphere microorganisms when compared with only heavy metals. Endochitinases were also included in the group of proteins modulated by stress although there are many evidences for their role in general physiological and morphological processes (Kasprzewska, 2003). A general decreased expression of endochitinases was observed upon the addition of both heavy metals and rhizosphere microorganisms. Furthermore, a drastic reduction of β-thioglucoside glucohydrolase (TGG2) expression, a myrosinase enzyme that degrades glucosinolates to produce toxins that deter herbivorous, was observed upon treatment with Cd and Zn. Finally, a reduction of porin expression was detected in plants treated with metals plus microorganisms compared to plant treated solely with Cd and Zn.

Several proteins identified as differentially expressed, play a significant role in cellular metabolism (Table 3.4). For example, fibrillin, a plastid-located protein, was found to be up-regulated by Cd and Zn, whereas tubulin 5 showed a down-regulation in plants treated with heavy metals plus microorganisms compared to control plants. Furthermore, two vegetative storage proteins (VSP1 and VSP2) were identified in four spots as strongly

inhibited by heavy metal treatment but induced by the addition of the rhizosphere microbes to the nutrient solution. VSPs have been included in the functional category of cellular metabolism since their role as temporary storage of amino acids, accumulating to high abundance in various vegetative storage organs, and in defence against herbivore insects (Liu *et al*., 2005). Similarly, β-glucosidase I, an enzyme implicated in cellular metabolism and defence, was reduced upon treatment with Cd and Zn. Finally, the treatment with rhizosphere microorganisms caused a down-regulation of a cobalamine independent methionine synthase and of a NADH-cytochrome b5 reductase.

3.6 - Western analyses

For five candidate proteins (PSBP1, Rieske, δ-subunit of plastidic ATPase, PSBO2 and MDAR), the regulation of the expression level was further confirmed by immunoblot analysis (Figure 3.6).

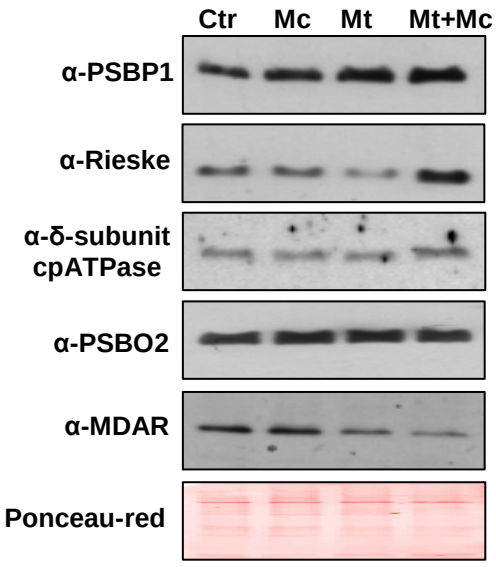


Figure 3.6 Western blot analysis showing the expression of five proteins identified as differentially expressed by proteome analysis. Each lane was loaded with 8 µg of total protein. Ponceau-red staining confirmed the equal loading in each lane.

The obtained signals underwent densitometric analysis (Figure 3.7). These investigations confirmed the expression pattern observed by 2D-analysis and provided further informations.

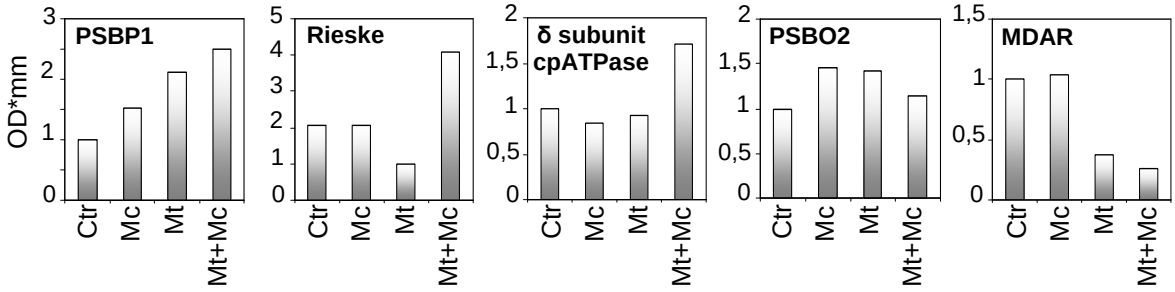


Figure 3.7 Fold changes of protein expression visualized as densitometric analysis of immunoblot signals**.**

4. DISCUSSION

Heavy metals are a class of pollutants deriving from industrial activity, agricultural practices and disposal of waste matter. They affect the metabolism of all types of organisms including plants and microorganisms. However, a great number of plant species has evolved an high tolerance to heavy metals. Tolerant plants are often excluders, limiting the entry and root-to-shoot translocation of heavy metals. Nevertheless, the important class of hyperaccumulator plants is able to combine an extremely high tolerance to a shoot accumulation of toxic elements. Consequently, hyperaccumulator plants have recently gained considerable interest because of their potential use in a phytoremediation approach (Pilon-Smits, 2005). Furthermore, hyperaccumulators constitute an exceptional biological material for understanding mechanisms regulating plant metal homeostasis as well as plant adaptation to extreme metallic environments. During the heavy metals hyperaccumulation process, it is important to underline the influence of the rhizosphere microbial population: this is under the influence of the plants which, in turn, are affected by soil microorganisms. Plants release photosynthesis-related compounds exploited as carbon sources by the microbial community (Olson *et al*., 2003) and, in return, rhizosphere microorganisms, through nitrogen fixation, phosphorous solubilization and phytohormone production, can promote plant growth and development (Dell'Amico *et al*., 2005). Moreover, rhizosphere microorganisms are able to antagonize soilborne plant pathogens through multiple mechanisms (Van Loon, 2007). Furthermore, it is known that plant uptake of mineral nutrients is facilitated by rhizosphere microorganisms. In particular, it has been observed that microorganisms derived from heavy metal contaminated sites stimulated metal uptake by plants (Salt *et al*., 1995), although in some cases they can prevent it (De la Fuente *et al*., 1997). However the exposure to heavy metals results in a gradual change in the quality and quantity of soil

microbial community with the development of tolerant population and loss of the more sensitive ones (Giller *et al*., 1998). In this study, a positive correlation between the presence of rhizosphere microorganisms and accumulation of Cd and Zn in the *A. halleri* shoots has been found. Compared to the native microbial population established in a heavy metal contaminated site (van Rossum *et al*., 2004), the rhizosphere microbial *consortium* was probably greatly changed during the experiment, due probably to the greenhouse culture conditions. However, the results of this work (see paragraph 3.3 section Results) suggest that populating the root zone with unselected microorganisms belonging to the autochthonous rhizosphere, is sufficient to enhance plant metal uptake and transport to the shoot. It has been proposed that the production of enzymes, siderophores, organic acids or biosurfactants by rhizosphere microorganisms can increase the soil metal mobility enhancing consequently the plant metal uptake (Braud *et al*., 2009). On the contrary, the massive *inoculum* with selected eight microbial strains, tolerant to Cd and Zn and isolated from rhizosphere of *A. halleri* plants grown on a contaminated soil, has caused a minor heavy metal accumulation (both Cd and Zn) in *A. halleri* above tissues. This phenomenon remains to be totally explained and the influence of these eight microbial strains on heavy metals uptake and translocation to the shoots is under investigation. However, several recent studies have demonstrated that negatively charged organic-acid functional groups present on bacterial cell walls can adsorb high concentrations of aqueous metal cations suggesting a key role in metal binding on microbial surface (Borrok and Fein, 2005). Thus, a first hypothesis is that these selected eight microbial strains could affect the plant metal uptake because of their biosorption capacity of metal ions on wall surface, influencing the availability of metals in the site. Usually, the mechanisms of biosorption may also involve processes like intracellular uptake and storage via active cationic transport systems or undefined systems (Gadd, 1990). For example, various microbial species, such as *Pseudomonas*, have been shown to be relatively efficient in the bioaccumulation of different heavy metals from

contaminated sites (Hussein *et al*., 2005). Although the most widespread heavy metal tolerance mechanism in resistance-microorganisms is the extrusion of metal ions from the cell by active transport, metal binding factors and enzymatic transformations (oxidations, reductions, methylation and demethylation) can also play significant roles as second lines of defence against toxic metals (Leedjärv *et al*., 2008). Some of these chemical modifications could be the reason of a alterated heavy metal mobility and the reduced uptake by *A. halleri* roots. For example, it was shown that the production of hydrogen sulphide, by the sulphate-reducing bacteria, can cause, for example, the precipitation of Cd(II) as the highly insoluble cadmium sulphide (CdS), minimizing thus its bioavailability (Sharma *et al*., 2000). All together these evidences could explain the minor Cd and Zn content in shoots of *A. halleri* plants treated with heavy metals plus the selected eight microbial strains if compared with contents observed in shoots of plants treated with only heavy metals or with the total community of rhizosphere. However, these preliminary results do not allow to speculate about the influence of these eight microbial strains on metal uptake and translocation to the shoots in *A. halleri* and therefore further molecular analyses are necessary. In particular, it is important to underline that even if these eight microbial strains were isolated from the total autochthonous microbial community of *A. halleri* rhizosphere, they had a dissimilar influence on heavy metal accumulation in this species if compared with the *inoculum* with the total microbial community. These conflicting observations might be due to the different title of microbial *inocula* for bioaugmentation tests. Furthermore, Cd and Zn concentration measured on shoots of the autochthonous *A. halleri* population confirmed the well-known metalliferous behaviour of this species.

Although proteomics is an important molecular tool for understanding biological systems, for example the metal hyperaccumulation physiology, there are few studies on stimulus-dependent change in plant proteome (Baginsky and Gruissem, 2006). Thus, to obtain insight into the effects of plant-microbe interactions on metal uptake and accumulation,

shoot proteomes of plants grown with or without rhizosphere microbes were compared. In this initial work, the attention was focused on the influence of the total rhizosphere microbial community and 2D-analysis was performed on total protein extract from *A. halleri* shoots without a protein fractionation and a subcellular localization of the gene product. From this molecular analysis, a consistent induction of proteins involved in the photosynthetic process was observed. It is worth to consider that one of the distinctive hallmarks of metal hyperaccumulator species is their ability to translocate most of the absorbed metals from the root to the shoot (Pilon-Smits, 2005). Conversely, non-accumulators, such as *A. thaliana*, normally sequester most of the uptaken heavy metals in roots or in stems of shoots, to avoid the excess toxic metals reaching the leaves, since photosynthesis is the most sensitive process negatively affected by heavy metals (Klein *et al*., 2008). When heavy metals do reach the leaves in non-accumulator species, photosynthesis typically decreases and plants show leaf chlorosis and reduced growth (Solti *et al*., 2008). In this study protein complexes responsible for light harvesting were overexpressed by metals and microorganisms treatments: these might be required for an enhanced energy demand of the entire cellular metabolism. Similarly, an increment of the complexes responsible for the electron transport (PSII and Cytb6/f) together with the ATP synthase was observed. It is in fact reasonable that an increased energy imput requires an enhanced downstream system to utilize ATP and reducing power, preventing thus the impasse of the photosynthetic chain. All these results could explain also the major chlorophyll content in shoots of *A. halleri* treated with heavy metals or with heavy metals plus rhizosphere microorganisms. Moreover, it was observed an increased expression of the Calvin cycle enzymes: the PRK, responsible for the regeneration of the ribulose 1,5-bisphosphate, the crucial substrate for Rubisco, and the fructose-biphosphate aldolase, catalyzing the condensation of the glyceraldehyde 3-phosphate with the dihydroxyacetone phosphate generating a molecule of fructose 1,6 biphosphate. According to these data an increased amount of Rubisco was detected mainly in metals and/or metals plus

microorganisms treated plants. In addition, it is well established that oxygenase activity is intrinsic to Rubisco and it is responsible for the formation of the 2-phosphoglycolate byproduct (Timm *et al*., 2008). The photorespiratory pathway represents a way to recover C atoms “wasted” by this side activity of Rubisco. The cycle requires the coordinated activity of chloroplasts, peroxisomes and mitochondria. In peroxisomes, HPR catalyses the last step of the pathway, reducing hydroxypyruvate to glycerate using NADH as electron donor. Reducing equivalents are indirectly imported into peroxisomes via the uptake of malate, subsequently oxidized to oxaloacetate by the MDH. Both HPR and MDH were significantly increased in heavy metals plus microorganisms treated plants and this could be due to the higher Rubisco content. In other words, it could be that higher amounts of Rubisco correspond to a higher rate of photorespiration that necessitates enhanced activity of HRP and MDH. Furthermore, a general increase in the thylakoid mechanisms could be related to the overexpression of fibrillin which doubled its expression level in metal-treated plants. Fibrillins are a component of plastoglobules, spherical structures found in all kind of plastids (Ytterberg *et al*., 2006). Plastoglobule organization is strictly correlated to stress phenomena that influence the lipid composition of thylakoid membranes, such as heavy metal stress (Simkin *et al*., 2007; DalCorso *et al*., 2008). It has been proposed that fibrillins could fulfill an indirect membrane-protection role influencing the formation and structure of plastoglobules. For instance, *in vitro* experiments showed that fibrillins counteract the inhibitory effects of uncouplers on plastidic-ATPase (Simkin *et al*., 2007). It was also reported that overexpression of a pepper fibrillin in *Nicotiana tabacum* conferred more robust growth under higher light intensities, together with an accumulation of plastoglobules (Rey *et al*., 2000). These data together suggest that the heavy metals hyperaccumulation process in shoots of *A. halleri* plants is extremely costly, it is thus reasonable that the plants can afford it by reinforcing the photosynthetic mechanism overexpressing proteins involved in photosynthesis

together with those components that act as scavengers of the unavoidable by-products that occur with such a massive energy use.

On the other hand, in this study it was observed that the addition of Cd and Zn caused a general down-regulation of proteins potentially involved in defence against herbivorous insects and pathogen attack. Several studies have indicated that metal hyperaccumulation within plant tissues has evolved as a self-protection against herbivorous insects or infection (Martens and Boyd, 1994; Boyd *et al*., 2002): in many plant *taxa* belonging to the *Brassicaceae* nickel hyperaccumulation could act as a plant-chemical defence (Martens and Boyd, 1994), inorganic and organic forms of selenium protect plants (e.g. *Brassica juncea*, *Medicago truncatula*) from herbivores (Freeman *et al*., 2007), Cd hyperaccumulation protects *Thlaspi caerulescens* from leaf feeding damage by thrips (Jiang *et al*., 2005) and Zn hyperaccumulation defends this species from caterpillars and slugs (Pollard and Baker, 1997). Moreover, it was also observed that Zn hyperaccumulation decreased the level of compounds related to plant defence mechanisms such as glucosinolates in shoots of *T*. *caerulescens* plants (Tolrà *et al*., 2001). Thus, this high metal concentration in shoots may be poison or act as deterrent against herbivores that can be adapted or not to high glucosinolates but are sensitive to high metal contents (Tolrà *et al*., 2001). In agreement with these data, in this work more than 10-folds inhibition of TGG2 was observed when plants were treated with heavy metals. This myrosinase enzyme breakdowns glucosinolates to produce a variety of degradation products toxic to herbivorous (Barth and Jander, 2006). When plants were treated with metals or with metals plus microorganisms a down-regulation of protein expression was also observed for two endochitinases of class I, involved in plant defense against pathogen attack and in general physiology and morphological processes (Kasprzewska, 2003). Treatment with Cd and Zn strongly inhibited VSPs expression that were conversely up-regulated by rhizosphere microorganisms. These proteins have been included in the functional category of cellular metabolism since they serve as temporary

amino acids stores during plant development, although it has been demonstrated their anti-insect acid phosphatase activity (Liu *et al*., 2005) and responsiveness to jasmonic acid (Matthes *et al*., 2008). Similarly, an inhibition of expression was detected for plant β-glucosidases when Cd and Zn alone or with rhizosphere microorganisms were added to the nutrient solution. These enzymes are involved in different biological processes, such as phytohormone activation, lignin synthesis and cell wall degradation, but an activity in defense mechanisms is also well documented (Mattiacci *et al*., 1995; Morant *et al*., 2008). Furthermore, the allene oxide cyclase, the first enzyme of the jasmonate biosynthetic, was down-regulated in plants treated with Cd and Zn plus rhizosphere microorganisms compared to plants treated with only microorganisms. Taken together, these results indicate that in shoots of plants treated with metals plus microorganisms, the complex signal networks that mediate plant defence responses against herbivores, insects and pathogen attacks is down-regulated. Effectively, metal uptake, transport and accumulation in shoots are energy-demanding processes, therefore if high metal concentration in shoots provides for a kind of protection system, other defence mechanisms are temporarily saved. These results also highlight a cross-talk between heavy metal signalling and defence signalling.

In contrast to response against biotic stress, an increased expression of protein involved in the response to abiotic stress was observed after treatments with heavy metals. Cd and Zn treatment caused an increase of Fe-superoxide dismutase, active in scavenging reactions that protect cells from the reactive oxigen species, inevitably formed during heavy metal stress (Romero-Puertas *et al*., 2004). Furthermore, in response to metals treatment, the glyoaxalase I is also up-regulated: indeed, it has recently been shown that this enzyme is overexpressed during salt stress and could mediate abiotic stress tolerance mechanisms (Singla-Pareek *et al*., 2006). This work also shows the modulation of two GST proteins belonging to the phi class. GSTF10 is up-regulated by heavy metals

and down-regulated by rhizosphere microorganisms, whereas the expression of GSTF2 is repressed by both heavy metals and microorganisms. The latter result was unexpected because GSTs are usually induced by heavy metals (Roth *et al*., 2006) therefore further investigation is required. In addition, a repression of MDAR was observed in heavy metals treated plants: indeed, it was reported that Cd exposure caused a reduction of ascorbate peroxidase (Michalak, 2006), responsible for the production of monodehydroascorbate, the substrate for MDAR activity.

In conclusion, this study indicates that the presence of a rhizosphere microbial population, adapted to heavy metal polluted sites, greatly enhances the accumulation of metals in shoots of the hyperaccumulator *A. halleri*. This suggests that metal hyperaccumulation in plant is an adaptive process requiring continuous interactions among co-occurring organisms (plant and rhizosphere microorganisms). Moreover, in plants, metal uptake, transport to the shoot and accumulation above tissues are energy-demanding processes and probably they are responsible for a general up-regulation of photosynthesis related protein. This increased energy requirement is counteract by thrift defence system, therefore if high metal concentration in shoots provides a kind of protection system, other defence mechanisms are temporarily saved, highlighting a cross-talk between heavy metal signalling and defence signalling.

Finally, future proteome analysis will focus on cellular pre-fractionation of protein extract to obtain insights into the regulation of low expressed proteins such as transcriptional factors, kinases, and transmembrane proteins of the pathways that lead to heavy metal hyperaccumulation.

CONCLUSIONS

The work reported in this PhD thesis was aimed to characterize some of the molecular components involved in heavy metal response in plants. In particular, with the purpose to obtain more information on the complex genetic network responsible for heavy metal accumulation and detoxification, the attention was focused on two important aspects: the role of transcription factors in response to heavy metal stress and the influence of microbial community of the rhizosphere on heavy metal uptake and translocation to the shoots.

- In **chapter I** the characterisation of *BjCdR15*, a *B. juncea* bZIP transcription factor involved in heavy metal tolerance and transport root-to-shoot in transgenic plants, has been described. The predicted cDNA full-length of *BjCdR15* was isolated from Cd-treated *B. juncea* plants by 5'- and 3'-RACE. *BjCdR15* sequence alignment showed an high sequence similarity with the *Arabidopsis TGA3* gene, coding a bZIP transcription factor. A role for BjCdR15 as a transcription factor was also deduced by its nuclear localization by protoplast transfection. Moreover, by means of northern and real-time PCR analyses, it was shown that *BjCdR15* transcription increases shortly in roots and shoots after Cd exposure and thereafter rapidly declines. Furthermore, *in situ* hybridization localized *BjCdR15* transcripts mainly in epidermal cells and in vascular system of roots and shoots of Cd-treated *B. juncea* plants. To verify whether BjCdR15 could be able to complement the function of TGA3 in the *Arabidopsis tga3* knockout mutant line and to test if its overexpression in transgenic plants influenced Cd tolerance and accumulation, BjCdR15 was overexpressed in *tga3-2* mutant and in *Arabidopsis* and tobacco WT plants. BjCdR15 overexpressing plants had a significantly higher Cd content in shoots than control plants. Moreover in presence of Cd, the BjCdR15 overexpressing plants grew better, showing an higher chlorophyll content and shoot fresh weight. Remarkably, it was observed that *tga3-2* shoots had a lower Cd content than all tested lines. However, the ectopic expression of BjCdR15 in *tga3-2* caused an increase of Cd transport to the shoot. As a

result 35S::*BjCdR15-tg3-2* plants showed the highest Cd content in shoots when compared to the other tests. A possible role of BjCdR15 in cellular mechanisms of Cd detoxification and transport to the shoot was therefore supposed. Moreover the expression of AtPCS1 was measured in the different transgenic lines to test the involvement of BjCdR15 in PCs synthesis and PC-Cd^{2+} complexation. The results obtained suggested that BjCdR15/TGA3 could be also involved in the regulation of PCS synthesis. Finally, to verify whether BjCdR15, and its putative orthologous TGA3, are involved in the control of the expression of genes responsible for root-to-shoot Cd transport and implicated in extrusion of Cd into the apoplastic and vacuolar compartments, the expression of some metal transporters was determined. Results confirmed that both genes are potentially involved, directly or indirectly, in the regulation of the expression of these membrane transporters.

- In **chapter II** a study, focused on the identification of genes involved in heavy metals tolerance and hyperaccumulation in *A. halleri* shoots in response to Cd and Zn and rhizosphere microorganisms, has been reported. In this research it was considered an ecotype of *A. halleri* (a well-known cadmium- and zinc-hyperaccumulator), adapted to grow on a contaminated site from heavy metals (Pb, Cd and Zn) in the North of France. Eight microbial strains, belonging to the autochthonous rhizosphere of *A. halleri* grown on polluted site, were isolated in axenic culture because of their ability to grow on Cd and Zn at the concentrations of 1 mM and 10 mM respectively. To obtain insight into the effects of plant-microbe interactions on metal uptake and accumulation, *A. halleri* plants were cultivated in Hoagland's nutritive solution in presence of heavy metals (Cd and Zn), with or without rhizosphere microbes, and in presence of the eight isolated microbial strains. At the end of the experiment, the total Cd and Zn content in shoots of plants subjected to different growth conditions was determined. The results confirmed that the total microbial community of the rhizosphere positively affects both the uptake and the root-to-shoot

transport of these heavy metals in *A. halleri*, whereas the *inoculum* with the eight selected microbial strains, tolerant to Cd and Zn, has caused a minor heavy metal accumulation of both heavy metals in the above ground tissues. Moreover, by a proteomic approach (2-DE), shoot proteomes of plants grown with or without rhizosphere microbes, and in presence of heavy metals, were compared and the differentially expressed proteins were identified. The 57 protein spots (up- or down-regulated) were classified in function of their biological activity: out of 39 identified proteins, 43.6 % are involved in photosynthesis, 33.3% were grouped as stress-responsive proteins, 18% in cellular metabolism and only 5.1% correspond to unknown proteins. This work could be a starting point to investigate which genes are responsible for Cd/Zn tolerance and hyperaccumulation in *A. halleri*, to well explain the molecular basis of phytoextraction, taking into account the influence of the rhizobacteria.

Future perspectives

A main aim will be the expression of *BjCdR15* under the control of *TGA3* native promoter, with the purpose of understanding the functional correlation between both genes and to confirm that BjCdR15 is really an orthologous of TGA3.

Furthermore, an other important aspect that will be consider is the molecular study of some of differentially expressed proteins in *A. halleri*, for a better understanding of the mechanisms of heavy metal tolerance and accumulation in this plant species.

Finally, the attention will be focused on the molecular study of the selected eight microbial strains tolerant to heavy metals. In particular, since these microorganisms can be considered a *reservoir* of important genes involved in detoxification of contaminants, the final goal of the project is the identification of some of these microbial genes, with the aim of transfer them into plants, obtaining thus plants with high phytoremediating efficiency.

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[2] **Farinati S**, DalCorso G, Varotto S, Fusco N, Furini A (2009). The *Brassica juncea* BjCdR15, an orthologous of *Arabidopsis* TGA3, is a regulator of cadmium uptake, transport and accumulation in shoots and confers cadmium tolerance in transgenic plants. *In prep*.

[3] DalCorso G, **Farinati S**, Maistri S, Furini A (2008). How plants cope with cadmium: staking all on metabolism and gene expression. *Journal of Integrative Plant Biology*. **50:** 1268-1280.

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· **Invited Review** ·

How Plants Cope with Cadmium: Staking All on Metabolism and Gene Expression

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Abstract

Environmental pollution is one of the major problems for human health. Toxic heavy metals are normally present as soil constituents or can also be spread out in the environment by human activity and agricultural techniques. Soil contamination by heavy metals as cadmium, highlights two main aspects: on one side they interfere with the life cycle of plants and therefore reduce crop yields, and on the other hand, once adsorbed and accumulated into the plant tissues, they enter the food chain poisoning animals and humans. Considering this point of view, understanding the mechanism by which plants handle heavy metal exposure, in particular cadmium stress, is a primary goal of plant-biotechnology research or plant breeders whose aim is to create plants that are able to recover high amounts of heavy metals, which can be used for phytoremediation, or identify crop varieties that do not accumulate toxic metal in grains or fruits. In this review we focus on the main symptoms of cadmium toxicity both on root apparatus and shoots. We elucidate the mechanisms that plants activate to prevent absorption or to detoxify toxic metal ions, such as synthesis of phytochelatins, metallothioneins and enzymes involved in stress response. Finally we consider new plant-biotechnology applications that can be applied for phytoremediation.

Key words: cadmium; heavy metals; metallothioneins; phytochelatins; phytoremediation; transporters.

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As sessile organisms plants have restricted mechanisms for stress avoidance and are subjected to environmental stresses that change growth conditions and alter (or sometimes disrupt) their metabolic homeostasis. Worldwide, these stresses are the most limiting factors for crop productivity: a large proportion of annual yield is lost due to pathogen attack and to unfavorable abiotic conditions such as drought, salinity and extreme temperatures. The average and record yields of many crops were compared in a classical study (Boyer 1982) and it was found that crop plants were reaching only 20% of their genetic yield potential. Diseases, insects and weeds contributed only in part, with the major yield reduction resulting from abiotic stresses. Therefore, understanding how plants cope with stresses and how, during the course of evolution, some plant species have acquired mechanisms of stress tolerance, will allow more stress-tolerant crops to be developed and significantly contribute towards increasing world food production to meet population growth requirements.

In recent decades, plant responses to stress conditions such as drought, salinity and temperature extremes have been the subject of intense molecular studies (Cushman and Bohnert 2000; Mittler 2006). Extensive expressed sequence tag (EST) collections and large scale EST sequencing initiatives for various crops have allowed these abiotic-stress genetic responses to be studied. By genetic engineering, the transfer of one or several structural genes controlled by a constitutive promoter has contributed towards protecting plants against environmental stresses (Smirnoff 1998). For instance, LEA (late embryogenesis abundant) proteins have been found in all plants in which they have been looked for and some are rapidly induced in vegetative tissues in response to water, cold or saline stress (Bies-Ethève et al. 2008). Their overexpression enhanced tolerance to salt and water stress (RoyChoudhury et al. 2007; Xiao et al. 2007). Improvement of freezing tolerance in transgenic plants has been achieved by expressing a

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cold-responsive (COR) gene of wheat (Shimamura et al. 2006). Furthermore, transcription factors play a crucial role in signal transduction pathways by controlling the expression of specific genes in response to environmental factors. Ectopic expression of the dehydration-responsive element (DRE)-binding protein DREB1A in *Arabidopsis* plants, resulted in improving freezing, salinity and drought tolerance and highlights the importance of regulatory controls for key stress-tolerance components (Kasuka et al. 1999). More recently, Nelson and co-workers demonstrated the potential of a transgene strategy for improving drought tolerance in crop plants (Nelson et al. 2007). They identified a transcription factor in *Arabidopsis* from the nuclear factor Y (NF) family, *AtNF-YB1*, that confers drought tolerance. A maize crop that constitutively expressed the orthologous gene *ZmNF-YB2* showed drought tolerance and improved yield under water-limited conditions.

Nowadays, plants also have to face rapid environmental changes mainly due to human activities causing air and soil pollution, acid precipitation, climate change etc. Plants thus need to adapt to changing environmental conditions in order to tolerate new stresses. Heavy metals, for instance, occur naturally in soils as rare elements. However, traffic, refuse dumping, and metal working industries contribute towards the spread of heavy metals in the environment. In agricultural soils, heavy metal pollution is an increasing problem due to soil amendment and the intense use of phosphate fertilizers that contain cadmium (Cd) as a contaminant (Polle and Schützendübel 2003). The latter is a highly toxic pollutant to prokaryotic and eukaryotic organisms also due to its solubility in water, which determines a rapid distribution in the environment. Uptake of Cd by crop plants is the main entry pathway into the food chain causing serious problems to human health (Buchet et al. 1990). Plants try to avoid Cd stress by preventing absorption; however the uptake depends on the soil metal concentration and pH. If taken up by plants, Cd is transported into the root by metal transporters or Ca channels (Perfus-Barbeoch et al. 2002). Within the cell Cd is detoxified preferentially by binding to S-containing ligands such as metallothioneins, glutathione and phytochelatins and the ligand-Cd complexes are most likely removed by sequestration from potentially sensitive organelles and structures (Cobbett 2000; Cobbett and Goldsbrough 2002; Clemens 2006). Nevertheless, exposure of plants to Cd stress may lead to the alteration of many cellular processes and structures (Hall 2002). Cd accumulation in plants causes reductions in photosynthesis, diminishes water and nutrient uptake (Sanità di Toppi and Gabbrielli 1999), inhibition of enzyme activities, disruption of cell transport processes, disturbance of cellular redox control (Clemens et al. 2001; Schützendübel and Polle 2002) and affects general root and shoot growth.

Cadmium is generally toxic to most plant species, and metallophytes in Cd-containing soils have evolved mechanisms of Cd exclusion by inhibiting its entry and hindering its transport to the above-ground tissue. However, a number of so-called hyperaccumulator plants, endemic to metal-rich soils, can accumulate Cd in their aerial organs to a level that is orders of magnitude higher than that normally found in plants, without showing any sign of phytotoxicity (Baker and Brooks 1989). The complex mechanisms evolved in these plants either to prevent Cd uptake or to enable metal extraction from soils, trafficking, allocation and cellular detoxification are research areas that have attracted the attention of many investigators. Indeed, to identify the genetic and physiological mechanisms of plants that are able to avoid Cd stress by preventing its absorption or translocation is needed to prevent Cd entering the food chain. Likewise, understanding the mechanism of Cd accumulation in the vegetative parts of hyperaccumulator plants is crucial to the promising approach of using plants as ecological remediation of Cd polluted environments (phytoremediation). In this review we have chosen to focus on plant mechanisms that allow Cd detoxification and absorption prevention. We will also discuss the potential biotechnological application in phytoextraction of Cd from polluted sites.

Cd Uptake and Transport

Higher plants can uptake Cd, depending on its availability and concentration, in soil or water; rather little is taken up directly from the atmosphere (Clemens 2006). Soil pH, the rhizosphere and presence of organic acids modulate the bio-availability of Cd (as well as of other heavy metals) for plant uptake (Benavides et al. 2005). For instance, it has been reported that Cd uptake in corn was lower in acid soils with high organic matter content (Benavides et al. 2005). The concentration of other nutrient elements (e.g. Ca, Zn and Fe) in the soil also influences Cd absorption; it has been shown that addition of Ca or Zn diminishes the Cd uptake (Cosio et al. 2004).

Since membrane potential, which might exceed −200 mV in root epidermal cells, provides a strong driving force for the uptake of cations (Benavides et al. 2005), toxic heavy metals compete with and gain access into the plant cell via the transport systems operating for micronutrient uptake: in particular, the uptake of Cd ions occurs via the same transmembrane carriers used to uptake Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+} (Roth et al. 2006; Papoyan et al. 2007). Due to its high mobility and water solubility, Cd readily enters the roots through the cortical tissue and can reach the xylem via an apoplastic and/or symplastic pathway, complexed to organic acids or phytochelatins (Salt et al. 1995). Once loaded into the tracheary elements, Cd complexes spread throughout the entire plant following the water stream. It has been hypothesized that Cd accumulation in developing fruits could occur via phloem-mediated transport, implicating a systemic diffusion of the heavy metals into the plant body (Benavides et al. 2005).

Cd Toxicity in Plants

Together with Ag, As, Hg, Pb and Sb, Cd is considered a non-nutrient element, since it has no known function in plant development and life, with the exception of the Cd-carbonic anhydrase of marine diatoms (Lane and Morel 2000). Furthermore, these heavy metals seem to be more or less toxic to eukaryotic organisms and microorganisms (Sanità di Toppi and Gabbrielli 1999; Benavides et al. 2005). The large majority of studies have been based on the application of extremely high Cd concentrations and so the consequences of acute Cd stress are well-documented. In higher plants, Cd negatively affects both plant growth and development, resulting in stunting and eventually plant death. The critical concentration, at which the metal causes injuries in plant physiology is in the range of 3 to 10 mg/kg dry mass (Bahlsberg-Pahlson 1989).

The bases of Cd toxicity are still not completely understood, but it might result from its high affinity for sulfydryls (e.g. threefold higher than Cu ions, Schützendübel and Polle 2002). Cd, binding to sulfydryl groups of structural proteins and enzymes, leads to misfolding, inhibition of activity and/or interference with redox-enzymatic regulation (Hall 2002). Another important toxicity mechanism is due to the chemical similarity between Cd^{2+} and functionally active ions situated in active sites of enzymes and signaling components. Thus, Cd^{2+} ions can interfere with homeostatic pathways for essential metal ions (Roth et al. 2006) and the displacing of divalent cations, such as Zn and Fe, from proteins could cause the release of "free" ions, which might trigger oxidative injuries via free Fe/Cu-catalyzed Fenton reaction (Polle and Schützendübel 2003).

It has to be noticed that *in vivo* Cd-related injuries on plants depend first on the plant species: hyperaccumulators or genetically resistant plants activate cellular mechanisms that weaken the impairment due to Cd stress. Moreover, time of Cd exposure and its magnitude together with external environmental conditions, contribute to modulating plant sensitivity to heavy metals (Sanità di Toppi and Gabbrielli 1999).

Photosynthesis and carbon assimilation

The most evident symptoms of Cd toxicity are leaf roll and chlorosis, water uptake imbalance and stomatal closure (Clemens 2006). Chlorosis might be due to changes in Fe : Zn ratio caused by Cd, as in corn leaves (Root et al. 1975) and to the negative effects on chlorophyll metabolism (Chaffei et al. 2004). At the cellular level, Cd damages the photosynthetic apparatus, particularly the light harvesting complex II and the two photosystems and causes a decrease in chlorophyll and carotenoid content (Figure 1A), leading to higher non-photochemical quenching (Sanità di Toppi and Gabbrielli 1999). Regarding stomatal closure, it has been shown that during Cd exposure, stomata close independently of water status. Stomatal closure can be actively driven by abscisic acid (ABA)-induced Ca^{2+} accumulation in the cytosol of the guard cells. The increase in cytosolic free Ca^{2+} promotes opening of plasma membrane anion and K^{+}_{out} channels. As more ions leave the cell, water follows and turgor is lost, with stomatal pore closure (MacRobbie and Kurup 2007). Being chemically similar to Ca ions, Cd probably enters guard cells through voltage-dependent Ca^{2+} channels and once in the cytosol, it mimics Ca^{2+} activity (Perfus-Barbeoch et al. 2002).

All together, stomatal closure, damage to the photosynthetic machinery and interference with pigment synthesis, cause a general depression of the photosynthetic efficiency lowering the effective quantum yield. Moreover, by inhibiting enzymes involved in CO_2 fixation, Cd decreases carbon assimilation (Perfus-Barbeoch et al. 2002).

Effects on nutrient uptake and root physiology

Cadmium, as do other heavy metals, imbalances the water uptake and nutrient metabolism (uptake, transport and use) at the root level interfering with the uptake of Ca, Mg, K and P (Benavides et al. 2005). The inhibition of the root Fe(III) reductase induced by Cd leads to a Fe(II) deficiency in cucumber and sugarbeet (Alcantara et al. 1994).

In different plant species (e.g. tomato, maize, pea and barley) Cd alters the activity of different enzymes involved in nitrogen metabolism (Nussbaum et al. 1988; Boussama et al. 1999). At the root level, the reduction of nitrate absorption may be due to transpiration inhibition. Moreover, both the nitrate reductase and nitrite reductase activity in roots and leaves are affected (Chaffei et al. 2004) as well as nitrate transport from roots to shoots (Sanità di Toppi and Gabbrielli 1999) leading to a reduced nitrate assimilation by the whole plant (Figure 1A). The activity of the enzymes responsible for the incorporation of ammonium molecules into the carbon skeleton (i.e. glutamine and glutamate synthetase) is also compromised (Chaffei et al. 2004). On the other hand, the activity of the glutamate dehydrogenase (GDH) is enhanced during Cd-stress (Boussama et al. 1999). Because high activity of GDH enzyme has been related with pathogen response and senescence induction (Osuji and Madu 1996; Masclaux et al. 2000) and changes in nitrogen metabolism due to Cd stress are similar to the ones induced during senescence, it has been hypothesized that Cd induces senescence-like symptoms at least in tomato, leading to nitrogen mobilization and a storage strategy (Chaffei et al. 2004).

Regarding sulfur metabolism, exposure to Cd induces a remarkable increase in the amount of thiol compounds, with a concomitant decrease in the activity of leaf Adenosine Triphosphate (ATP)-sulfurylase and O-acetylserine sulfurylase (Figure 1A), the first and the last enzymes involved in the sulfate assimilation pathway (Astolfi et al. 2004). It is noticeable that also nitrogen

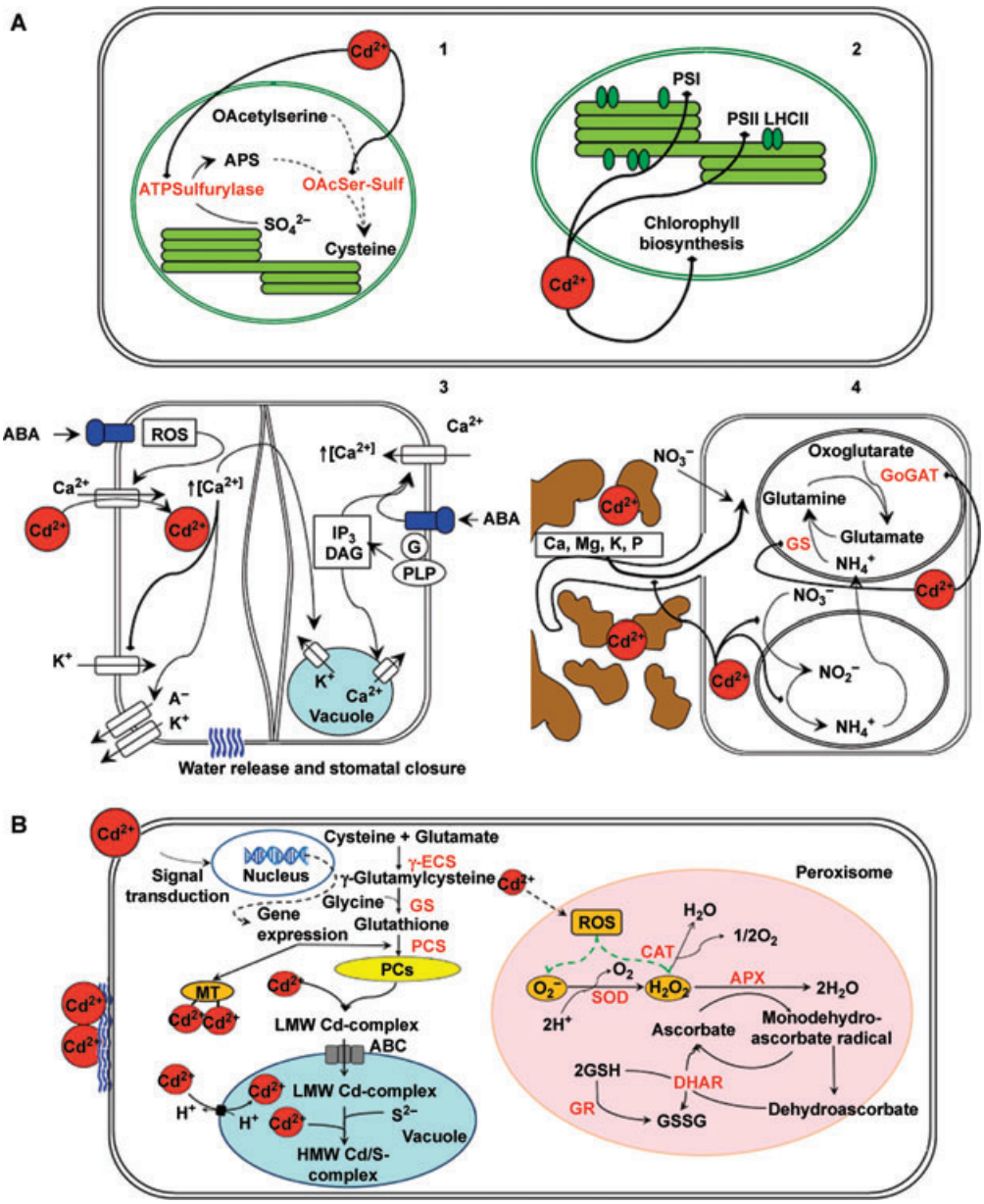

Figure 1. Main effects of Cd in plant physiology and plant responses to Cd stress.

(A) Effects of Cd on shoot cells: after uptake into the cell (through a still unidentified metal transporter) Cd inhibits sulfur metabolism **(1)** photosynthesis and chlorophyll biosynthesis **(2)**. **(3)** Mimicking Ca^{2+} ions, Cd enters stomatal guard-cell and activates the opening of the plasma membrane anion and K^{+}_{out} channels. As more ions leave the cell, water follows and turgor is lost, with stomatal pore closure (see the text for further explanation). **(4)** Cd induces inhibition of root enzymes involved in nitrogen assimilation: both nitrate and nitrite reductase activity are inhibited. Moreover, Cd interferes with activity of both GS and GOGAT enzymes, involved in ammonium assimilation.

(B) Constituents of the cell wall can immobilize Cd ions preventing cytosolic uptake. Once Cd enters the cytosol, it stimulates the synthesis of phytochelatins and probably metallothioneins. After complexation with Cd, the low-molecular-weight (LMW) complex enters the vacuole via a tonoplast-localized ATP-binding-cassette (ABC) transporter and further complex in high-molecular-weights (HMWs). On the right, particular of the Cd-induced activation of ROS-scavenging cycle taking place mainly in peroxisomes.

ABA, abscissic acid; APX, ascorbate peroxidase; CAT, catalase; DAG, diacylglycerol; DHAR, dehydro-ascorbate reductase; G, G-proteins, activating PLP enzyme; GOGAT, glutamate synthase; GR, glutathione reductase; GS, glutamine synthase; LHCII, light harvesting complex II; MT, metallothioneins; PCs, phytochelatins; PCS, phytochelatin synthetase; PLP, phospholipase protein, involved in DAG and IP_3 mediated signaling; PSI, photosystem I; ROS, reactive oxygen species; IP_3, inositol-3-phosphate; SOD, supeoxide dismutase; γ-ECS, γ-glutamylcysteine synthetase.

fixation and primary ammonia assimilation decreased in nodules of soybean plants during Cd treatments (Balestrasse et al. 2003).

Phenotypically, Cd exposure inhibits root growth and lateral root formation while it induces differentiation of numerous root hairs both in *Arabidopsis* and tobacco plants (S Farinati et al., unpubl. data, 2008). In tomato, Cd-treated roots were thicker and stronger and the root biomass was less affected than the leaves (Chaffei et al. 2004).

Symptoms on cellular homeostasis

In different plant species, such as bean and wheat, cytotoxicity of Cd exposure appears as chromosomal aberrations and inhibition of mitotic processes with consequent altered cell cycle and division (Benavides et al. 2005). Furthermore, Cd causes high mutation rates in *Arabidopsis thaliana*, floral anomalies (banding), poor seed production and malformed embryos (Ernst et al. 2008). It also induces vacuolization and mitochondrial degeneration (Silverberg 1976) affecting cell metabolism and aerobic respiration.

Although Cd does not participate directly in cellular redox reactions (i.e. Cd ions do not alter their oxidation state, since they do not take part in Fenton and Haber-Weiss reactions, Clemens 2006), its exposure drives oxidative injuries, such as lipid peroxidation, which leads to alteration in the membranes functionality, and protein carbonylation (Schützendübel et al. 2001; Romero-Puertas et al. 2002) and converges into a general redox homeostasis impairment. Cd unbalances the activity of antioxidative enzymes and affects catalase and super-oxide dismutase (SOD) activity triggering H_2O_2 and O_2^- (reactive oxygen species, ROS) over-accumulation (Romero-Puertas et al. 2004). It is still not clear if the over-production of ROS during Cd treatment is the cause of redox cellular imbalance or if this is a specific stress mechanism activated by the plant cell to cope with the heavy metal ions (Romero-Puertas et al. 2004). It was also shown that Cd induces peroxisome-senescence in leaves activating the glyoxylate cycle enzymes, malate synthase and isocitrate lyase, as well as peroxisomal peptidases, the latter being well-known as leaf senescence-associated factors (Chaffei et al. 2004).

Furthermore, Cd interferes with plasma membrane ion transporters and ATPase (Sanità di Toppi and Gabbrielli 1999) disturbing ion and metabolite movement and accumulation. In addition, Cd exposure inhibits the activity of metabolic enzymes such as glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, malic enzyme, isocitrate dehydrogenase, Rubisco and carbonic anhydrase (Sanità di Toppi and Gabbrielli 1999).

Cd effects on intracellular signaling

A secondary effect due to the accumulation of ROS in the cell compartments is the alteration of the signaling mediated by H_2O_2 and other oxygen species. It was widely accepted that H_2O_2 can play a role as signal molecule in triggering the induction of defense mechanisms against both abiotic stresses, such as temperature and ozone (Dat et al. 2000; Sharma et al. 1996) and pathogen attack as infections due to bacteria or powdery mildew fungi (Thordal-Christensen et al. 1997; Bestwick et al. 1998). Interfering with H_2O_2 accumulation, Cd meddles with the signal transduction pathways in which ROS are involved. Being chemically very similar to Zn^{2+}, Cd^{2+} ions can hamper the activity of Zn-finger transcription factors, substituting Zn ions and consequently interfering with transcription mechanisms (Sanità di Toppi and Gabbrielli 1999). With similar mechanisms, Cd^{2+} replace Ca^{2+} ions in calmodulin proteins, causing the perturbation of intracellular calcium level and altering the calcium-dependent signaling (Ghelis et al. 2000; Perfus-Barbeoch et al. 2002).

Plant Responses to Cd

Plants, like all other organisms, have evolved a complex network of homeostatic mechanisms to minimize the damages from exposure to nonessential metal ions. To avoid Cd toxicity, land plants developed active and passive strategies of exclusion of the heavy metal ion from the cellular environment. As first defense to Cd stress, plant exudates such as malate or citrate bind to metal ions in the soil matrix excluding them from the root absorption (Delhaize and Ryan 1995). Second, the cell wall (through pectic sites and hystidyl groups) and extracellular carbohydrates (callose, mucilage) can play a significant role in immobilizing toxic ions and preventing their uptake into the cytosol (Sanità di Toppi and Gabbrielli 1999). Nevertheless, as soon as the concentration of the toxic element rises above the physical adsorption limit of these barriers, active metabolism takes charge producing chelating compounds (phytochelatins and, in some cases, metallothioneins) involved in the detoxification and compartmentalization of the heavy metals in specific cellular compartments. Moreover, as for other abiotic stresses, Cd resistance involves the synthesis of stress-related proteins and signal molecules (heat shock proteins, salicylic and abscisic acids, ethylene) (Sanità di Toppi and Gabbrielli 1999). The signal transduction pathway is characterized by a complicated interaction of genes in which transcription factors have essential roles since regulation of their expression may strongly affect plant stress response (Uno et al. 2000). With the recent introduction of genomics technology it has been possible to identify numerous putative genes involved in response to Cd-stress: for example, several genes induced in *Brassica juncea* after Cd treatments were identified by cDNA Amplified Fragment Lenght Polimorfism (AFLP) (Fusco et al. 2005). Changes in the transcriptome of *Arabidopsis* plants exposed to Cd and Pb were studied by Affymetrix DNA array (Kovalchuk et al. 2005).

Cd induces modulation of gene expression

Responses to heavy metal stress depend on an intricate signal transduction pathway within the cell that begins with the sensing of heavy metal (or heavy metal associated symptoms) and converges in transcription regulation of metal-responsive genes (Singh et al. 2002). Still much remains unknown about the molecular components of the metal-induced signal transduction, and only recently thanks to differential-expression analyses has it been possible to identify transcription factors (TFs) putatively responsive to heavy metal stress (Fusco et al. 2005). As commonly found for other stress-related TFs, heavy metal responsive TFs also share the same signal transduction pathway and are therefore activated by abiotic stresses such as cold, dehydration, Salicylic Acid (SA) and H_2O_2 (Singh et al. 2002). In addition, cross-talk also exists between Cd tolerance mechanisms and pathogen defense signaling (Suzuki et al. 2001).

Cadmium affects the expression of ERF proteins that belong to the APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP) family. Members of these TFs can bind to several pathogenesis-related promoters and dehydration-responsive elements (DRE motif) (Singh et al. 2002). It has been shown that *ERF1* and *ERF2* genes are induced after 2 h of Cd-treatment in *A. thaliana* roots (Weber et al. 2006). Moreover, it has been reported that DREB2A is induced by Cd: DREB2A specifically interacts with the DRE motif in the promoter region of the rd29A and activates its transcription in Cd-exposed plants. Rd29A is already known to be induced by cold, salt and dehydration stresses (Suzuki et al. 2001). OBF5, a bZIP-type DNA binding protein, was shown to be Cd-induced: it binds to the promoter region of the gene coding for the glutathione S-transferase, an enzyme involved in ROS scavenging and xenobiotic detoxification (Suzuki et al. 2001). Furthermore, it has recently been demonstrated that the expression of BjCdR15, a bZIP protein identified in *B. juncea*, is induced after short Cd treatment (Fusco et al. 2005). This TF controls the expression of several metal transporters, is involved in long distance root-to-shoot Cd transportation and its overexpression in *A. thaliana* and tobacco plants enhances Cd tolerance and accumulation in the shoot (S Farinati et al., unpubl. data, 2008).

WRKY53, a TF belonging to the WRKY family, was isolated as being differentially expressed in Cd-treated *Thlaspi caerulescens* plants. This gene is also modulated by other environmental stresses such as salinity, drought, cold and salicylic acid and seems to participate in the stress-related signal transduction pathway regulating the activity of other TFs rather than directly activating gene expression (Wei et al. 2008).

MYeloBlastosis Protein (MYB) proteins, and in particular members of the R2R3 MYB subgroup also respond to heavy-metal stress: in *A. thaliana* MYB4 is more highly expressed after Cd and Zn-treatment (Van de Mortel et al. 2008), while MYB43, MYB48 and MYB124 proteins are specifically induced by Cd in roots (Weber et al. 2006). In *T. caerulescens* MYB28 is strongly expressed under Zn deficiency and high Cd concentrations and is involved in the regulation of glucosinolate (GSL) synthesis (Van de Mortel et al. 2008). GSL plays an important role as a storage form of sulfur and its biosynthesis responds to changes in nutritional status, biotic and abiotic stresses (Hirai et al. 2007). As already mentioned, Cd interferes with nutrient uptake and sulfur metabolism.

The modulation of TF belonging to different groups may indicate the complexity of the response of plants to Cd, from the signal perception to the intracellular transduction cascade triggering the activation of genes responsible for Cd uptake, transport and detoxification.

Phytochelatins

Cadmium can induce the synthesis of small metal-binding peptides defined as phytochelatins (PCs). PCs have the general structure $(\gamma\text{-Glu-Cys})_n\text{-X}$ where n is a variable number from 2 to 11 and X an amino acid such as Gly, β-Ala, Ser, Glu or Gln (Cobbett and Goldsbrough 2002). Due to the presence of the thiolic groups of Cys, PCs chelate Cd and form several complexes with molecular weight of about 2 500 or 3 600 Da, protecting the cytosol from free Cd ions (Cobbett 2000). Glutathione is the building block for PCs synthesis, which is catalyzed by the cytosolic PCs synthetase (PCS). It has been shown that PCS is constitutively expressed and post-translationally activated by heavy metals (Cobbett and Goldsbrough 2002).

Due to their metal ion affinity, PCs are supposed to play a role in cellular homeostasis and trafficking of essential nutrients such as Cu and Zn (Thumann et al. 1991) and they are required for detoxification of toxic metals, particularly to Cd, as confirmed in both *Arabidopsis* and *Schizosaccharomyces pombe*, by the Cd-sensitive phenotype of *cad1* mutants defective in PCS activity (Ha et al. 1999). Regardless, an excessive amount of PC does not confer, *per se*, any hyper-tolerance; indeed, although an enhanced PCs synthesis seems to increase heavy metals accumulation in transgenic plants (Pomponi et al. 2006), an excessive expression of *AtPCS* genes determines hypersensitivity to Cd stress (Lee et al. 2003). After synthesis, PCs bind the heavy metal ions and facilitate their transport as complexes into the vacuole (Clemens 2006) where they eventually form high-molecular-weight (HMW) complexes (Figure 1B). Several studies demonstrated that in *Arabidopsis* the transport of HMW complexes across the tonoplast is mediated by ATP-binding-cassette (ABC) transporters (Cobbett and Goldsbrough 2002). It was also reported that PCs play a role in Cd transport from root to shoot and it was demonstrated that a PCs-dependent "overflow protection mechanism" would contribute to keeping Cd accumulation low in the root, causing extra Cd transport to the shoot (Gong et al. 2003).

Metallothioneins

Metallothioneins (MTs) are other cysteine-rich peptides with a low molecular weight able to bind metal ions by means of mercaptide bonds. Differently from PCs, MTs are products of mRNA translation, induced in response to heavy metal stress (Cobbett and Goldsbrough 2002). MT proteins in vertebrates are characterized by a stretch of 20 Cys residues highly conserved, whereas plant and fungi isoforms do not contain this structure (Cherian and Chan 1993).

Regarding their metal binding activity, the pea MT (PsMTa) can bind Cd, Zn and Cu when expressed in *Escherichia coli* (Tommey et al. 1991). Moreover, *Arabidopsis* MTs restore tolerance to copper in MT-deficient yeast strains (Zhou and Goldsbrough 1994). Although the role of plant MTs in Cd tolerance is still almost unknown, there is some evidence that supports their participation in Cu homeostasis (Cobbett and Goldsbrough 2002). Moreover, overexpression of mouse MT in tobacco plants enhances Cd tolerance *in vitro* (Pan et al. 1994), whereas *Brassica juncea* MT2, ectopically expressed in *Arabidopsis thaliana*, confers increased tolerance to Cd and Cu (Zhigang et al. 2006).

In terms of transcript amount, many plant MT genes are expressed at very high levels in all tissues. *Arabidopsis* MT1a and MT2a seem to accumulate in trichomes, being involved in sequestration of heavy metal ions in these structures (Salt et al. 1995). Since *Arabidopsis* MT expression has been detected in phloem elements, a role in metal ion transport has been postulated (Garcia-Hernandez et al. 1998). Finally, MT genes are expressed during various stages of plant development and in response to different environmental conditions (Rauser 1999). For instance, the MT gene of wheat and rice can be induced by a variety of metal ions, such as Cu, Cd and Al, and abiotic stresses, such as high temperature and deficiency of nutrients (Cobbett and Goldsbrough 2002). A number of MT genes have been isolated from ripening fruits (Rauser 1999) and they probably have a role in normal development processes.

Metal ion transporters

Land plants possess a highly effective metal ions uptake system that allows the acquisition of metal ions and other inorganic nutrients from soil by plant roots. Therefore metal transporters, situated in the tonoplast or plasma membrane, play a central role in the maintenance of metal homeostasis within physiological limits. In fact, Cd tolerance is correlated with its extrusion or intracellular compartmentalization mediated by the activation of specific transport processes.

Generally, metal transporters appointed to ion import show low selectivity. For example A*t*IRT1 (localized in the plasma-membrane of root cells) is the primary root iron uptake system in *Arabidopsis* but can transport significant amounts of Cd (Korshunova et al. 1999). On the other hand, intracellular transporters that export metal ions from the cytosol to both vacuoles or outside the cell, are highly selective. For instance, tonoplastic transporters A*t*MTP1 and A*t*MTP3 specifically export Zn into the vacuole (Krämer et al. 2007).

An important group of metal transporters is the ZIP (ZRT, IRT-like protein) family, plasma-membrane transport proteins that are induced both in roots and shoots of *Arabidopsis* in response to Zn-limiting conditions. ZIP members have now been identified in several plant species, as well as in bacteria, fungi and animals and results indicate that they are involved in divalent cations transport across the membranes (Lòpez-Millàn et al. 2004). Members of this family are thought to be implicated in Cd uptake from the soil into the root cell and in cadmium root-to-shoot transport, being involved in the xylem unloading process (Krämer et al. 2007). Enhanced root metal uptake mediated by ZIP transporters seems to be a factor necessary, but not sufficient, for hyperaccumulation in the model species *Arabidopsis halleri* and *T. caerulescens* (Krämer et al. 2007) and accumulation capacity in these plants varies with the expression of these proteins. For instance, in *A. halleri*, ZIP9 has a high expression level in roots already under Zn-sufficient conditions, while it is upregulated in shoots in response to Zn-deficiency (Krämer et al. 2007). On the contrary, in *A. thaliana*, ZIP9 is induced during Zn-deficiency in both root and shoot. Similarly, ZIP6 is highly expressed in hyperaccumulators under Zn-deficiency, whereas it is not induced in *A. thaliana* (Becher et al. 2004; Filatov et al. 2006).

The family of natural resistance-associated macrophage protein (NRAMP) metal ion transporters represents another important group of transmembrane protein involved in metal transport and homeostasis. These transporters are considered as "general metal ion transporters" due to their ability to transport Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} (Nevo and Nelson 2006). Like the ZIP transporters, members of the NRAMP family share remarkable protein sequence identity among plants, yeast and mammalians (Nevo and Nelson 2006). By cDNA microarray, it has been shown that the expression level of NRAMP genes is higher in hyperaccumulator species (Chiang et al. 2006). They are expressed in both root and shoot and are implicated in the transport of metal cations across the plasma-membrane into the cytosol or across the tonoplast (Krämer et al. 2007). In *A. thaliana* these metal transporters participate principally in Fe homeostasis. In heterologous systems, three members of the *Arabidopsis* NRAMP family, *At*NRAMP1, *At*NRAMP3 and *At*NRAMP4, can mediate uptake of Fe, Mn and Cd (Curie et al. 2000). Interestingly, the overexpression of *AtNramp3* results in Cd hypersensitivity of *Arabidopsis* root growth and in an increased accumulation of Fe (Thomine et al. 2000). These results lead us to suppose that NRAMP metal transporters are able to transport both Fe and Cd *in planta* (Thomine et al. 2000).

Concerning efflux systems, metal transporters P_{1B}-ATPases (HMA) translocate metal ions out of the cytoplasm (both

outside the plasma membrane or into the vacuole) hydrolyzing ATP. As already mentioned, export metal-transporters are more selective than import-transporters: indeed, HMA members (e.g. HMA2, HMA3 and HMA4) export Zn and Cd exclusively (Krämer et al. 2007). Recent works highlighted that members of this family (*Ah*HMA4, *Ah*HMA3 and *Tc*HMA4 deriving from hyperaccumulator species *A. halleri* and *T. caerulescens* respectively) are able to confer Cd or Zn tolerance when expressed in yeast (Bernard et al. 2004; Papoyan and Kochian 2004). Therefore, it has been proposed that *Ah*HMA4, *Tc*HMA4 and probably *At*HMA4, its homolog in *A. thaliana*, may contribute to Cd and Zn homeostasis extruding the metal ions from the cytosolic compartment (Krämer et al. 2007). Furthermore, their expression mainly in the vascular system of root and shoot suggests an implication of these transporters in metal root-to-shoot transport (Verret et al. 2004).

Recently, ABC transporters have been shown to be implicated in a range of processes that encompasses polar auxin transport, lipid catabolism, disease resistance, stomatal function, xenobiotic and metal detoxification (Kim et al. 2006; Rea 2007). Examples are the ABC family of the mitochondria in *Arabidopsis* (*At*ATM). It has been found that *At*ATM3 is upregulated in roots of plants treated with Cd and Pb. Moreover, *At*ATM3-overexpressing plants were more tolerant to Cd, whereas *AtATM3* mutants showed increased sensitivity. The *At*ATM3 homolog in *Schizosaccharomyces pombe* (HMT1) is a tonoplast transporter exporting Cd-phytochelatin complexes. Similarly, it has been hypothesized, but has still to be demonstrated, that *At*ATM3 has a role in extruding Cd-GSH complexes formed in the mitochondria and that the sensitivity of the mutant is due to the oxidative damage of Cd accumulation in this organelle (Kim et al. 2006). *At*PDR8 is another ABC transporter in *A. thaliana* involved in metal homeostasis: it was demonstrated that *At*PDR8 participates in both Cd tolerance and pathogen resistance (Kobae et al. 2006; Stein et al. 2006). Not only is its expression induced by Cd, but its overexpression induces lower Cd accumulation in root and shoots. *At*PDR8 is mainly localized in the membrane of root hair and epidermis (Kim et al. 2007). It is proposed that *At*PDR8 might confer Cd tolerance by pumping it out of the plasma membrane to the apoplast (Kim et al. 2007).

Finally, members of the "cation diffusion facilitator" (CDF) transporter group seem to mediate vacuolar sequestration, storage and transport of metal ions from the cytoplasm to the outer compartment (Krämer et al. 2007). CDF transporters have been characterized in both prokaryotes and eukaryotes and can transport across membranes divalent metal cations such as Zn, Cd, Co, Fe, Ni or Mn (Montanini et al. 2007).

Enzymes

As already mentioned, toxicity of heavy metals determines altered activity and accumulation of different enzymes (Prasad 1995). For example, Cd inhibits the activity of enzymes involved in carbon assimilation (e.g. Rubisco) probably through reaction with Sulphidric Groups (SH) groups of the protein interfering with its folding or activity (Prasad 1995). Furthermore, treatment with Cd increases Mg dependent ATPase activity and induces diacylglycerol (DAG) kinase in roots of *B. juncea*, suggesting that Cd may activate the lipid signaling pathway (Lang et al. 2005). It has been reported that Cd can affect protein kinase expression in *Arabidopsis* (Suzuki et al. 2001) and that mitogen activated protein kinase (MAPK) cascade is involved in the Cd-signaling pathway in rice and alfalfa plants (Romero-Puertas et al. 2007). Under Cd stress, enzymes involved in primary nitrogen assimilation and nitrogen mobilization are impaired (Chaffei et al. 2004). It has been shown that the total glutamine-synthetase (GS) activity decreases (Figure 1A). Specifically, in shoots the plastidic GS isoform is decreased both in activity and expression, whereas the gene transcription of the cytosolic isoform is increased. In roots, the mRNA of the cytosolic GS isoform accumulates. This suggests that when Cd affects the plastidic-GS activity, plants induce the cytosolic isoform to compensate and maintain glutamine biosynthesis (Chaffei et al. 2004). On the other hand, a response mechanism to overcome heavy metal stress is the production of PC by PCS. PCS is activated, both *in vivo* and *in vitro*, by a wide range of metals and metalloids, such as Cd, Ag, Pb, Cu, Hg, Zn, As and Au (Schat et al. 2002). The activation mechanism is still unknown and PCS was believed to sense heavy metals directly binding the metal ion, but it has been proved that its catalytic activation does not depend on this binding (Vatamaniuk et al. 2000). The Cd-induced expression of PCS genes has been examined in *A. thaliana* and in *B. juncea* and the results are, in most cases, contradictory. Cazale and Clemens (2001) demonstrated that the *AtPCS1* and *AtPCS2* genes are constitutively expressed and not transcriptionally regulated by Cd, whereas other authors found that the *AtPCS1* level of transcript, but not of protein, is responsive to Cd (Lee et al. 2002). Furthermore the level of PCS protein was enhanced in leaves, but not in roots, of *B. juncea* after prolonged Cd exposure (Heiss et al. 2003) suggesting that the effects of Cd on PCS expression may also vary with the plant organ and species.

Cadmium causes oxidative stress by inducing generation of ROS and by disturbing the antioxidative systems in their scavenging (Schützendübel et al. 2001; Romero-Puertas et al. 2004). Catalase (CAT) represents a key enzyme for the defense responses against oxidative stress (Figure 1B). It is present only in peroxisomes and catalyzes the H_2O_2 breakdown (Buchanan et al. 2000). In *B. juncea* four distinct CAT sequences have been cloned and it has been shown that Cd exposure causes an increase of *CAT3* transcript. This induction could be useful to limit high H_2O_2 concentration in order to protect the cell from oxidative stress (Lang et al. 2005). In pea plants, Cd-induced oxidation of CAT protein determines reduced CAT activity and

protein content. As a compensatory mechanism, in response to Cd, CAT transcription is upregulated (Romero-Puertas et al. 2007).

The cycle ascorbic acid-glutathione is activated as a ROS scavenging mechanism. The main enzymes of these reactions are modulated by Cd, which induces increased activity of the ascorbate peroxidase (the first enzyme of the cycle) in *Phaseolus vulgaris* and in *Pisum sativum* (Romero-Puertas et al. 2007). In addition, another enzyme taking part in the cycle, the glutathione reductase (GR), is differently induced in roots and leaves of Cd treated pea plants (Yannarelli et al. 2007).

Super-oxide dismutase enzyme plays a role in protecting cells against ROS accumulation. SOD activity was induced in tomato seedlings after prolonged Cd treatment (Dong et al. 2006). Moreover, a significant increase of SOD activity was shown in wheat leaves, but only under exposure to high Cd concentration, probably due to the high production of superoxide (Lin et al. 2007). Nevertheless, it has to be considered that previous studies showed that SOD activity decreased in response to Cd toxicity in pea plants (Romero-Puertas et al. 2007).

Hyperaccumulator Plants: A New Frontier of Plant Biotechnology

Heavy metal hyperaccumulators are a unique group of plants that can accumulate high amounts of various toxic elements in their tissues (Reeves and Baker 2000). Hyperaccumulation is an active process that depends on an internal hypertolerance mechanism to resist the cytotoxic levels of the accumulated metals and on a powerful scavenging mechanism for the efficient uptake of the pollutants (Salt 2006). To date, there are approximately 400 known metal hyperaccumulator plants (Eapen and D'Souza 2005). Most of them are Ni and/or Zn hyperaccumulators, whereas only a few species are known to hyperaccumulate Cd. The most common are *T. caerulescens*, *Thlaspi praecox*, *A. halleri* and *Sedum alfredii* (Van de Mortel et al. 2008). *Thlaspi* species are polymetallic hyperaccumulators known to accumulate high amounts of Zn, Cd, Ni and Pb (Mari et al. 2006), whereas *A. halleri* is able to tolerate Zn, Cd and Pb and hyperaccumulates Zn and Cd (Van Rossum et al. 2004). *S. alfredii* has been identified as a Zn hyperaccumulator, and recently it was confirmed to also hyperaccumulate Cd (Zhou and Qiu 2005).

Non-hyperaccumulator plants normally accumulate heavy metals in roots, whereas hyperaccumulator plants are able to transport most of the absorbed toxic elements to the shoots (Lasat et al. 1998). Metal translocation from root to shoot through the xylem is therefore a key determinant of the hyperaccumulation phenotype. In this respect, it has been recently demonstrated that the metal transporter HMA4 is essential for the root-to-shoot transport. Interestingly, HMA4 is expressed at higher levels in the hyperaccumulator *A. halleri*, in comparison with non-tolerant *A. thaliana* (Hanikenne et al. 2008).

At the molecular level, amino and organic acids have been proposed to play a role in heavy metal hyperaccumulation or tolerance (Sharma and Dietz 2006), although no clear mechanisms of metal long-distance trafficking related to metal hyperaccumulation have been described (Mari et al. 2006). Phytoremediation is an emerging *in situ*, cost-effective and ecological technology that exploits the ability of plants to accumulate heavy metals in their above-ground tissues to reclaim polluted environments (Alkorta et al. 2004). In this respect, hyperaccumulator plants have a direct performer role of pollutant removal or indirectly represent sources of genes for the improvement of non-hyperaccumulator plants. Ideal hyperaccumulator plants, in fact, should have some specific features such as high biomass, rapid growth rate, highly branched and extended root apparatus and easy harvesting. However, natural hyperaccumulator plants have generally low biomass and slow growth rate. This restriction may be overcome by transferring the genetic potential responsible for hyperaccumulation from hyperaccumulator species to plants with appropriate traits for phytoremediation, to confer enhanced capacity for pollutant accumulation and tolerance. Poplar has recently emerged as a model system (its genome is under sequencing) and a good candidate for phytoremediation purposes. The transgenic yellow poplar expressing a bacterial mercury reductase, developed for enhanced mercury phytoremediation is well known (Rugh et al. 1998). Moreover, Indian mustard (*B. juncea*) is also a suitable target species, because of its large biomass production, a relatively high metal accumulation and the already well-established transformation technology.

Typically, chelation of the metal ion, transport of metal or its complexes and subsequent compartmentalization in vacuoles are the processes where biotechnology can play a part in enhancing the phytoremediation capacity of plants. For example, transferring a single gene involved in metal transport, such as *HMA4*, from *A. halleri* to *A. thaliana* has increased the shoot metal uploading in this non-accumulator species (Hanikenne et al. 2008). Regarding metal-conjugates transport, plants overexpressing specific transport proteins (such as members of the CDF group, Krämer et al. 2007) might acquire higher detoxification and compartmentalization of GS-Cd conjugates into the vacuoles. Transgenic *B. juncea* plants engineered to produce more glutathione and phytochelatins accumulated significantly more Cd than wild-type plants (Bennett et al. 2003). *A. thaliana* and tobacco plants engineered with the MT gene information developed Cd tolerance and accumulation (Eapen and D'Souza 2005). Furthermore, Cd tolerance and accumulation is also enhanced by overexpressing the γ-glutamylcysteine synthetase, an enzyme with an important role in controlling glutathione synthesis and therefore metal chelation (Figure 1B) (Zhu et al. 1999). Another study revealed that the expression of the *AtPCS1* gene increased Cd and As

tolerance and accumulation in *B. juncea* (Gasic and Korban 2007) and in tobacco plants (Pomponi et al. 2006). Recently, it has been verified that a bZIP transcription factor isolated as differentially expressed in response to Cd treatment in *B. juncea* (Fusco et al. 2005), enhances Cd accumulation and tolerance in transgenic *Arabidopsis* and tobacco plants (S Farinati et al., unpubl. data, 2008). Moreover, the comparison between hyper-accumulator with non-accumulator sister species (e.g. *A. halleri* with *A. thaliana*) suggests that the hyper-accumulating features could reside in sequence mutations, gene copy number and/or in different expression levels of the proteins that contribute to the metal tolerance (Plaza et al. 2007; Hanikenne et al. 2008). These findings highlight that probably part of the genetic potential for metal detoxification is already present in most plant species and those small sequence changes that influence both metal sensing and activation of appropriate responses make the difference.

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