Cloning and Functional Characterization of Putative Heavy Metal Stress responsive (*Echmr*) gene from *Eichhornia crassipes (Solm* L.*)*

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By

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STATEMENT

I hereby declare that the work presented in this thesis is original and was obtained from the studies undertaken by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the supervision of **Prof. Lingaraj Sahoo.**

As per the general norms of reporting research findings, due acknowledgements have been made wherever the research findings of other researchers have been cited in this thesis.

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CERTIFICATE

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph.D degree of The Indian Institute of Technology Guwahati by **Ganesh Thapa** is his original work. The matter presented in this thesis incorporates the findings of independent research work carried out by the researcher himself. The entire research work and the thesis have been built up under my supervision. The matter contained in this thesis has not been submitted elsewhere for the award of any other degree.

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.....Dedicated to Baba, Nomi & Bhraaj

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31st Dec, 2012 **Ganesh Thapa**

CONTENTS

ABBREVIATION

ADH	Alcohol Dehydrogenase
Amp	Ampicillin
AP2	APETALA type 2
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BSA	Bovine Serum Albumin
bZIP	Basic region leucine zipper
cAMP	Cyclic adenosine monophosphate
Cm	Chloramphenicol
DCW	Dry cell weight
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DREB	Dehydration responsive element-binding protein
DTT	1, 4-Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ERF	Ethylene responsive factor
GFP	Green fluorescent protein
GSH	Glutathione reduced
gshA	encoding the γ-Glu-Cys synthetase
gshB	encoding the GSH synthetase
GSSH	Glutathione disulfide
h/min/s	Hours/minutes/ second
HM(s)	Heavy metal(s)
IPTG	Isopropyl β-D thio galactopyranoside
Kb	Kilobase (s)
kDa	Kilodalton
Km	Kanamycin
L/ml/µl	Litres/milliliters/microlitres
LB	Luria broth
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
MRE	Metal responsive element
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
ng	Nanogram
nm	Nanometer
NO	Nitrous oxide
OD	Optical Density
ORF	Open reading frame
ori	Origin of replication
P/Pi	Phosphate, Phosphorus / inorganic phosphate
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
PC	Phytochelatin
PCS	Phytochelatin synthase
PEP	Phosphoenolpyruvate
RNA	Ribo nucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
µM/mM/M	Micromolar/Millimolar/Molar
SDS	Sodium Dodecyl Sulphate
SMQ	Sterilized MilliQ water
sp.	species
STRE	Stress responsive element
TCA	Tricarboxylic acid
TF	Transcription factor
Tris	Tris (hydroxymethyl) aminomethane buffer
WT	Wild type
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D galactopyranoside
Δycf1	Yeast cadmium factor 1 mutant

Note: The full forms of several rarely used abbreviations have been described within the text.

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ABSTRACT

The water hyacinth (*Eichhornia crassipes* (Mart.) Solm-Laubach, *Pontedericeae*) is a floating macrophyte, thrives in polluted water and well-known for their capacities to hyper accumulate heavy metals (HMs). The present study is carried out with the objectives to assess the morphological, biochemical and ecotypic validation of the *Eichhornia* as potential gene source for HM and abiotic stress, cloning of *Echmr* gene from *Eichhornia* and its bioinformatics analysis, expression and functional characterization of the *Echmr* gene in prokaryotes and yeast system and the subcellular localization study of the EcHMR protein in *Arabidopsis* protoplast for possible use of the isolated gene in phytoengineering.

The *E. crassipes* plant, soil and water samples were collected from the HMs contaminated and control areas. The high level of HMs contamination in the soil, water and different plant parts were ascertained and quantized by X-Ray Diffraction (XRD), Energy Dispersive X-ray Elemental Spectrometry (EDX) and Atomic Absorption Spectrometry (AAS) study. The XRD and EDX analysis of the soil samples revealed the presence of Fe, Cu, Zn, Hg, Pb, Ni, As, Cd, Cr, Mn and Co. The AAS analysis of plant samples quantized that HMs Pb, Cr, Ni, Cu and Cd accumulated to higher extent. Furthermore, high expression of alcohol dehydrogenase (ADH), peroxidase (POX) and altered regulation of esterase (EST) were observed in root tissue during stress. The enhanced accumulation of metals in *Eichhornia* and altered regulation of proteins in shoot and enzymes in root tissues during stress strengthen it to be used as biomarker of heavy metal contamination.

We also report here the isolation and *in silico* analysis of HM stress responsive (*Echmr*) gene from *Eichhornia*. The isolated 850 bp *Echmr* gene revealed its homology with different salt and water stress unknown ESTs from soybean, groundnut, finger millet and lotus. The presence of various stress responsive transcription elements (STREs) in its untranslated regions viz., DRE, ABRE, HSE and calmudilin binding element, suggest its possible role in abiotic stress tolerance. The function and structure prediction of EcHMR has recognized it to have transition metal ion or divalent inorganic cation transmembrane transporter activity as yeast Mg^{2+} channel protein Mrs2 and to be localized at cell periphery, intrinsic to membrane. This study provides an directions for *Echmr* gene functional characterization through subsequent wet lab assays.

The detoxification of HMs frequently involves conjugation to GSH prior to compartmentalization and catabolism in higher plants. Hence, the functional characterization of *Echmr* gene was done in Cd sensitive *E. coli* Δ*gsh* mutants for tolerance against HMs such as Cd, Pb, heat, cold and UV-B stress. The recombinant *E. coli* Δ*gsh* mutant cells showed better growth and survival than control cells under Cd

Abstract

(200 µM), Pb(200 µM), heat shock (50°C), cold stress at 4°C for 4 hrs, and UV-B (20 min) exposure. The enhanced expression of *Echmr* gene revealed by northern analysis under above stresses further advocates its role in multi-stress tolerance and this demonstrates that *Echmr* is functional.

Furthermore, expression of *Echmr* in Cd(II) sensitive yeast *ΔYcf1* mutant cells complemented the cells to survive in the excess Cd(II) concentration of 1000 µM. The enhanced expression of the *Echmr* in the stressed complemented ΔYcf1 cells was maintained during Cd(II) stress. *Echmr*-induced desiccation tolerance was also observed in yeast FY3 strain that enables it to sustain well even under *in vitro* conditions of 4% polyethylene glycol. These results demonstrate that *Echmr* enhances Cd(II) and desiccation tolerance in yeast *ΔYcf1* mutant and FY3 cells through the possible mechanisms of cation transport.

The transmembrane class of proteins that predominantly localize at intracellular and plasma membrane plays a crucial role in the influx/efflux of cations in plants. Therefore, EcHMR recombinant protein with green fluorescent protein (GFP) fusions were employed to examine the subcellular localization in the protoplast of *Arabidopsis*. The subcellular localization study of the EcHMR protein showed discrete localization within the plasma membrane or intrinsic to membrane and cytosol as predicted by the bioinformatics tools. The subcellular localization study ascertained EcHMR protein within the plasma membrane or intrinsic to membrane and cytosol indicated possibly role of EcHMR in transmembrane transporter activity. The isolated gene from *Eichhornia* and its integrated functions can be harness for the stress tolerance engineering of plants in near future and phytoremediation approaches.

1.1 INTRODUCTION

Metals like zinc, iron and copper are essential micronutrients required for a wide range of physiological processes in all plant organs for the activities of various metal-dependent enzymes and proteins. However, they become toxic depending on the oxidation state, complex form, dose and mode of exposure (Beyersmann and Hartwig, 2008). Metals like arsenic, mercury, cadmium and lead are nonessential and potentially highly toxic. Once the cytosolic metal concentration in plant turns out of control, phytotoxicity of heavy metal inhibits transpiration and photosynthesis, disturbs carbohydrate metabolism, and drives the secondary stresses like nutrition stress and oxidative stress, which collectively affect the plant development and growth (Krämer and Clemens 2005). Heavy metal pollution of soil is a significant environmental problem with a negative potential impact on human health and agriculture. 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, 2004). 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).

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. Therefore, plants need to adapt to changing environmental conditions in order to tolerate these stresses. Plants have developed a complex network of highly effective homeostatic mechanisms that serve to control the uptake, accumulation, trafficking, and detoxification of metals. Components of this network have been identified continuously, including metal transporters in charge of metal uptake and vacuolar transport; chelators involved

in metal detoxification via buffering the cytosolic metal concentrations; and chaperones helping delivery and trafficking of metal ions (Clemens, 2001).

The hyperaccumulation of heavy metals in some plants has been recorded during last few decades (Barman et al., 2000) and this has emphasized the importance of further advanced research in molecular basis of phytoremediation technology. The hyperaccumulation of heavy metals depends on the plant species, soil condition (pH, organic matter content, cation exchange capacity etc.) and types of heavy metal (Barman et al., 2001; Spinoza-Quinones et al., 2005; Xian and Shokohifard, 1989; Otte et al., 1993). In recent years, major scientific strides have been taken in understanding the soil chemical and plant molecular-genetic mechanisms that drive metal hyperaccumulation in plants. The complexity of plant–metal interactions and influences of the environment, and specific matrix factors that control the chemical speciation of the metal, and interactions of other toxicants that may be present at the site all add to the strategy of phytoremediation (Cobbett, 2002; Banuelos et al., 2007). Hyperaccumulator plants represent a resource for remediation of metal polluted site, as they are able to extract wide range of metals and to concentrate them in their upper parts with the character of metal tolerance. In some plant species, the concentrations accumulated in aboveground biomass of metals or metalloids are more than one and up to four, orders of magnitude higher than in other adjacent plants (Baker and Brooks, 1989; Reeves and Baker, 2000). This unique extent of accumulation of heavy metals, have been reported in a total of approximately 500 plant species to date (Kramer, 2010).

Water hyacinth (*Eichhornia crassipes*) is a staggeringly efficient hyperaccumulator capable of accumulating very high level of heavy metals i.e., Cd, As, Cr, Zn, Cu and Ni, and cyanide (Das and Jana, 1999). Therefore, it represents an excellent plant species for investigation of the molecular mechanisms and candidate genes involved in underlying heavy metal detoxification process. However, the molecular mechanisms of adaptation to toxic metal

tolerance and hyperaccumulation in water hyacinth are unknown, and genes related to metal detoxification have not yet been identified.

1.2 OBJECTIVES

The present investigation has been carried out to explore the possibilities of cloning genes from Water hyacinth which may have role(s) in heavy metal stress tolerance and other abiotic stresses. The work has been undertaken with the following objectives:

1. Study of morphological changes in various tissues of *Eichhornia crassipes* grown in heavy metals contaminated area and evaluation of metal stress induced changes in protein and isozymes.
2. Cloning and sequence analysis of heavy metal stress responsive (*Echmr*) gene from *Eichhornia crassipes*.
3. Expression and functional characterization of *Echmr* gene in prokaryotic system.
4. Functional validation of *Echmr* gene in yeast.
5. Studies on sub-cellular compartmentalization of EcHMR protein in protoplasts of *Arabidopsis.*

2. 1 Abiotic stress and tolerance mechanism(s) in plants

Abiotic stress responses are vital for plants in order to survive under hostile environment. The term 'abiotic stress' includes numerous stresses caused by complex environmental conditions, e.g. strong light, high and low temperatures, freezing, drought, salinity, HMs and hypoxia. The global climate change is going increase abiotic stresses in the years to come which would affect crop production by around 30% (Ciais et al., 2005). Therefore, understanding abiotic stress responses is now thought to be one of the most important topics in plant science. Application of molecular biology in plant science has resulted in isolation of many abiotic stress-inducible genes and functional characterization in transgenic plants. The availability of these data broadened and deepened our view of abiotic stress responses and tolerance in plants.

The availability of genome information has allowed monitoring of expression profiles for all predicted genes at a single time by means of microarrays and facilitated identification of potential *cis*-regulatory elements and *trans*-factors. Information from all-gene data has allowed examination of all the metabolic pathways in plant cells, and has provided a way to assign polypeptides detected in mass spectrometry-based protein analyses. Combination of these 'omics' data is now necessary to elucidate cellular and whole-plant processes. In this section, we summarize recent progress in abiotic stress and offer a new prospective on the research directions for the next decade.

2.1.1 Overview of Abiotic Stress Responses

Abiotic stresses have been shown to cause accumulation of many intracellular substances, including nucleic acids, proteins, carbohydrates and amino acids. After the introduction of molecular biological techniques into plant biology, a great deal of effort went into the identification of stress-inducible genes, such as *RD29A*, using differential screening or differential display techniques for various plant species, including *Arabidopsis*. These studies succeeded in isolating genes that are presumed to function in stress responses and tolerance. Over-expression of some of these genes *in planta* confers some abiotic stress tolerance (Bartels

and Sunkar, 2005; Umezawa et al., 2006). More importantly, using the expression of such inducible genes as markers, an overall scheme of transcriptional regulation was developed. The various induction phases for stress-inducible genes are due to their varying dependency on *de novo* synthesis of proteins or signaling molecules, such as abscisic acid (ABA) (Yamaguchi-Shinozaki and Shinozaki, 2006). These findings suggest that abiotic stress responses are never simple, and that each induction phase may be controlled by a different signaling mechanism and different transcription factors. Identification of the relevant factors in each pathway has been addressed using responsive genes as markers. For example, an abiotic stress-responsive *cis*-element, dehydration responsive element (DRE)/C-repeat (CRT) (A/GCCGAC), was identified, which in turn has triggered important studies to identify the transcriptional regulating factors, DRE-binding protein (DREB)/C-repeat binding factor (CBF), and their post-translational regulatory mechanisms (Thomashow, 2001; Shinozaki and Yamaguchi-Shinozaki, 2007). In addition, genetic screens for mutations that affect the expression of stress-inducible genes have allowed the identification of novel components in the regulatory system (Chinnusamy et al., 2002). These results have provided a basic picture of gene regulatory networks in abiotic stress responses in plants. However, the stress-inducible genes isolated in this way were largely restricted to those with higher expression levels, and thus our knowledge on gene expression profiles was limited.

Complete determination of the genomes of *Arabidopsis*, *Oryza sativa* spp. *japonica* cv. Nipponbare, and other plants has changed the situation dramatically. The complete genome sequence has enabled genome-wide gene expression profiling in response to various abiotic stresses (e.g. using AtGenExpress, Kilian et al., 2007). Comprehensive transcriptome analysis revealed the relationships among stress-regulated transcripts, and enabled the prediction of their *cis*-regulatory elements (Kilian et al., 2007; Weston et al., 2008). Ma and Bohnert (2007) showed a clear correlation between expression profiles and the 5′ regulatory motifs of stress-regulated

genes. These analyses indicated that stress-regulated genes are controlled by a complicated regulatory network.

Determination of the function of stress-inducible genes has been addressed by the reverse genetic approach, aided by the use of insertional mutation lines. The products of stress-inducible genes identified in vast microarray experiments can be classified into two groups (Shinozaki et al 2003): one containing mainly proteins functioning in direct abiotic tolerance [e.g. late embryogenesis abundant (LEA) proteins], and the other consisting of regulators for intracellular signaling and stress-inducible gene expression (e.g. protein kinases such as MAP kinases, phosphatases, phospholipid metabolic enzymes, and various types of transcription factors). The identification of stress-inducible signal transducers gave rise to the idea that plants have developed flexible cellular response mechanisms to efficiently respond to various abiotic stresses (Figure 2.1).

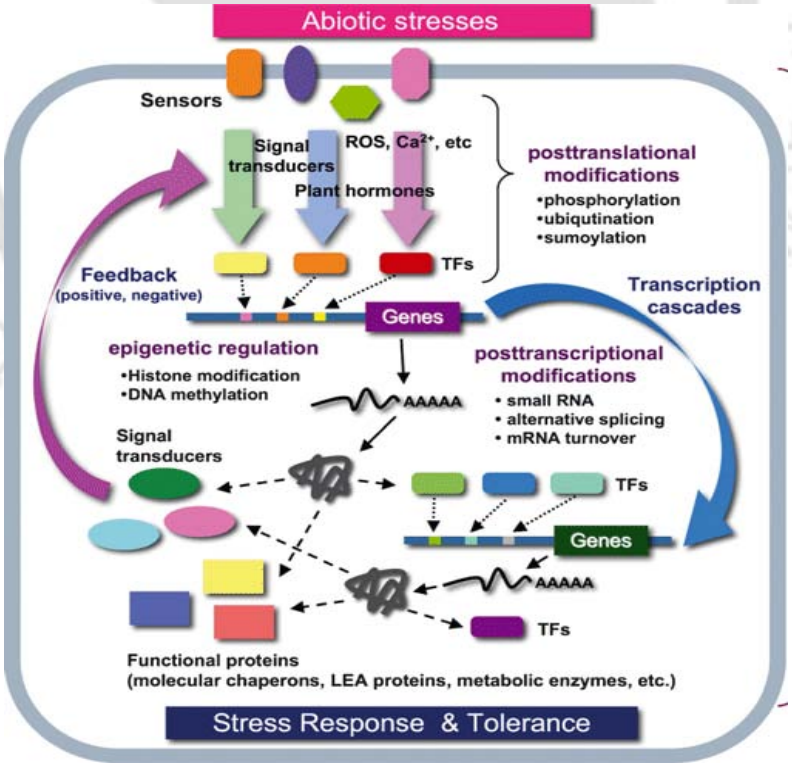

Fig.2.1 Model of a plant abiotic stress response

2.1.2 Regulation of Transcription in Abiotic Stress Responses

Many transcription factors involved in stress responses have been identified. The transcriptional regulatory system for cold and drought (or osmotic) stresses are depicted in Figure 2.2. The DREB1/CBF family comprises APETALA2 (AP2) type transcription factors that recognize DRE/CRT and function in cold stress responses. Expression of *DREB1A*/*CBF3* or *DREB1C*/*CBF2* is regulated at the transcriptional level by INDUCER OF CBF EXPRESSION 1 (ICE1) (Chinnusamy et al., 2003) or calmodulin binding transcription activator (CAMTA) (Doherty et al., 2009), respectively. ICE1 is a MYC-type transcription factor that has also been shown to regulate stomata formation (Kanaoka et al., 2008). CAMTA transcription factors recognize Conserved Motif (CM) sequences providing a link with the Ca^{2+} signaling that is activated in the abiotic stress response, as CAMTA proteins have a calmodulin-binding domain. ZAT12, a zinc finger protein, is also implicated in the regulation of *DREB/CBF* expression (Vogel et al., 2005). DREB2, another AP2-type transcription factor that recognizes DRE/CRT, is involved in drought or salinity stress responses. Osmotic stress activates several other transcription factors, including zinc finger homeodomain (ZFHD) proteins and NAM ATAF CUC2 (NAC). ZFHD1 binds the CACTAAATTGTCAC motif, named ZFHDR, in the promoter region of *EARLY RESPONSE TO DEHYDRATION 1* (*ERD1*). NAC proteins recognize a MYC-like target sequence and activate *ERD1* (Tran et al., 2004; 2006). Osmotic stress increases the ABA level, which in turn activates sets of genes. ABA-responsive transcription factors (AREB/ABF), with a bZIP type DNA-binding domain that binds the ABA-responsive element (T/CACGTGGC), have a pivotal role in ABA-dependent gene activation (Choi et al., 2000; Uno et al., 2000). MYB and MYC transcription factors are synthesized *de novo* under osmotic stress conditions, and cooperatively activate stress-inducible genes such as *RD22* (Abe et al., 2003).

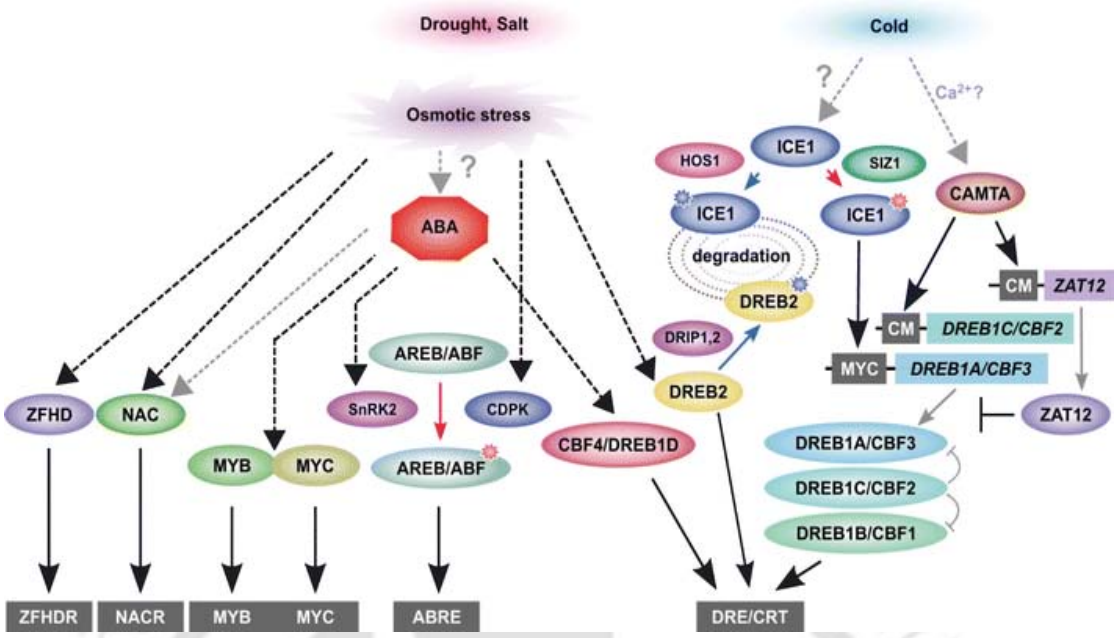

Fig. 2.2 Transcriptional regulatory network functioning in drought, salinity and cold stress responses

Some stress-associated transcription factors are themselves regulated at the transcriptional level, constituting a transcriptional cascade. Other transcription factors are regulated by post-translational modifications. Such regulation of transcription factors may be required for rapid and fine-tuned regulation under abiotic stress conditions. There is much evidence showing that ubiquitination, which usually induces degradation of the target protein, plays a pivotal role in abiotic stress responses (Vierstra, 2009). DREB2 was shown to be regulated by DREB-INTERACTING PROTEIN 1 and 2 (DRIP1 and DRIP2), which are RING finger E3 ligases, through ubiquitination (Qin et al 2008). ICE1 was demonstrated to be under the control of HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), another RING finger protein (Dong et al., 2006). Sumoylation is also involved in the regulation of transcription factors in abiotic stress responses. In contrast to ubiquitination, sumoylation regulates the activity of target proteins. A recent study showed that a SUMO E3 ligase, SIZ1, sumoylated ICE1 and enhanced its activation of DREB1A/CBF3 (Miura et al., 2007).

As in other organisms, transcription factors in plant systems are regulated by phosphorylation. The ABA-responsive transcription factors ABI5 and AREB/ABF of *Arabidopsis* and TRAB1 of rice are regulated by the phosphorylation of multiple Ser/Thr residues. SNF1-related kinase 2 (SnRK2)-type protein kinases (Kobayashi et al., 2005; Furihata et al., 2006) and Ca^{2+}-dependent protein kinase (CDPK) are good candidates for regulators of AREB/ABFs (Choi et al., 2005; Kaplan et al., 2006; Zhu et al., 2007). SnRK2s are activated by osmotic stress or ABA (Boudsocq et al., 2004), while CDPKs are activated by increased intracellular Ca^{2+} levels induced by various stimuli (Harper et al., 2004). Presumably, such transcription factors function as a hub component that integrates multiple signal inputs under abiotic stress conditions.

2.1.3 Post-Transcriptional Regulation of Abiotic Stress-Inducible Transcripts

Recent studies have indicated that post-transcriptional regulation contributes to stress responses more than was previously thought. Transcribed RNA goes through various modifications: addition of a 5′ cap structure, splicing, and 3′ polyA addition. After these processes, mRNA is exported actively from the nucleus to the cytoplasm, where translation occurs and unnecessary or abnormal mRNA is promptly degraded. Each step is regulated coordinately (Houseley and Tollervey, 2009). Genetic studies on *Arabidopsis* mutants exhibiting an abnormal response to abiotic stress or ABA revealed that mRNA processing and metabolism have a close link with stress responses (Fedoroff, 2002; Kuhn and Schroeder, 2003; Hirayama and Shinozaki, 2007). For example, RNA helicases are implicated in abiotic stress responses in various organisms including plants (Owttrim, 2006). In addition, Lida et al (2004) showed that alternative splicing, which enables production of diverse polypeptides from one gene, is regulated by various abiotic stresses, such as cold stress. Moreover, cold stress changed the alternative splicing profiles of splicing factors, suggesting that complex multi-step regulation controls the splicing profiles in abiotic stress responses (Lida et al., 2004; Reddy, 2007). Alternative splicing events are

considerably conserved between *Arabidopsis* and rice, indicating their importance (Wang and Brendel, 2006).

2.1.4 Small RNA-Dependent Gene Regulation in Abiotic Stress

An emerging topic in gene regulatory systems in abiotic stress responses is the regulation of gene expression by small RNAs. The evidence shows that the basic mechanisms of small RNA synthesis and function are common to many eukaryotes but that numerous organism-specific mechanisms are involved (Siomi and Siomi, 2009). Presumably, plants, as sessile organisms, have developed unique features of small RNA regulatory mechanisms (Brodersen and Voinnet, 2006; Jones-Rhoades et al., 2006). During abiotic stress, the levels of several small RNAs change in *Arabidopsis*, indicating their function in abiotic stress responses (Sunkar and Zhu, 2004). Comprehensive transcriptome studies have revealed that more than 7000 transcriptional units that have the potential to produce endogenous siRNA (called natural antisense transcript-associated siRNA, nat-siRNA) (Yamada et al., 2003). Abiotic stress induces the accumulation of novel antisense overlapping transcripts (Matsui et al., 2008) and transcripts from transposons or pseudogenes, which are a source of siRNAs (Matzke et al., 2007; Zeller et al., 2009), implying a role for siRNAs in abiotic stress responses in plants. Borsani et al (2005) showed that nat-siRNA produced from overlapping mRNAs for Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH) and SIMILAR TO RCD ONE 5 (SRO5) regulated the expression of these genes. The authors showed that, under salt stress conditions, *SRO5* is induced and siRNAs are produced, which in turn down-regulates *P5CDH* and leads to accumulation of the osmoprotectant proline. This *P5CDH–SRO5* regulatory system appears to function only in *Arabidopsis* and not in other plants.

2.1.5 Stress-Tolerance Mechanisms in Diverse Plant Species

Knowledge obtained from the study of plant species living under extreme environmental conditions has provided important information on stress tolerance mechanisms. One of the most studied groups is halophytes, such as mangroves, which grow under high-salinity conditions.

Generally, the mechanisms by which halophytes tolerate high salinity differ among species, but common features can be found in their tight regulation of internal Na^+, K^+ and Cl^- content (Flowers and Colmer, 2008). However, halophytes exhibit tolerance to other abiotic stresses as well. Among them, *Thellungiella halophila*, or salt cress, has been used as a model plant because it is closely related to *Arabidopsis*. Detailed analysis of salt cress revealed that this halophyte has additional features, including the accumulation of more proline under stress conditions, high selectivity for ions, and sensitive stomatal regulation (Inan et al., 2004). Given the high similarity to *Arabidopsis*, transcriptome analyses of salt cress have been performed by adapting the genome-related information on *Arabidopsis*, revealing both common and distinct expression profiles of stress-related transcripts (Taji et al., 2004; Gong et al., 2005). Detailed analysis of the differences between glycophytes and halophytes will provide information on the molecular basis of salinity tolerance and other stress tolerance attributes of halophytes. Du et al (2008) attempted to identify salt stress genes that confer salinity stress tolerance to *Arabidopsis* and obtained several candidates. Such an approach is useful to understand the molecular basis of salt tolerance in halophytes.

Quantitative trait locus (QTL) analysis among tolerant and intolerant species of crops is now receiving much attention. A major benefit of QTL-based approaches is that they may enable production of stress-tolerant crops by combining or 'pyramiding' QTLs for various stress tolerances. Several QTL studies relating to various abiotic stress tolerances have been reported (Takeda and Matsuoka, 2008). For example, Ren et al (2005) identified the *SKC1* locus encoding a high-affinity K^+ transporter (HKT)-type sodium transporter by analyzing a QTL for salinity tolerance using salt-tolerant and salt-susceptible rice varieties. Such genes or loci can be used to improve the salinity tolerance of rice.

2.1.6 Strategies in improving abiotic stress tolerance potential

One purpose of studying abiotic stress responses in plants is to improve the abiotic stress tolerance of crops by means of genetic manipulation. In brief, ectopic expression of components

involved in abiotic stress responses has led to improved stress tolerance. However, constitutive ectopic expression of these components often causes reduced plant growth, presumably due to an adverse effect of accumulated factors on cellular functions or energy consumption. Such unfavorable effects can be suppressed by using appropriate promoters, such as tissue-specific or abiotic stress-inducible ones (Umezawa et al., 2006). Alternatively, careful manipulation of transcription factors can also be used. Some transcription factors or signal transducers may be converted to constitutive active forms by deleting the inhibitory domains or changing phosphorylation-accepting amino acid residues to phosphorylation-mimicking amino acid residues (e.g. serine to asparatic acid). Such strategies are promising techniques to improving stress tolerance.

However, improvement of crops using the above strategies will require further research. The biggest concern is the differences in the conditions between laboratories and crop fields. In the field, plants are subjected to various stresses simultaneously and the constraints extend throughout their lifetimes in some cases. Combined abiotic stresses have been reported to cause unexpected physiological changes in plant cells (Larkindale et al., 2005; Mittler, 2006). The molecular basis for this interaction has not yet been elucidated. Therefore, transgenic crops harboring a gene or genes designed to improve the tolerance to a specific stress might encounter unexpected problems. To overcome these difficulties, the whole stress-response system of plants needs to be fully understood.

2.1.7 Perspectives

As described above, our understanding of abiotic stress responses has taken a big leap forward in the last decade, namely the post-genome era. However, we still have several critical problems to overcome in molecular breeding of stress-tolerant plants. Dinneny et al (2008) clearly demonstrated that different differentiated cells in roots responds differently to various abiotic stresses, suggesting that different cell types respond differently to abiotic stresses. Our understanding of the whole-plant stress response mechanism is very limited. Keeping the

complexity of abiotic stress intact, we will try to review specifically the heavy metal stress response mechanisms in plants in the forthcoming section.

2.2 Molecular mechanistic model of plant heavy metal tolerance

HMs effluence has ever been a significant environmental predicament with a negative probable impact on human health and plant productivity. Even after the natural occurrence as rare elements, diverse anthropogenic practices and refuse dumping has contributed to spread of HMs in the environment. The toxicity mechanisms of HMs vary from plant to plant, and even several endeavors have not been successful to understand fully at the molecular level. In order to cope with stress signals, plants require a coordination of complex physiological and biochemical processes, gene expression, protein modifications, changes in metabolite's compositions leading to proper stress signal perception and tolerance (Urano et al., 2010). Though there has been a recent development in the HMs stress signaling cascades, gene expression modulation and stress responsive non-coding RNA (Borsani et al., 2005; Matsui et al., 2008). Hence, these findings may facilitate the understanding of complex stress response of plants and radically contribute towards escalating development of HMs stress tolerant plants.

" HMs" are elements with a specific gravity of atleast five times higher than that of water (specific gravity of water is 1 at 4°C). Some well-known toxic HMs with a specific gravity that is 5 or more times that of water are As, 5.7; Cd, 8.65; Fe, 7.9; Pb, 11.34; and Hg, 13.546 (Lide 1992). Transition metal as such bears all the characteristic properties of metals with incomplete d sub-shells leading to variable oxidation states (IUPAC, 1997) and it has been reviewed for the use of term transition metal and d-block (Jensen, 2003). Among all the HMs reported, Cd is considered to be the most phytotoxic because of its high solubility and absorption by plants and radical introgression into the food chain causing serious human hazards (Buchet et al., 1990; Lux et al., 2010; Gill et al., 2011). The high degree of negative effects on mineral nutrition and homeostasis in plant shoot and root growth and development even at low concentrations uptake

makes Cd the most toxic HM to plants (Macek et al., 2002; Metwally et al., 2005; Farinati et al., 2010). The mode of HM action, toxicity mechanisms, its accumulation in subcellular level and different strategies followed by plants to combat HM stress has been reviewed extensively but key factors at different signaling stages that lead to HM tolerance has not been studied well. In general, HMs stress in plants is characterized by synthesis of stress proteins, signaling molecules and chelators like organic acids and GSH mediated phytochelatins. Though the mode of action of each metal is specific, but some toxicity mechanisms may be shared. Correspondingly, cells may mount both customary and metal-specific responses to counteract HMs toxicity. Because of this reason only a relatively diminutive set of hyperaccumulator plants is capable of sequestering HMs in their shoot tissues at elevated concentrations. So, we focus on the progress on the molecular key components of HM induced response pathways in plants. Here in this section, a review of the progress on key components of HM tolerance regulatory network together viz., sensors, kinases, cis-regulatory elements, transcription factors, aquaporins/transporters, nitrous oxide signaling, etc. has been made to understand the HM induced signaling network and crosstalk with other stresses.

2.2.1 Heavy metal stress response: From sensing to gene regulation

Plants possess a range of potential mechanisms that may be involved in the detoxification of HMs and response under metal stresses. Hence, a coordinated network of molecular processes such as reduced uptake or increased plant internal sequestration along with multiple metal-detoxifying mechanisms, repair capabilities and signaling molecules governs the process from HM sensing to plant HM stress tolerance.

The cellular toxicity of HMs depends on the chemical properties, incompletely filled d-orbital (belonging to the sequence s, p, d, f orbitals) and on the production of O^{-2} and H_2O_2 via Fenton's type reactions (Lund et al., 1993; Shi and Dalal 1993; Imlay et al., 1988). The high affinity of Cd for sulfydryl groups of structural proteins and enzymes may lead to misfolding, inhibition of activity and/or interference with redox enzymatic regulation (Hall, 2002;

Sch¨utzend¨ubel and Polle (2002). Another important toxicity mechanism may be due to chemical similarity between Cd^{2+} and functionally active ions at active sites of enzymes and signaling molecules. There are numerous reports of such inhibition, *viz,* displacement of Mg^{2+} in ribulose1, 5 bisphosphate carboxylase/oxygenase with Co^{2+}, Zn^{2+} and Ni^{2+} has resulted in loss of function (Wildner and Henkel, 1979; Van Assche and Clijsters, 1986). The phosphodiesterase activity has been reported to get inhibited in radish by the displacement of Ca^{2+} with Cd^{2+} in cell signaling protein calmodulin (Rivetta et al., 1997). Furthermore, Cd^{2+} replaces Ca^{2+} in the photosystem II reaction center, causing the inhibition of PSII photoactivation (Faller et al., 2005). Thus, Cd^{2+} ions can cause cellular homeostatic imbalance of essential metal ions (Roth et al., 2006) or the displacement of divalent cations, such as Zn and Fe from proteins releasing "free" ions, leading to production of ROS (Polle and Sch'utzendu'bel, 2004). Thus, HMs toxicity may be due to blockage of functional group, displacement of metal ions from regulatory biomolecules or production of ROS by auto oxidation.

There are various reports of co-transportation of the metals ions by metal transporters in plants, which may be the prime cause of metal toxicity. Specific transporters might be involved in metal ion uptake, such as in the case of Cd uptake in Alpine Penny-cress (*Thlaspi caerulescens*) (Lombi et al., 2001). The ZIP transporter family members, IRT1(iron regulated transporter) expressed in *Arabidopsis* iron deficient plants (Zhao and Eide, 1996) and ZRT1 and ZRT2 (zinc regulated transporter) high and low affinity zinc transporters respectively of yeast were found to uptake other HMs apart from transporting Fe(II) across the membrane (Zhao et al., 1996). An *Arabidopsis* iron transporter (AtIRT1) was found to functionally complement yeast mutant (*fet3fet4*) defective for iron uptake (Zhao and Eide, 1996). Hence IRT1 through complementation and uptake studies in yeast confirmed the ability to transport both Mn and Zn along with Fe. Even (Guerinot, 2000) also reported that in Fe deficient plants the IRT1 gene get expressed and once expressed it facilitates the transport of Mn and Zn along with Fe. Furthermore, IRT1 isolog from pea (RIT1) and tomato (LeIRT1 and LeIRT2) when expressed in

yeast Fe transport deficient (*fet3-fet4*) and Zn transport deficient (*zrt1zrt2*) mutants were found to high affinity uptake of Fe and Zn along with low affinity Cd uptake (Cohen et al., 1998). Hence, the co-transportation of HMs may be one of the numerous reasons of HM toxicity in plants.

Plants with time have evolved a complex network of homeostatic mechanisms to minimize the damages due to HM ions. As primary defense to Cd stress, plant exudates such as malate or citrate bind to metal ions to avoid root absorption (Delhaize and Ryan, 1995) or physical interaction of HM with cell wall may immobilize toxic ions and prevents their uptake into the cytosol (Sanit`a di Toppi and Gabbrielli, 1999). Nevertheless, the high mobility and water solubility of Cd enable it to readily enter the roots through the cortical tissue and reach the xylem via an apoplastic and/or symplastic pathway (Salt and Rauser, 1995). As soon as the concentration of the toxic element rises above the threshold limit in cytosol, production of chelators involved in the detoxification and sequestration of the HMs in specific cellular compartments become imperative. It is well known that GSH functions as an HM-ligand (Ca´novas et al, 2004) and an antioxidant. Upon HM exposure, GSH concentrations drop as a consequence of initiated phytochelatin (PC) biosynthesis. The constitutively elevated GSH biosynthesis in different *Thlaspi* species was reported to be the indicator of their tolerance to Ni stress via mitigation of Ni dependent oxidative stress (Freeman et al., 2004). The production of PCs in response to As stress may play a significant role in As tolerance in plants but still needs a thorough study (Panda et al., 2010). It has been reported that the ability of high-rate PC synthesis is insufficient to cope with the metal load if the functionality of the antioxidant system is simultaneously hampered. Similarly, the GSH content was inversely linked to Cd sensitivity when comparing ten pea genotypes showing differing Cd sensitivity (Metwally et al., 2005). Moreover, as for other abiotic stress, HMs especially Cd resistance involves the synthesis of stress-related proteins and signal molecules (Sanit`a di Toppi and Gabbrielli, 1999) (Figure 2.3). The signaling pathway involves complex 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). HMs interferes with cell signaling pathways in all the biotic systems. Even in animals and humans, HMs activates the transcription factors nuclear factor kB (NF-kB) and activating protein 1 (AP-1), which control cell survival, differentiation, inflammation and growth (Valko et al., 2005). In plants, convincing evidence demonstrates interference of Cu, Pb, Zn and Cd with mitogen kinase signaling cascades. Plant jasmonic acid, salicylic acid and ethylene levels increase upon exposure to HMs (Metwally et al., 2003; Maksymiec et al., 2005; Rodriguez-Serrano et al., 2006). These plant hormones play controversial roles in HM tolerance, and both beneficial effects- the counteraction of Cd and Mn toxicity by the activation of antioxidants (Metwally et al., 2003; Shi and Zhu, 2008) and negative effects such as enhancement of H_2O_2 generation (Zawoznik et al., 2007) have been reported. By modulating components of the cellular signaling network, mostly through application of established inhibitors or effectors and overexpression of HM transporter, a systematic effort needs to be made to enhance the HM tolerance of the plants.

The databank of numerous putative genes involved in response to Cd-stress has been increasing with the genomics studies: for example, in *Brassica juncea*, receptor like kinase protein induction as metal sensor was reported during Cd stress (Fusco et al., 2005). Further, the activation of MAPK family kinases during Cd and Cu stress suggests the involvement of phosphorylation cascade in Cd signaling (Jonak et al., 2004). Cd stress was also found to stimulate Ca concentration leading to changes in calmodulin proteins that may at least regulates Cd tolerance in plants (Yang et al., 2003; DalCorso et al., 2008). In fact, Ni and Pb tolerance has been achieved in transgenic plants overexpressing tobacco calmodulin like protein (Arazi et al., 2000). Although HM induced gene regulation has been known for decades, the microarray analysis done in various plants viz., *Arabidopsis* (Kovalchuk et al., 2005; Herbette et al., 2006; Weber et al., 2006), pea (Romero-Puertas et al., 2004), and barley (Tama´s et al., 2008) indicates the mode of action of HM in different organs, depending on metal used and its concentration. The

genes (*AtFer1- AtFer4*) which encode ferritin were found to be differentially expressed during high Fe treatment in *Arabidopsis* roots and leaves (Connolly and Guerinot, 2002).

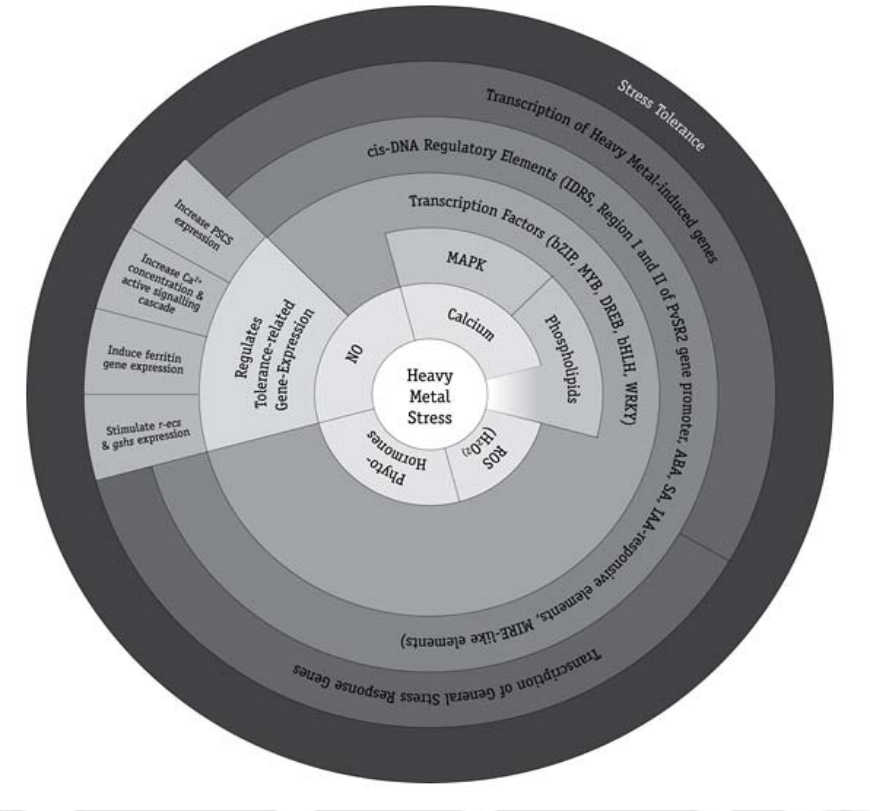

Fig. 2.3 Probable mechanisms of HM induced response in plants

The increased accumulation of jasmonates due to Cu and Cd exposure in mature leaves of *Arabidopsis* and *Phaseolus coccineus* suggests the transcriptional control of GSH biosynthesis genes during HM stress (Maksymiec et al., 2005). The specific up regulation of genes for zinc finger (C_2H_2) proteins, metal transporters (ZIP9), calmodulin-binding protein and ethylene receptor-related protein in *Arabidopsis* roots by Cd stress only not by Cu indicate specificity of plant response to organ and metal type (Weber et al., 2006). The transcriptome analysis of the rice roots during Cr (IV) stress suggested the vulnerability to short-term exposure of higher Cr concentration than environmentally relevant high concentration. Furthermore, it may be clear that GSH plays an important role for detoxification of Cr-stress as most of the genes related to GSH

metabolism, transport, and signal-transduction were differentially expressed upon Cr (VI) stress (Dubey et al., 2010). Recently, the Cd-responsive miRNAs were identified in rice upon Cd stress and the mRNA levels of several target genes were negatively correlated with the corresponding miRNAs under Cd stress suggesting their function in HMs tolerance in plants (Ding et al., 2011). Hence, HMs stress response in plants involves a number of pathways and stress signals which differs with organ, metal and concentration that overlap and transduce/crosstalk to impart HM tolerance. The overall stress network is poorly understood which needs further study to get a clear picture of plant HM response.

2.2.2 Key players of HM tolerance/sensitivity engineering

Plants respond to the HMs stress in specific and discrete manner to each HM via its varied response mechanisms. Understanding the plant-based molecular mechanisms is important to isolate plant genes responsible for the expression of the remediating phenotype. The identification and isolation of these genes may open the opportunity to ameliorate plants for environmental cleanup. For example, transferring a single gene involved in metal transport, such as HMA4, from *A. halleri* to *A. thaliana* has enhanced the leaves metal uploading potential of *A. thaliana*, a non-accumulator species (Hanikenne et al., 2008). Regarding metal-conjugates transport, plants overexpressing specific transport proteins such as CDF proteins might acquire elevated detoxification and compartmentalization of GS-HM conjugates into the vacuoles (Kramer et al., 2007). 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 developed Cd tolerance and accumulation (Eapen and D'Souza, 2005). Furthermore, Cd tolerance and accumulation is also enhanced by overexpression of γ-glutamylcysteine synthetase, an enzyme with an important role in controlling GSH 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). Recently, it has been

validated 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. Moreover, the comparison between hyperaccumulator with non-accumulator species (e.g. *A. halleri with A. thaliana*) suggests that the hyper-accumulating features could be due to sequence mutations, gene copy number or due to varied expression levels of the metal stress proteins (Plaza et al., 2007; Hanikenne et al., 2008). This indicates that genetic potential for metal detoxification is already present in plant genomes and those mutations probably affects both metal sensing and activation of appropriate responses, which make the difference. Depending upon the recent progresses made in the field of HM response mechanisms, we herewith discussed the upcoming key players of HM tolerance engineering.

2.2.3 Heavy Metals sensors

Plant root is the main site of HMs access has been proved by electron microscopy localization study of the majority of HMs accumulates in root cells than the cytoplasm (Andruini et al., 1999). Cd and Ni exposure has resulted in synthesis of apoplastic space localized proteins suggesting cell wall as prime metal sensing site (Blinda et al., 1997). Hence, primarily cell wall is the site of functional signaling molecule and metabolite in response to HMs (DalCorso et al., 2010). Study on HM sensing and signaling pathways in plants have been limited to the extracellular signal receptor-like protein kinases. It has been reported that the gene coding for lysine motif receptor-like kinase in barley is shown to be induced by Cr^{2+}, Cd^{2+}, Cu^{2+} during leaf senescence (Gleba et al., 1999). Further, the expression of metal induced barley receptor-like kinase was also reported to be facilitates by Ca^{2+} level. The proteomic study on Cd-treated rice roots indicates the induction of putative receptor protein kinase. However, more detailed study on the function of other Cd induced sensor has to be done.

2.2.4 Heavy metals induced signaling protein kinases

The HM stress responses in plants were reported to encompass calcium level changes, mitogen-activated protein kinases (MAPK) cascades and transcriptional modulation of the stress-

responsive genes (Shao et al., 2008, 2009). It was hypothesized that some HMs (Cd, Ni, Co) may cause a perturbation in the intracellular Ca^{2+} level and interferes with calcium signaling by substituting Ca^{2+} in calmodulin regulation (Kim et al., 2007). Yeh et al (2006) has reported that Cd^{2+} and Cu^{2+} induce ROS production and Ca^{2+} accumulation in rice roots and thereby activate the MAPK signaling pathways. The Cd^{2+} and Cu^{2+}induced MAP kinase activation required the involvement of Ca^{2+}-dependent protein kinase (CDPK) and phosphatidylinositol 3-kinase (PI3 kinase) as shown by the inhibitory effect of a CDPK antagonist, W7, and a PI3 kinase inhibitor, wortmannin, respectively . Hence, using a Ca^{2+} indicator, it was demonstrated that Cd^{2+} and Cu^{2+} induces Ca^{2+} accumulation in rice roots. Although CDPK and PI3 kinase may be involved in both Cd^{2+}- and Cu^{2+}-induced MAP kinase activities, Cd^{2+} and Cu^{2+} induce MAP kinase activation via distinct ROS-generating systems in rice roots, and their responsiveness may differ depending on the types of ROS involved (Yeh et al., 2006). Taken together, these findings implicate that Cd^{2+} and Cu^{2+} induce MAP kinase activation through distinct signaling pathways may lead to HM tolerance in plants.

Hsu and Kao (2003) have reported that ROS, Ca^{2+} and abscisic acid (ABA) were significantly accumulated in TNG67(Cd^{2+} tolerant) than in TN1(Cd^{2+} sensitive) during Cd^{2+} treatment and the accumulated ROS and Ca^{2+} subsequently might activate MAP kinase which may provide Cd^{2+} tolerance to rice plants. The fact that ABA plays crucial role in MAP kinase activation in plants was further strengthen by the report on MAP kinase activation in barley aleurone and pea leaves (Knetsch et al., 1996; Burnett et al., 2000), Thus, low levels of MAP kinase activity of TN1 may also be due low levels of ABA content in TN1 and the HM induced Ca^{2+} accumulation and MAPK activation in tolerant TNG67 rice plants may be permanent as compared to transient accumulation in sensitive plants. Furthermore, it has been reported that the treatment of tobacco cells and Scots pine roots with Cd and lupine roots with Pb^{2+} caused the generation of H_2O_2 (Rucińska-Sobkowiak et al., 2006). The Cd-producing oxidative burst in tobacco in response to Cd is mediated by calmodulin and/or calmodulin-dependent proteins

(Haydon and Cobbett, 2007). Thus, available data suggest the involvement of Ca/calmodulin pathway in signaling of metal response in plants. The MAPK pathway is involved in the transduction of extracellular signals to intracellular targets in all eukaryotes (Li et al., 2006). MAPKs pass on the cytoplasmic signal to nucleus, where they activate other protein kinases, specific transcription factors and regulatory proteins (McCully, 1999; Shao et al., 2008). It was recently indicated that Cd and Cu activate four different MAPKs (SIMK, MMK2, MMK3, and SAMK) in *Alfalfa,* whereas Cd induces one such kinase (AtMEKK1) in *Arabidopsis* and one (OsMAPK2) in rice (Shao et al., 2010). However, it is not clear if activation of MAPKs occurs by these HMs or ROS or it occurs via action of other mediators and therefore, the MAPK responsiveness may differ depending on the type of metals and ROS involved.

2.2.5 Heavy metals induced NO signaling

Xiong et al (2010) reported that HM toxicity lead to changes in the endogenous nitric oxide (NO) content in plants and thereby NO plays various roles in alleviating the HM toxicity. *In vitro*, an increase in NO production has been observed in *Arabidopsis* cell suspension cultures under a 300 µM Fe stress (Arnaud et al, 2006). Similarly, soybean cells treated with 4 or 7 µM Cd^{2+} for 72 h exhibit a dose dependent and rapid production of NO, which may suggest that NO functions as a signal molecule involved in alleviation of the HM stress (Kopyra et al., 2006). Many authors have reported contradictory results like in cell suspension that a short HM treatment period promoting a NO burst and a long treatment decreases NO generation. Groppa et al (2008) propose that these opposite results could be explained by the use of different HM concentrations, variable age of sample and plant tissue, duration of treatment used.

Recent transcriptomic studies indicate that genes regulated by NO modulate a diverse set of cellular functions, a pleiotropic role of this molecule in plant physiology (Zago et al., 2007). Although a detailed study has been reviewed on stress-related genes modulated by NO (Grün et al., 2006), little is known about heavy metal tolerance-related genes modulated by exogenous NO. Exogenous NO was thought to attenuate oxidative stresses by decreasing the H_2O_2 content

enhancing the activity of antioxidant enzymes like SOD, APX, and CAT. Whether the enhanced activity follows from increased gene expression or from the post-translational medication of the respective proteins has not been investigated (Grün et al., 2006). HM-induced accumulation of NO also appears to be responsible for HM toxicity. These convicting results on the relationships between NO and HM toxicity are attributed to the impacts of HM on NO content and the various pathways of NO production in plants. Further studies may facilitates to understand the networks involved in plant defenses against HM stress and the roles of NO in regulating both ion homeostasis and cellular responses to heavy metals (Xiong et al., 2010).

2.2.6 Hormonal *cis*-DNA regulatory elements

The promoter analysis has revealed that the respective *cis*-DNA regulatory elements for abscisic acid (ABA), salicylic acid (SA), and auxin (IAA) were detected in HM-induced genes suggesting the involvement of such signaling in HM response. The auxin-responsive mRNA was detected in Cd-treated *Brassica juncea* plants (Minglin et al., 2005). Proteomic analysis of Cd-treated *A. thaliana* showed the induction of nitrilase protein, which is involved in auxin biosynthesis and the transcription activation of the gene (SAMT) involved in biosynthesis of SA was detected in pea treated with Hg (Shao et al., 2010). It is known that Cd induces the biosynthesis of ABA and ethylene, which in turn evoke various stress responses (Polle and Schützendübel, 2004). Furthermore there are reports in other systems where the interference between HMs and growth hormones affects gene expression. Vergani et al (2009) reported that in fish RTH-149 cell line, the crosstalk between HMs and growth hormones regulate metallothionein expression which does not strictly depend on Ca^{2+} signaling but activates ERKs which enhances metallothionein because of differential recruitment of transcription factors. These results suggest that phytohormones may have a role to play in plant response network to HM. However, further endeavours need to be made in this direction to dissect out whether these hormones helps in signaling for the activation of HM-responsive genes, or serve as effectors of certain HM-obligatory reactions or participate in both processes (Cobbett and Meagher, 2002; Gasic and Korban, 2007; Doty, 2008).

2.2.7 Heavy metals responsive transcription factors and cis-regulatory elements

The modulation of transcriptional processes in plants in response to HMs and its functional link between signaling pathways and responses has not been deeply studied. The transcriptome analysis of plants treated with various HMs has revealed that HMs can induce transcription factors that regulate corresponding transcriptional processes (LeDuc et al., 2006). The Cd-induction of transcripts for basic region leucine zipper (bZIP) and zinc finger transcription factors has been detected in *A. thaliana* and *Brassica juncea* (Ramos et al., 2007). Members of the ERF subfamily are reportedly involved not only in ethylene signaling but also in plant responses to various biotic and abiotic stresses. The AP2/ERF superfamily is a large gene family of transcription factors characterized by a unique AP2/ERF domain (Nakano et al., 2006). It has been reported that ERF1 and ERF5 were induced by Cd in *A. thaliana* (Herbette et al., 2006). Induction of ERF genes by Cd in *A. thaliana* and *A. halleri* has also been reported (Weber et al., 2006). Diverse patterns of ERF gene expression induced by Cd suggest that each of the ERF proteins might be specific to different genes that respond to various levels of Cd stress. It has been reported that dehydration-responsive element-binding protein (DREB) transcription factors gets down-regulated after HM treatment. DREB1A, DREB1B, and DREB1C are involved in induction of cold stress-responsive genes, and DREB2 is involved in osmotic stress-responsive gene expression in *A. thaliana* (Nakashima and Yamaguchi-Shinozaki, 2006). Although it is unclear why these stress-related transcriptional regulators were down-regulated under Cd-stressed environments, they might play an important role in transcriptional regulation of stress- responsive genes during the Cd acclimation process in plant roots. This phenomenon may be explained as when the plant senses the HM stress, the DREB gene family gets down regulated to maintain a normalized osmotic potential across the cell membrane so as to reduce the inflow of HM contaminated water. This will mitigate the HM concentrations at the cellular level and the loading of HM to transport vessels to avoid phytotoxic affects. This may provide a preliminary line of defense of plants to sustain in hostile HM contaminated environments.

Screening of Cd-responsive genes in *A. thaliana* indicates that DREB2A gene is upregulated by Cd (Bennett et al, 2003; Curie and Briat, 2003). Qi et al (2007) reported Cd-induced transcription factors (OBF5) which bind to the promoter region of the GSH transferase gene (GST6) in *Arabidopsis* which was known to be induced by auxin, SA and oxidative stress. Despite existing data on the HM-induction of different transcription factors, it is still not clear if these activations are specific to a particular HM, common to most of the metals, related to oxidative stress. The process of ROS mediated transcription activation of factors is thought to be a common link in different stress responses in plants. Therefore, among all possible pathways, ROS seems to play a key role, but not the only role, in activation of HM induced transcription factors in plants. Other organisms, such as yeast and animals, contain specific HM induced transcription factors which bind to the HM responsive element present in promoters of HM responsive genes. It has been reported that MTF-1 controls HM load by binding with MREs (TGCRCNC) present in metallothioneins and other HM genes from insects to mammals. In contrast, it has been reported that copper starvation has led to evolution of *Drosophilidae* specific regulation of the *Ctr1B* copper importer via MREs/MTF-1(Selvaraj et al., 2005). This indicates to a regulatory mechanism where the same transcription factor exerts two diverse functions in response to the opposite environmental situation (Selvaraj et al., 2005). Whereas in *Saccharomyces cerevisiae*, it has reported that the two extremes of Cu availability requires different transcription factors. The homologs of Ctr1 that import copper are activated upon copper starvation by the Mac1 transcription factor (Yamaguchi-Iwai et al., 1997); the activation of metallothionein genes upon copper load is driven by the transcription factor Ace1. Furthermore, in mammals, there exists Ctr1 and Ctr2 (Rutherford and Bird, 2004); neither of which is regulated at transcriptional level by Cu availability, and there are no MREs in their promoter region (Selvaraj et al., 2005). Dormer et al (2000) presented evidence that the transcription factors Met-4, Met-31, and Met-32 are involved in Cd induced gene regulation whereas the DNA-binding protein Cbf1 acts as negative regulation of *GSH1* expression. It is seen here that HM responsive

transcription factors is involved in various gene expression and other cellular functions. Rutherford et al (2004) described the varied roles of HM induced transcription factors in his excellent review that focuses on the role that HM-responsive transcription factor have in regulating trace metal metabolism, sensing HM and coordinating the expression of genes that are involved in the HM stress response.

The *cis*-acting elements related to HM responsive elements have been found within promoters of a few plant genes, including metallothionein-like genes; however, there is no evidence that these sequences confer HM responsiveness on these genes. So far, Deckert (2008) reported existence of *cis*-DNA elements in plants, which may be functional in HM response viz., iron-dependent regulatory sequences (IDRS) responsible for the Fe-regulated transcription of genes and recently identified element in the promoter region of the *PvSR2* gene from *Phaseolus vulgaris*, whose expression is upregulated by Hg, Cd, As and Cu. The HM-responsive elements were localized within two regions of the *PvSR2* gene promoter- Region I (similar to MRE of metallothionein genes) and region II (represents a novel HM responsive element in plants and has no similarity to other *cis*-acting DNA elements) (Cunningham et al., 1995; Fox and Guerinot, 1998; Shao et al., 2009). It seems obvious that plants employ a wide array of mechanisms to activate the genes required to cope with the HM stress and to confer other regulatory functions. Depending on the available information of possible molecular mechanisms of plant response to HM signaling pathways and transcription regulation, a conceptual model has been postulated (Figure 2.4). This proposed model simply put forward the putative targets where the further experimental endeavour may be directed to develop HM tolerant plants.

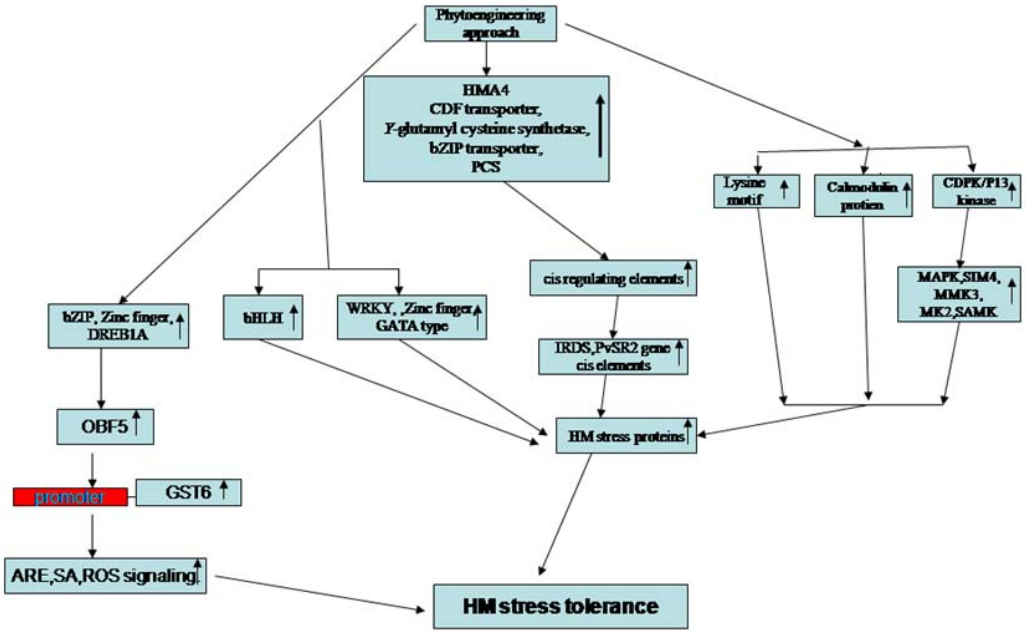

Fig. 2.4 A regulatory target based conceptual model for HM tolerance phytoengineering. (↑ indicates upregulation of the target genes)

2.2.8 Heavy metal stress signaling and crosstalks

The plant prefers to change gene activation patterns after sensing the HM stress stimuli through various signaling cascades (DalCorso et al., 2010). Several molecular techniques have been established to study the response mechanisms in a global view against abiotic stress in plants. Studies on HM responses have also seen the emergence of proteomic analysis as a promising tool. The recent findings of proteomic studies on the effects of metal ions showed the importance of transporter proteins in roots, the effect on primary metabolism, most noticeably the importance of the sulfur assimilation and metabolism in roots, as well as phytochelatin and GSH synthesis (Roth et al., 2006; Aina et al., 2007; Kieffer et al., 2008; Sarry et al., 2008). The plants response to combined environmental stresses is unique and need thorough understanding to predict and avoid underscoring of the effects of crosstalks (Zurbriggen et al., 2010).

To discuss the HM induced proteomic changes in the plant system, we would like to corroborate with the excellent study done by Kieffer et al (2009) in poplar against Cd stress.

Their study reveals the early accumulation of many typical stress-related proteins like heat shock proteins (HSPs), or glutathione-S-transferases, while most proteins from the primary metabolism (glycolysis, TCA cycle, nitrogen and sulfur metabolism) were severely decreased in abundance due to Cd stress and the important metabolic proteins regulation has been depicted in Figure 2.5. The upregulation of the molecular chaperones and PCs in the early stage may be due to avoid the misfolding of crucial proteins and enzymes and to repair the HM degraded proteins. Further, the increased synthesis of PCs may lead to faster chelation of the HMs to mitigate the cellular damage.

Furthermore, the increased abundance of the tricarboxylic acid (TCA) cycle proteins at later stages of HM stress may be to provide energy supplement to help plants acclimatize in hostile environments. Thus, it seems that plants follows a definite pattern of prophylactic and avoidance measures in the early stages of HM stresses but when the HM stress prolongs, the plants preferred to opt for survival and acclimatization by conserving energy and switching off large fraction of proteins. This varied expression of proteins and gene regulation suggests that HM stress response is a coordinated complex process which brings together the coherent action of different cellular processes. Since roots are the first tissue system to counter the HM ions and consequently, proteomic changes may be seen earlier than in leaves (Kieffer et al., 2009). Furthermore, the energy conserving phenomena in Cd treated plants may be due to Cd stress, where Cd affects leaves later by blocking CO_2 uptake or by interfering with the guard cell regulation via Ca^{2+} channels (Perfus-Barbeoch et al., 2002), and it also has a direct impact on electron transport in chloroplasts by damaging photosystems I and II (Sanita' di Toppi and Gabbrielli, 1999). This would greatly increase the ROS production in chloroplasts even though the overall Cd content in leave is less. Just to summarize the importance of validating observed protein changes, it can be said that the changes can be related to an increase or decrease in abundance, but they can also result from posttranslational modifications (PTM) or may be related

to degradation processes. Having said these, the detailed proteomic study during HM stress will definitely provide new leads to tackle HM stress and it definitely seeks attention for further study.

The HM stress was reported to alter the cellular level of Ca and calmodulin proteins, which regulate ion uptake, gene activation, metabolism apart from contributing to Cd tolerance suggesting a link to crosstalks (Yang and Poovaiah, 2003). Furthermore, the reduced GSH level increases during Cd stress with parallel upregulation of antioxidant enzymes and response genes (Romero-Puertas et al., 2007). The increased accumulation of the jasmonic acid, ethylene and salicylic acid (SA) in roots of Cd stressed roots indicate towards the existence of crosstalk between biotic stress and HM stress (Maksymiec, 2007). The induction of the phytoalexins biosynthesis in response to both HM stress and ROS-mediated biotic stress points to sharing of common signaling pathways (Mithoefer et al., 2004). It is important to mention here that HM signaling in plants is specific for each HM and it differs for each metal. Furthermore, the elevation of NO level in response to HM stress does suggests the HM signaling share common pathway in plant defense response also (Xiong et al., 2010).Taken together all, elevation of Ca level, calmodulin and MAPK activation, GSH mediated regulation, ROS and NO signaling and stress related hormone signaling all culminate through diverse pathways to activate transcription factors to activate stress responsive genes to impart HM tolerance.

2.2.9 Conclusion

There is a dearth of data in HM extracellular signal perception, and now it is believed to be mediated by receptor like protein kinase during HM stress. The induction of putative receptor like kinase during Cd treated rice root proteomic study points towards its role as metal sensors (Yeh et al., 2007). The plant HM response and accumulation seem to vary with each HM type, time and exposure of HM stress, organs and transport process to shoots, which signifies role of various HM response mechanisms in plants (Savenstrad and Strid, 2004; Haydon and Cobbett, 2007). The up regulation of an array of stress-related proteins like HSP, proteinases and PR-related proteins and GSH metabolism related proteins, signaling proteins upon HM stress suggest the involvement

of diverse networks of stress pathways ultimately leading to plant HM tolerance. Further, some crucial questions still remain to be answered *viz.*, which mechanisms link HM stress intimately to redox imbalances and oxidative stress? Why during HM stress, even in the presence of effective scavenging and export mechanisms, metals conversely bind to high-affinity targets? How HM-binding can disturb cellular metabolism? What governs the plant organs to behave in a specific way to HM stress? Does the plant conserve energy during HM stress or is it due to the HM stress? Does the HM tolerance engineering of plants demands modulation of single regulatory gene or a family of multigene in a coordinated network? A systematic understanding of implicated mechanisms is unavailable, and it certainly should be a major goal for future research prospects to answers the raised questions.

Further we need to look deep into the HM signaling and crosstalks with other stresses and its eventual effects. It has been reported recently that the exogenous NO plays role on alleviating heavy-metal toxicity in plants. However, the understanding of how the NO alleviates heavy-metal toxicity and its role in plant defense mechanisms has to be studied. To explain such differences, a better understanding of the redox network of the plant cell is mandatory. We need to answer the question that why do the signaling cascades fail to activate appropriate compensation mechanisms when subjected to HM stress? Further research directions might concern how the plants redox mechanism behaves during multi metals stress as practically there exist numerous HMs in soils? The varied HM stress induced proteins expression suggests that a coordinated network of proteins and genes work together to finally reach at HM response. This require effort from all the specialized laboratories and institutes throughout the world to work as unit to converge at a point of the master regulator, which can be modulated to achieve HM stress tolerance.

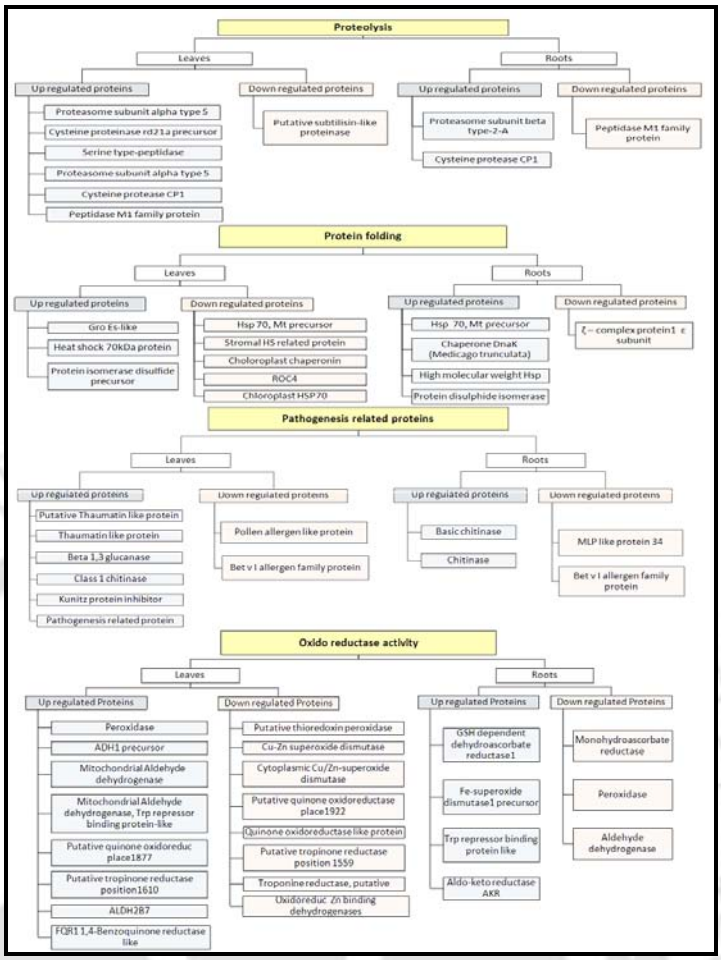

Fig 2.5 Varied proteomic regulatory expression profile of Cd treated poplar plant showing the evidence HM stress affects different functional networks. (Kieffer et al 2009).

2.3 Plant heavy metal stress responsive proteins and *Eichhornia crassipes*

HM toxicity poses major environmental and health problems. Cd, for example, is a non-essential HM which is toxic to living cells at very low concentration. Cd^{2+} ions displace Ca^{2+} or Zn^{2+}in proteins and can cause oxidative stress (Stohs and Bagchi, 1995; Goyer, 1997). In humans, Cd^{2+} is a suspected carcinogen (Lemen et al., 1976). It has been reported that uterus and breast cancer can be induced by exposure to Cd even at the so called safe levels (1 mg/kg.d) stipulated by the World Health Organization (Cuypers et al., 1999). Using plants to remove HM pollution from the environment is known as phytoremediation (Raskin et al., 1994; Cunningham et al., 1995; Salt et al., 1998), and this is a technique that shows promise for the future. One possible approach for

phytoremediation is to use natural heavy metal hyper accumulators. More than 400 species of plants have been reported to accumulate high levels of HMs in their above ground parts (Baker and Brooks, 1989). However, most of them are not suitable for direct phytoremediation because they are slow-growing, have low biomass, and are difficult to manage. Genetic engineering has been used to modify plants in recent years so as to improve the efficiency of phytoremediation. Thus, the main concern now is the selection of candidate genes and host plants to isolate HM stress responsive genes for phytoengineering.

2.3.1 Glutathione and heavy metal stress tolerance in plants

GSH has been detected virtually in all cell compartments such as cytosol, chloroplast, endoplasmic reticulum, vacuole, and mitochondria. It is the predominant cellular non-peptide thiol supplier of the cells. The chemical reactivity of the thiol group of GSH makes it particularly suitable to serve a broad range of biochemical functions in all organisms This reactivity along with the relative stability and high water solubility of GSH makes it an ideal biochemical to protect plants against stresses including oxidative stress, HMs and certain exogenous and endogenous organic chemicals (Millar et al., 2003; Foyer and Noctor, 2005; Rausch et al., 2007). Several studies have indicated that exposure of plants to high level of HMs induces ROS, either directly or indirectly by influencing metabolic processes. GSH participate in the control of H_2O_2 level of plant cells (Foyer and Noctor, 2005; Shao et al., 2005). Change in the ratio of its reduced (GSH) to oxidized (GSSG) form during degradation of H_2O_2 is important in certain redox signaling pathways (Millar et al., 2003). It has been suggested that the GSH/GSSG ratio, an indicative of the cellular redox balance, may be involved in ROS perception.

Reduced GSH acts as an antioxidant and involve directly in the reduction of most ROS generated during stress (Millar et al., 2003; Foyer and Noctor, 2005; Shao et al., 2008). Additionally, GSH plays fundamental role in many cellular detoxification processes of xenobiotics and HMs. GSH does this by prior activation and conjugation with such compounds (Marrs 1996; Alfenito et al., 1998). The conjugation of GSH with such molecules is governed by

glutathione S-transferase (Edwards et al., 2000; Edwards and Dixon, 2005). The conjugates are subsequently transported to the vacuole and protects plant cell from their harmful effect (Klein et al., 2006; Yazaki, 2006). But the massive use of reduced GSH in xenobiotic or HM detoxification results, at least transiently, in decrease of cytosolic GSH content. This impinges directly on the GSH/ GSSG redox potential, generating a redox signal in stress-exposed cells (Nocito et al., 2006). Consequently, any massive upgrading of GSH-based detoxification processes will impact on cellular redox poise. Therefore, under such circumstances maintenance of GSH/GSSG ratio become very crucial for the survival of plants. Under standard growth conditions, ROS levels in a plant cell are under tight control of scavenging systems that include GSH. Excess ROS formed within cells can provoke oxidation and modification of cellular amino acids, proteins, membrane lipids and DNA. These changes lead to oxidative injuries and result in the reduction of plant growth and development (Ogawa and Iwabuchi, 2001). Role of GSH in ROS detoxification starts at an early stage of plant development. This has been known from a recent study on T-DNA insertions in *AtGSH1*, a gene encoding γ-glutamylcysteine synthetase. Loss of function of this enzyme results in a recessive embryo-lethal phenotype in *Arabidopsis* (Cairns et al., 2006). Additionally, GSH is also indirectly involved in the glutaredoxin (Grx)-mediated redox control of many cellular proteins. Multiple forms of Grx are kept in the reduced state by NADPH, GSH reductase and GSH (Rouhier et al., 2005). Grx is one of the important components in redox-mediated developmental processes, such as flowering (Xing et al., 2006). As described above, one protective role of GSH in plants during HM stress exposure is the quenching of ROS. Secondly, GSH acts as a precursor for the synthesis of PCs which is discussed in following paragraph. This function makes GSH an important factor contributing towards HM tolerance in plants.

2.3.2 Phytochelatins and Plant metal hypertolerance

PCs play an important role in metal detoxification in plants and fungi. PCs are effective at binding peptides with bivalent cations such as Cd^{2+}, Cu^{2+} and Co^{2+}. PCs have the structure (γ-

Glu-Cys)n-Gly (n = 2–11) (Rauser, 1990); the Cys thiol group of PCs is bound to free Cd^{2+} ions via a coordinate bond, and then the PC-Cd complex is transported into vacuoles in plant or yeast cells. In this way, Cd is sequestrated in vacuoles through chelation and compartmentation to protect the cell from harm caused by Cd (Rauser, 1995, 1999). Detoxification mediated by PCs is one of the most important and general mechanisms in plants (Cobbett, 2000 a, b). PCs are synthesized by phytochelatin synthase (PCS) from the substrate GSH. This enzyme is activated by a number of metal ions, including Cd^{2+}, Cu^{2+} and Co^{2+}. Among these, Cd is the most effective (Cobbett, 2000a, b). Glutathione synthetase (GS) and gamma-glutamylcysteine synthetase *(γ-ECS*) are the key enzymes in the last two steps of GSH synthesis. This is achieved through transpeptidation of the c-Glu-Cys moiety of GSH, via c-Glu-Cys dipeptidyl transpeptidase (PCS, EC .3.2.15), to another GSH or to a growing PC peptide (Cobbett, 2000a, b). PCs form complexes with HMs followed by subsequent compartmentalization of these complexes into vacuoles in plant cells (Zenk, 1996). PCs have been extensively studied in terms of their biosynthesis, metal-binding properties, identification of Cd-activated PCS activities, and ATP-dependent transport of PC–metal complexes into vacuoles for sequestering and compartmentalization of toxic HMs (Grill et al., 1989; Rauser, 1999; Cobbett 2000a). The PCS is constitutively expressed in plants and its enzyme activity appears to be self-regulated as newly synthesized PCs chelate enzyme-activating metals, thereby terminating the enzyme reaction (Loeffler et al., 1989).

Detoxification of xenobiotic compounds and HMs is a pivotal capacity of organisms, in which GSH plays an important role. In plants, electrophilic herbicides are conjugated to the thiol group of GSH, and HM ions form complexes as thiolates with GSH-derived PCs. In both detoxification processes of plants, PCS emerges as a key player. The importance of the PC pathway for the detoxification of Cd^{2+}, arsenite and arsenate prompted the question- even before the function was unequivocally shown. Whether PC synthesis is also involved in the naturally selected metal hypertolerance shown by several plant species? Hypertolerance, i.e. the capacity to

sustain growth in the presence of much higher toxic metal concentrations than tolerated by plants possessing basal tolerance levels (Clemens et al., 1999), is found not only among metal hyper accumulators. Studies on various *Silene vulgaris* accessions and on As (V) hyper tolerant *Holcus lanatus* clones (Schat et al., 2002). When Cd/Zn- or Cu-hyper tolerant *S. vulgaris* lines were compared to *S. vulgaris* lines from non metallicolous soil, it was consistently found that the more tolerant plants showed significantly smaller PC accumulation rates when compared at same-effect concentrations or conditions of equal uptake rates (de Knecht et al., 1994). This was interpreted as evidence for the expression of other efficient sequestration pathways in the hyper tolerant populations which would reduce the amount of toxic metals available for activation of PC accumulation. For As, on the other hand, a positive correlation between degree of tolerance and rate of PC production was determined (Hartley-Whitaker et al., 2001). These findings were re-evaluated and extended in a recent study by Schat et al (2002). Appropriate contrasting pairs of plant accessions were analyzed to determine whether altered PC synthesis contributes to hyper tolerance of Cd, As, Ni, Zn, Cu and Co. Tolerance was measured in the absence and presence of the γ-glutamylcysteine biosynthesis inhibitor BSO. The results can be summarized as follows: As hyper tolerance is indeed PC-dependent whle Cu, Cd, Zn, Co, and Ni hyper tolerance are not. Closely connected to these findings and a consequence of the interest in phytoremediation that sparked some of the work leading to the molecular elucidation of PC synthesis is the question, whether PCS genes could be exploited to enhance metal tolerance and/or accumulation rates in transgenic plants. The outcome of most respective studies so far is, that a simple overexpression of PCS genes results in only a slightly enhanced metal tolerance (Pomponi et al., 2006) even though expression in cells that normally do not produce PCs can lead to a dramatic enhancement of Cd^{2+} tolerance-as seen for *S. cerevisiae*. On the contrary, *AtPCS1* over expression in *A. thaliana* was reported to result in higher Cd^{2+} sensitivity (Lee et al., 2003).

2.3.3 Heat shock proteins and heavy metals

The heat shock response and the metal-induced stress share many features at the molecular level. Both phenomena induce HSPs ranging from sHSPs to the larger HSPs, such as HSP105. The central component of the heat shock response is oxidative stress, which in fact is also a typical As -related effect. These stimuli lead to up-regulation of heat shock factor (HSF) phosphorylation and hence, HSP induction. However, it is suggested that the pathways of HSF phosphorylation induced by heat or arsenite (As) are different, implying distinct mechanisms of transcriptional control (Elia et al., 1996). AP-1-binding elements have been implicated in $CdCl_2$ and As induction, whereas STREs and AP-1 elements are involved in response to a range of stresses in yeast (Ruis and Schuller, 1995; Grably et al., 2002).

HMs induce the expression of a number of genes. It is known that Cd activates stress response genes including those coding for metallothioneins (MTs) and HSPs, as well as many other genes (Yamada and Kiozumi, 2001; Seok et al., 2006). Metal response is mediated by a conserved DNA element, MRE (metal responsive element) usually present in multiple copies in the upstream regions of MT genes. It has been shown that the activation of a Zn finger transcription factor MTF-1 (MRE-binding transcription factor-1) is essential for MRE-mediated transcriptional induction by metals. Although MREs are associated with a number of metal-inducible genes, there also exist many genes that are inducible by metals but lack MRE, suggesting that such genes are probably controlled by distinct mechanism. Transcription of the human hsp70 gene, known to be activated by heat stress, is also induced by a variety of heavy metals including Zn, Cd, Cu, Hg and Ag (Murata et al., 1999). It has been shown that the induction of the hsp70 gene by HMs as well as by heat shock is mediated by binding of HSF1 to its cognate recognition sequence; HSE (Mosser et al., 1988; Williams and Morimoto, 1990). Uenishi et al (2006) for the first time showed that two metal responsive transcription factors, MTF-1 and HSF1 were able to interact with each other.

2.3.4 Metallothioneins

Higher plants when exposed to heavy metals leads to production of two major types of cysteine-rich, metal-binding peptides, the metallothioneins (MTs) and the PCs (discussed above). Differently from PCs, which are enzymatically synthesized from GSH, MTs are low-molecular-weight proteins which contain cysteine-rich domains at the amino- and carboxy-terminal regions (Cobbett and Goldsbrough, 2002). MTs are gene-encoded polypeptides that are usually classified into two groups. Class 1 MTs possess cysteine residues that align with a mammalian (equine) renal MT; Class 2 MTs also possess similar cysteine clusters but these cannot be easily aligned with Class 1 MTs (De Miranda et al., 1990; Robinson et al., 1993; Prasad 1999). Since they were first purified from horse kidney (Margoshes and Vallee, 1957), MT genes and proteins have been discovered in many prokaryotic and eukaryotic organisms. Many genes encoding MTs have been isolated in plants and information about their putative function was obtained from gene expression studies. MT genes are part of a multigene family whose members are differentially regulated in relation to developmental stages and are implicated in the response to wounding, pathogen attack and oxidative stress (Zhou et al., 2005).

It has been reported that MT2 mRNA was strongly induced in *Arabidopsis* seedlings by Cu, but only slightly by Cd and Zn (Zhou and Goldsbrough, 1994); when genes for MT1 and MT2 from *Arabidopsis* were expressed in an MT-deficient yeast mutant; both genes complemented the mutation and provided a high level of resistance to Cu. There is further interesting report which states that MT genes can be induced by Cu, and that the expression of MT2 RNA is increased in a Cu-sensitive mutant of *Arabidopsis* that accumulates high concentrations of Cu (Van Vliet et al., 1995). 10 ecotypes of *Arabidopsis* were surveyed and a clear correlation between the Cu sensitivity of seedlings and the expression of MT2 RNA was shown (Murphy and Taiz, 1995a, b). By contrast, in a study of the effects of Cd exposure on *Brassica juncea*, it was reported that MT2 expression was delayed relative to PC synthesis (Haag-Kerwar et al., 1999) and they argued against a role for MT2 in Cd detoxification. Thus the role of MTs remains to be established. They could clearly play a role in metal metabolism, but

their precise function is not clear; they may have distinct functions for different metals (Hamer, 1986). It has been hypothesized that the cysteine residues found in MTs might be involved in ROS detoxification and maintenance of the redox level. In animals, MTs are known to function as ROS scavengers and to confer protection against oxidative DNA damage (Nury et al., 2005). In plants, many genes and cDNAs encoding MTs have been isolated, with functions involved in the response to HM-induced stress. In *Arabidopsis*, rice and tobacco the expression of Type 1 MT is induced by copper (Cobbett and Goldsbrough, 2002) while *Silene vulgaris* populations with Cu tolerance are characterized by higher mRNA levels and gene copy number of a Type 2 MT gene (Vah Hoof et al., 2001). MTs have been recently identified and characterized also in poplar. Six different genes are present in the hybrid *P. trichocarpa* x P. *deltoides genome* (Kohler et al., 2004). The occurrence of multiple MT genes in poplar might reflect their different roles in HM sequestration and in other mechanisms essential for plant growth. In a recent study, marker free transgenic lines of *P. alba* cv. '*Villafranca*' expressing a *Pisum sativum* Type 2 metallothionein (PsMT), produced using the MAT (Multi-Auto Transformation) technology, showed increase tolerance to HM toxicity (Balestrazzi et al., 2009). Since overexpression of MTs has been generally recognized as an effective approach to improve stress tolerance *in planta*, further investigation are required to better define candidate MT genes suitable for genetic engineering.

2.3.5 Heavy metals induced aquaporins/transporters

Aquaporins are membrane localized such as of tonoplast and plasma membranes, and function as water channels and play a role in water managements in plants (Kaldenhoff and Fischer, 2006). The aquaporins namely TIPs, PIPs, SIPs, and a NIP respectively, were reported to be significantly down-regulated in Cd stress (Hirotaka et al., 2010). So far, little information is available regarding transcriptional regulation of aquaporins under Cd stress except for a report on induction of a PIP gene in barley roots by Cd (Tama ´s et al., 2008). In *A. thaliana*, the expression of TIP and PIP genes were reported to be down-regulated by salt and drought stresses (Alexandersson et al., 2005; Boursiac et al., 2005). The down regulation of aquaporin genes may be due to the fact

that exposure to HM might leads to a potential dehydration stress in plant root, which possibly may be attributed to the unavailability of aquaporins leading to disruption of water and solutes exchange across the membranes. Consequently, this will inhibit the HM contaminated water transport into the cell and hence the plant may strive to adapt in the HM polluted areas. Hirotaka et al (2010) reported diverse range of unigenes regulation in *Solanum torvum* Sw. cv. Torubamubiga (TB) roots at low Cd 0.1µM concentration (Fig 2.6), showing down regulation of dehydration-related transcription factors and aquaporin isoforms. Together with the transcriptional regulation of dehydration-related transcription factors and other regulators, including DREB and ERF proteins, these implies that drought stress is one of the important constituents of the impediments caused by HM stress in roots. The inhibitory effect of HM on aquaporins may explain the transcriptional regulation of dehydration-related genes in plant roots during HM stress.

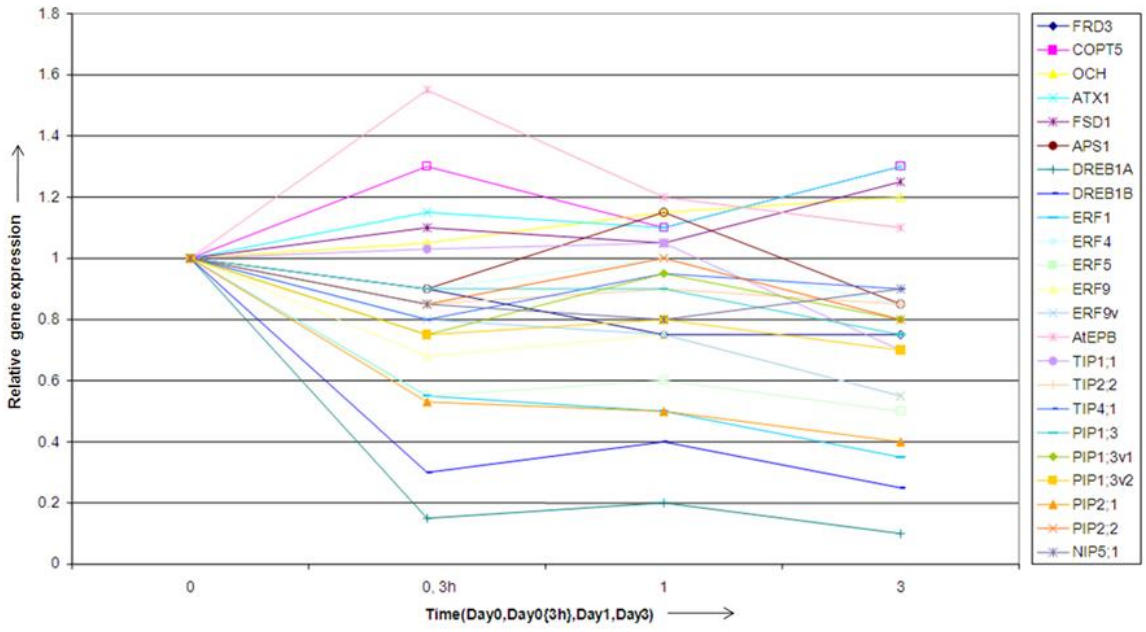

Fig. 2.6 Expression profile of various unigenes showing varied role in HM response in plant roots. The Cd treatments of 0.1µM are depicted as 0 day, 0 day, 3 h, 1 day and 3 day.

It has been reported that low loading of Cd into xylem in the roots is responsible for the reduced translocation of Cd to the shoots (Mori et al., 2009). The transcriptional regulation of metal transporter genes that might be involved in the reduced amount of Cd and acclimation processes during Cd treatment was examined. Members of the P1B-ATPase family are involved in the transport of HM ions in higher plants (Baxter et al., 2003; Williams and Mills, 2005). In *Arabidopsis*, eight family members were identified, and heavy metal ATPases, HMA1–HMA4 and HMA5–HMA8 were predicted to transport Zn/Cd/Pb/Co and Cu/Ag, respectively. HMA2 and HMA4 are exporters of Zn and Cd, and their involvement in HM tolerance and metal hyper accumulation has been characterized (Mills et al., 2003; Hussain et al., 2004; Courbot et al., 2007). It is expected that Cd ions are taken up via divalent cation transporters such as ZIP transporters. In addition, a member of the ABC transporter AtPDR8 has been reported to contribute to HM tolerance by mediating efflux of Cd from plasma membrane of root epidermal cell (Kim et al., 2007). Recently, expression of another family class of metal transporters NRAMP (natural resistance-associated macrophage protein), AtNRAMP6 in yeast resulted in Cd mobilization from storage organelle or accumulating Cd to cellular compartment where it is toxic (Cailliatte et al., 2009). This suggests that NRAMP transporters contribute to Cd toxicity. Furthermore, HMs were also reported to induce CDF (cation diffusion facilitator) family associated with cytoplasmic efflux and vacuolar sequestration of Zn, Cd, Co, Ni and Mn (Kramer et al., 2007).

2.3.6 Water hyacinth-A hyperaccumulator

The water hyacinth (*Eichhornia crassipes* (Mart.) Solm-Laubach, *Pontedericeae*) *is* a floating macrophyte, thrives in polluted waters and literally takes over whole ecosystems. Continued massive herbicide and chemical use have added another level of pollution with little net benefit. On the other hand, Hyacinth has high affinity and capacity for metals, is used as a biomarker and is introduced in wetlands for water phytoremediation (Ismail and Abael-Sabour, 1996; Zhu et al., 1999). Data on the metal-binding capacity and selectivity of Hyacinth and its metal binding

components can help in assessing its environmental functions, impact and risk as nutritional biomass. However, its enormous biomass production rate, its high tolerance to pollution, and its HM and nutrient absorption capacities (Jayaweera and Kasturiarachchi, 2004) qualify it for use in wastewater treatment ponds. These attributes of *E. crassipes* and its availability in the mining regions makes the water hyacinth an appropriate candidate for the treatment of HMs effluents.

E. crassipes as heavy metal hyperaccumulator has long been validated (Kay et al., 1984; Prasad et al., 2001a, b). It takes up HMs actively from their surroundings at higher capacity than non-hyperaccumulaters. Thus, *E. crassipes*, among other aquatic macrophytes have long been and continue to be rather popular candidate for rhizofiltration of metal-polluted aquatic environments (Suñe et al., 2007). Hence, *E. crassipes* will certainly serve as the better host plant system to study the HM stress.

2.3.7 Perspectives for heavy metal stress tolerance

Efforts for improving the tolerance of plants to HM stress by genetic engineering or breeding have not been successful to a great extent, due to the lack of mechanistic understanding of genetic complexity and the inherent diversity of the HM stress-response pathways. For example, genes encoding PC synthase from *Arabidopsis thaliana* (*AtPCS1*), wheat (*TaPCS1*), and other species have been recently cloned (Clemens et al., 1999; Vatamaniuk et al., 1999, 2000). However, all of these *pcs* genes were cloned from plants that are not tolerant to HMs. Pomponi et al (2006) overexpressed the *Atpcs* gene in tobacco plants showing transgenic plants enhanced tolerance to Cd than wild-type plants. Li et al (2006) produced transgenic tobacco lines carrying *Cdpcs1*, which was cloned from the HM-tolerant plant *Cynodon. dactylon*, had a greater effect than earlier reports. This shows that *pcs* gene isolated from natural hyperaccumulators had higher impact than the genes from non-accumulators. Although the two studies were not completely identical with respect to experimental conditions, more research on HM-tolerant plants, especially related hyperaccumulators, will help us to maximize the effectiveness and efficiency of

phytoremediation. Hence, isolation of *pcs* gene from *E. crassipes* will serve as better target gene for HM stress study.

Despite the intensive work on aquatic macrophytes for their potential use in phytoremediation, the relative contribution of the diverse mechanisms leading to metal detoxification and tolerance, as well as the interspecific differences in defense strategies, have been given scanty consideration so far. Furthermore, keeping in view the ever increasing problem of HM contamination and health hazards, the use of crucial genes from natural hyperaccumulator viz., *E. crassipes* provides immense promise for future phytoremediation.

Recent developments in the field of structural/functional genomics and proteomics provide ample opportunities for the isolation and characterization of novel HM stress-responsive/associated genes, proteins and pathways. The evolutionary conservation of stress response has shown that functionally analogous stress associated determinants exist in both unicellular and multicellular organisms. Elucidation of components of stress-response pathways in simpler systems like bacteria and yeast may help in finding overlapping mechanisms in higher plants. High throughput sequencing programs supported by genome-wide transcript profiling has a great potential in isolating differentially expressed and functionally important stress-regulated genes. Hence, isolation and functional characterization of stress responsive genes from *E. crassipes* will add strength to the endeavour towards unraveling HM stress response mechanism in hyperaccumulator plants.

3.1 INTRODUCTION

E. crassipes is perennial aquatic macrophytes spread all over the world and considered noxious and extremely invasive for freshwater environments (source: USDA, 2004). Its potential as HMs hyperaccumulator has long been validated (Kay et al, 1984; Delgado et al., 1993; Maine et al., 2001; Prasad et al., 2001a, b). It takes up HMs actively from their surroundings at higher capacity than non-hyperaccumulator. *E. crassipes* has been a popular candidate for rhizofiltration of metal-polluted aquatic environments (Suñe et al., 2007). Moreover, because of its high biomass production, wide distribution, and tolerance to cyanide (CN) and metals, it can be harnessed for phytoremediation and use as a model to study HMs stress response (Williams, 2002; Singhal and Rai, 2003; Ghabbour et al., 2004; Jayaweera and Kasturiarachchi, 2004).

HMs as such are essential for normal plant growth and development, but their elevated concentrations may lead to toxicity symptoms and growth inhibition. Metal ions such as Magnesium (Mg), Cu, Zn, Mn, Ni and Fe are essential for plant growth, in processes that range from respiration to photosynthesis, but deleterious when present in excess amounts. Others such as Cd, Aluminium (Al), and Pb have no nutritional value and are toxic. When present in large amount in the soil, metals interfere with the uptake of essential ions, biosynthesis of chlorophyll and nucleic acids, and lipid metabolism, thus profoundly affecting plant growth and development (Ouariti et al., 1997; Dykema et al., 1999). With the necessity to regulate metal ion uptake and achieve metal ion homeostasis, plants have evolved a range of potential cellular mechanisms and proteins that may be involved in detoxification of HMs to achieve tolerance (De Vos et al., 1991; Dietz et al., 1999). The high degree of deleterious effects of HMs is contributed by its longer persistence in soil and absorption through plant roots (Tomas et al., 2008; Vollmannova et al., 2008). Increasing emissions of HMs has become a threat for human health as the entry of toxic metals into the food chain has become more flexible (Lantzy and Mackenzie, 1979; Galloway et al., 1982; Angelone and Bini, 1992). So, the recovery of degraded soils and the reclamation of industrial sites need stress-tolerant hyperaccumulator plants. Efforts are on to improve plant stress

tolerance and pollutants hyper-accumulation potential with the aim of using plants for soil remediation (Salt et al., 1995). In order to devise new strategies for phytoremediation and improved tolerance, a model system is needed to understand the basic principles of pollutants toxicity mechanisms at the cellular and tissue level.

Plants subjected to HMs stress undergo biochemical changes producing ROS such as superoxide anion radical (O^{2-}), hydrogen peroxide and hydrogen radicals (^{-}OH) (Cho and Park, 2001). The expression of antioxidation enzymes like, superoxide dismutase (SOD), aldehyde dehydrogenase (ADH), esterase (EST), peroxidise (POX) and catalases (CAT) play a crucial role in scavenging ROS and providing sustenance to stressed plants (Gupta et al., 1999). A significant induction of antioxidant enzymes (POX and CAT) along with an increase in the levels of protein, lipid peroxidation and GSH was noted in response to cadmium chloride ($CdCl_2$) in rice seedling (Yu et al., 2000). HMs may cause alteration either in the expression of stress proteins or enzyme activity. Although, *Eichhornia* as biosorbent of heavy metals ions has been reported (Schneider et al., 1995; Sabale et al., 2010), but correlative studies with soil HMs contamination and plant changes in morphological, protein and antioxidant enzymes level has not been done. In this research article, an endeavour was made to assess the morphological indicative changes in various tissues of a hyperaccumulator plant, *Eichhornia* grown in HMs contaminated area. This is followed by an effort to evaluate the metal stress induced changes in protein and isozymes level to see the possibility of usage of *Eichhornia* as organic indicator and model system for metal contamination and stress study respectively.

3.2 Materials and Methods

3.2.1 *Eichhornia* plant, soil and water samples

The *Eichhornia* root and shoot samples were collected on site from the HPLC Paper Mill dumping area, Jagir road, Assam and stored in liquid nitrogen. Laterally, a control set of *Eichhornia* plant samples were collected and maintained for further analysis. The soil and water

samples were also collected from site for preliminary analysis to reveal the varied composition of HM. The soil samples were collected with the help of auger to depth ranging from 20 cm to 100 cm in November 2008. A total of 18 soil samples with 3 replicates for each sample of varying depth and 3 replicates per samples were collected from site in randomized block experimental design.

3.2.2 Physical parameters of the samples

3.2.2.1 Sieving analysis of soil

The HMs contaminated soil (waste) sample collected from dumping of Hindustan Paper Company, Jagir Road was used throughout this research. Due to moisture content, the clod soil was first crushed into small pieces to homogenize and then incubated in hot air oven at 80˚C overnight for drying. Then, it is ground by a bar mill and was subjected to sieve analysis by passing through sieves of various size (425, 300, 150, 75 µm) respectively for the well microencapsulation process. The 75 µm sieve passed was used for further downstream analysis of HMs composition. The sieve retained and passed soil samples was analyzed for its physical characteristics and aggregation.

3.2.2.2 AAS analysis

Water hyacinth naturally growing in the HPLC Paper Mill dumping area, Jagir road, Assam, was harvested for AAS analysis. After being rinsed with deionized water, the roots and shoots were further repeatedly washed with double distilled water to remove adhered soil particles. Shoots and roots were oven-dried at 65˚C for 4 days and digested by USEPA method (1997). The water samples collected from contaminated dumping site and control areas were filtered prior to use. The soil samples were properly homogenized and dried in an oven at 105°C prior to analysis. The 75 µm sieve passed soil samples were eextracted with 20 mL of 0.01 M ethylene diamine tetraacetic acid (EDTA) solution by shaking in plastic centrifuge tubes for 15-20 minutes. The samples were centrifuged and the supernatant was decanted and filtered into fresh test tubes for analysis. To avoid metal precipitation from the dissolved liquid phase into the solid phase, which

causes error in the accuracy of the metal concentration measurement, the leachate pH was reduced to less than 2.0 by the addition of nitric acid before the metallic analysis, because metals remain dissolved in pH less than 2.0. Metal concentration was measured by flame AAS using Air-Acetylene or Nitrous Oxide- Acetylene Burner (AAS; Varian). Concentrations of the metal ion standard solutions were adjusted to cover a range of 0 to 25 mg/L (ie. 0 to 25 ppm) and quality control metal Standard Reference were used (NIST, Gaithersburg, MD). The same sample solution was used to analyze number of metal ions. Three replicates of each samples were taken for analysis. Some samples which reported to be in a concentration "OVER RANGE" were diluted appropriately and re-measured.

3.2.2.3 XRD analysis

The HMs composition of the dumping waste soil sample was characterized using an AXS D8 ADVANCE Fully Automatic Powder XRD following non-destructive technique. Here, the crushed and homogenized 75 µm sieve passed soil sample baked overnight in hot air oven at 80˚C was days were ground into fine crystalline powder and analyzed by the XRD device for the reflection angle (2θ) from 10˚ to 70˚. To identify the crystalline phases, the results were compared with the Joint Committee on Powder Diffraction Standards (JCPDS).

3.2.2.4 SEM analysis

A scanning electron microscope (SEM, Leo 1430vp) was used to consider the microstructure of the HMs contaminated soil. Flat surface pieces from previously crushed and 75µm sieve passed soil sample, which had been cured overnight in hot air oven at 80˚C, was selected and treated with acetone-alcohol solution to stop hydration reactions (Asavapisit et al., 1997). Gold was used as a conducting medium to coat the surface of samples under vacuum conditions in the Sputter Coater device. Selected 75 µ sieve passed soil sample was further characterized using a SEM, (Leo 1430vp), equipped with EDX. Vacuum-dried soil sample was mounted on carbon stubs using double stick tape and then coated with carbon. The analysis of soil sample necessitated a 30 min vacuum time to obtain a sufficient vacuum.

3.2.2.5 Morphological imprinting

The growth of the *Eichhornia* plants growing in the industrial dumping site and control areas was monitored and compared in terms of shoot and root length, root hair proliferation, bulb girth and discolouration of the bulb and apical parts. Observation for evident HMs stress symptoms like leaf rolling, chlorosis and discolouration of bulb were also recorded. Triplicate readings for each parameter were taken and the average mean value was considered for comparisons between the morphological changes of stressed and control plants.

3.2.3 Biochemical parameters of the samples

3.2.3.1 Protein and isozyme analysis

Protein was extracted from *Eichhornia* shoot and root tissues samples collected from naturally grown plants under dumping site contaminated and control area for protein and isozymes analysis respectively. The leaf total proteins were extracted by using homogenized sample powder suspended in a buffer consisting of 30 mM Tris-HCl (pH 8.5), 1 mM ascorbic acid, 1 mM EDTA, 5 mM magnesium chloride ($MgCl_2$), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Zivy et al., 1983). Polyvinyl polypyrrolidone (PVPP, 50 mg g^{-1} fresh weight of the tissue) was added while homogenizing the samples. Crude homogenate was spun and the supernatant was transferred and centrifuged (13,000 rpm, 4°C). Proteins in the supernatant were precipitated by adding 8 volumes of chilled acetone containing 10 mM of 2-mercaptoethanol at -20°C. Following centrifugation (15,000 rpm, 10 min, 4°C), the precipitate was dissolved in Laemmli buffer [0.625 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 10% glycerol]. An aliquot of the supernatant was used for estimation of protein amount by Bradford method (Bradford 1976). Dissolved proteins denatured by heating at 100°C for 5 min in a water-bath. 20 µg of the protein was electrophoresed on a 12% discontinuous SDS-polyacrylamide gel at 50 mA constant current (Laemmli, 1970) and stained.

For Alcohol dehydrogenase (ADH) assay, the root samples were ground in prechilled pestle and mortar with 0.1 M Tris-HCl, pH 7.4 and 10 mM DTT at 4ºC. The supernatant obtained after centrifugation (15,000g, 25 min) was used for further assay. For analysis of esterase, the sample was ground in prechilled pestle and mortar with 50 mM Tris-Cl buffer (pH 7.6) containing 5mM β-ME and 5 mM EDTA in the ratio of 1:2 (w/v). For peroxidase, 50 mM Tris-Cl buffer (pH 7.6) alone was used for extraction. The ground mixture was then centrifuged at 15,000 rpm for 20 min at 4ºC using SIGMA Centrifuge. All operations unless mentioned otherwise, were carried out at 4ºC. The supernatant obtained was immediately used for anionic polyacrylamide gel electrophoresis to separate the isozymes as mentioned by Davis (1964). The staining of the ADH was carried out in Tris-Cl buffer (50 mM, pH 8.0) containing 0.02 % NAD^+, 0.03 % NBT, 0.004 % PMS and 5 % absolute alcohol (Dongre, 1988) and stored in 7% acetic acid solution. The staining of EST isozyme was carried out by modified Brewer's method (1970). The staining of POX isozyme was done in solution containing 2.08 % Benzidine, 18 % acetic acid, 3 % H_2O_2 (Reddy and Gasber, 1971). After stopping the enzymatic reactions, the gels were fixed in 7% acetic acid solution and documented for banding patterns.

3.2.4 Statistical analysis

Data for the control and stressed trials were pooled together and analyzed statistically using the analysis of variance and the mean of the treatments with the computer software SPSS and subjected to ANOVA.

3.3 Results and Discussion

3.3.1 Mineralogical characterization of soil samples

State of the *Eichhornia* plants growth in the contaminated dumping site was as scribed in the legend to Figure 3.1 and control soil was primarily checked for pH before starting all the characterization analysis. It was found to be acidic around pH 4.8. Primarily, the soil samples were analyzed by passing through various sieves and the 75 µm passed soil was used for SEM study which reveals the difference in physical characteristics and clumping of soil.

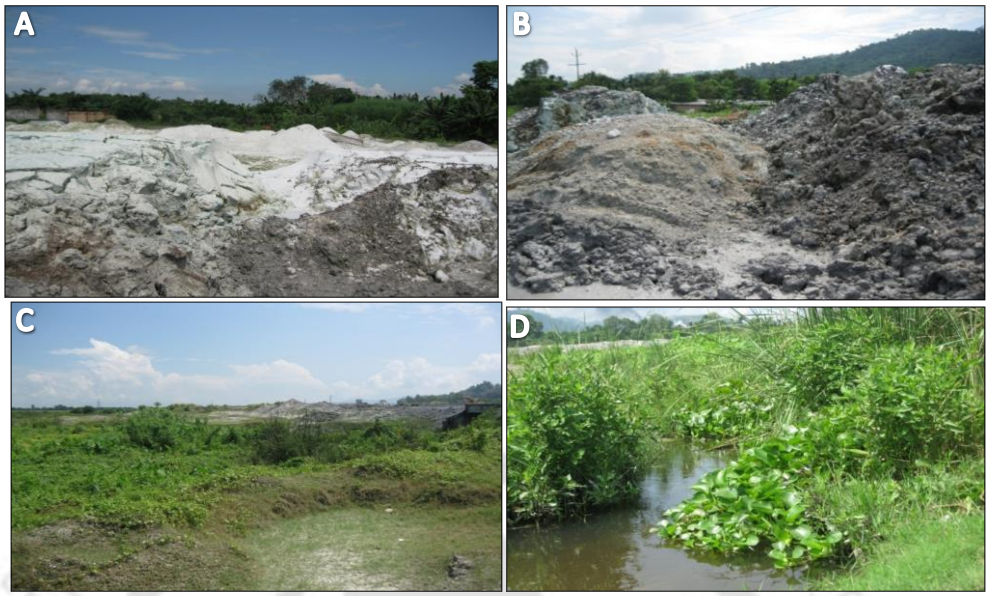

Fig 3.1. A, B: Industrial waste dumping area; C, D: *Eichhornia crasssipes* sample collection site

The soil structure and texture was deciphered after passing the sieve (Fig. 3.2a, 3.2b). XRD patterns of the contaminated dumping site soils are shown in Fig. 3.3A. Data for control soil are not shown. A distinctive peak at 36.10 A in the contaminated soil sample corresponds to Cd and Co presence in the tested soil. Various peak at 48.7 A, 28.85 A, 23.05 A, 33.0 A suggested in situ formation of a ZrSiO4, CaF_2, Cristoballite and Platternite-like mineral in the HMs -contaminated soil. The presence of Hg was ascertained by the characteristic weak peak at 66.1 A and Zincite like mineral was also found in the contaminated soil. The presence of substantial amount of such minerals has been reported in contaminated garden, and urban and motorway roadsides (Cotter-Howells, 1996). However, further research is needed to identify these unknown minerals. Discrete presence of HMs in the dumping site soil was further documented by EDX analysis of 75 µm sieve passed soil sample (Fig. 3.3B).

Fig 3.2a. Micrograph of heavy metals contaminated soil after passing various sieve

The EDX analysis reveals the presence of HMs such as Hg, Pb, Ni, Cd, Co, Cu and As contamination in the dumping site soil. Among all these metal contaminants, the contaminated soil sample was found to follow decreasing Co>Pb>Hg>Cd>As>Cu intensity wise order. HMs contamination following Pb>Cd>Zn>Cr>Cu order has also been reported in Lead-Acid factory dumping site of Nigeria (Adejumo et al 2011).

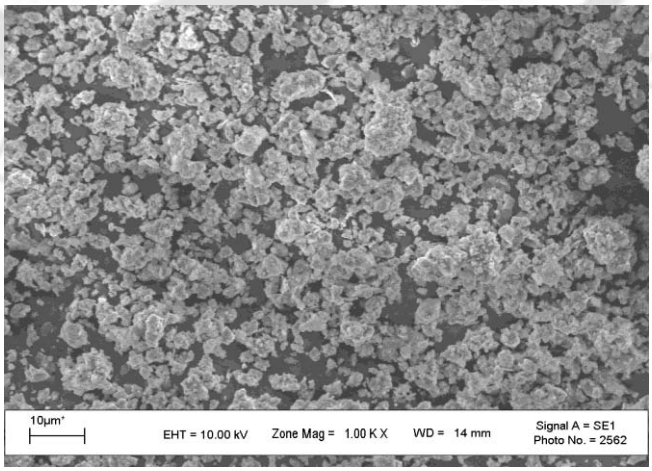

Fig 3.2b. SEM micrograph of heavy metals contaminated soil 200X micrograph after physical changes and passing sieve number 75 µm

The high level of presence of these HMs contaminations in the soil may be because these metals were used in Paper Mill (Fig. 3.3 A, B). Further, the low pH of the soil may have contributed to the HMs contamination as they are more soluble in acidic conditions (Smejikalova et al., 2003; Ogundiran, 2007).

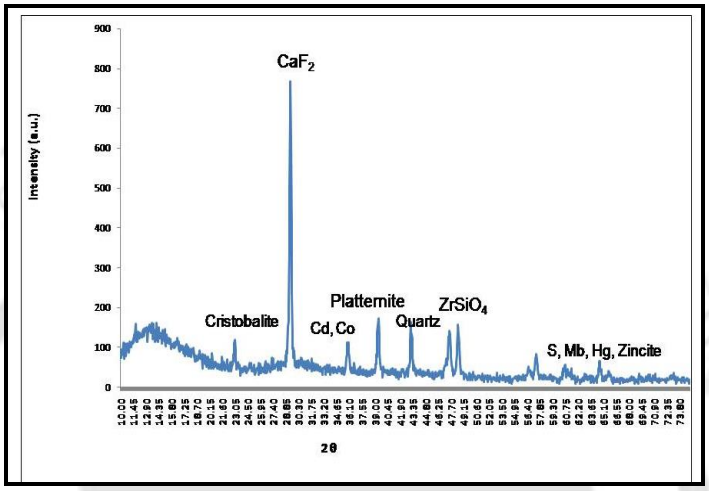

Fig 3.3A. X ray Diffractogram of contaminated soil sample

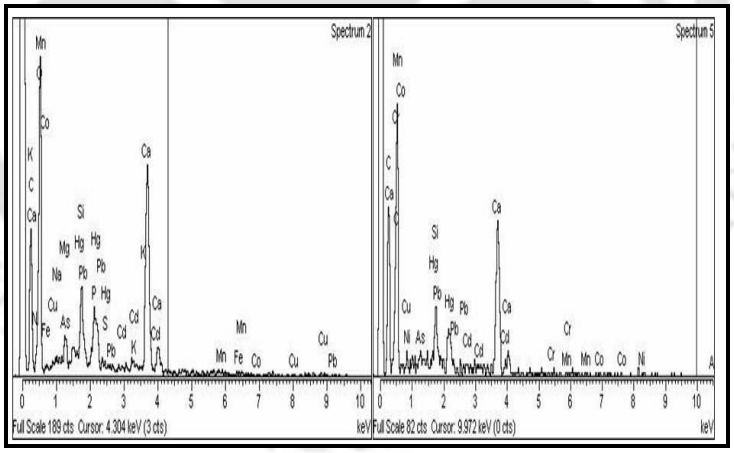

Fig 3.3 B. EDX spectrum of soil sample

The AAS analysis of the soil and water sample collected from dumping site and control areas reveals that there is significant difference in the HMs concentration ($P<0.05$). The degree of HMs contamination in the dumping site soil was found be in Pb>Cr>Cu>Ni>Cd order. Whereas, the water analysis shows the HMs get accumulate in water bodies in following order

Pb>Cr>Ni>Cu>Cd (Table 3.1). The Cd concentration in soil seems to be 8 times higher than control whereas in water, Cd level is 5 times more than control. The Cr, Cu and Pb contamination level were found to be in higher side (>1 mg/l, $P<0.05$) whereas Ni concentration level was higher in water than soil. In fact, the analysis of the HMs concentration in soil and water reveals that the dumping site and water bodies were totally contaminated with HMs in hazardous levels.

AAS analysis to confirmed the high level of heavy metals contamination in soil and water of the *Eichhornia* habitat						
Samples		**Concentration (mg/L)**				
		Cd	**Cr**	**Ni**	**Cu**	**Pb**
Soil	**Control**	**0.014**	**0.225**	**0.066**	**0.792**	**1.940**
	Treated	**0.117**	**2.101**	**0.125**	**1.078**	**2.170**
Water	**Control**	**0.035**	**0.033**	**0.046**	**0.233**	**0.452**
	Treated	**0.198**	**1.671**	**0.265**	**0.692**	**1.720**

Table 3.1. Analysis of stressed and control soil and water samples through AAS for heavy metals accumulation

3.3.2 Metal phytoaccumulation

In this study, HMs contamination in the dumping site soil and water was ascertained, resulting in more HMs uptake by the naturally growing *Eichhornia* in the site. Although in all plants, the metal concentration in the tissues increased with the external metal concentration, and metal concentrations were generally higher in roots than in shoots, there was tissue specific variation in the metal accumulation for different plant parts (Table 3.2). Cd concentrations were significantly higher in stressed root as compared with the other plant parts used ($P<0.05$). Cd accumulation in shoots and bulb was not significantly different in lower range. Other studies have also validated that this plant is a good accumulator of Cd (Muramoto and Oki 1983, Odjegba and Fasidi 2007). As for Cr accumulation, among the plant parts analysed the lowest Cr values was observed in

both roots (P<0.05) and bulb (P<0.05) and highest accumulation of (0.323 mg/l) was found in shoot. This is in accordance of previous reports of Cr accumulation by *Eichhornia* (Lytle et al., 1996).

Samples		Concentration (mg/L)				
		Cd	Cr	Ni	Cu	Pb
Root	Control	0.007	0.210	0.053	0.053	0.240
	Treated	0.214	0.228	0.124	0.292	0.280
Bulb	Control	0.011	0.049	0.010	0.084	0.190
	Treated	0.041	0.126	0.048	0.107	0.220
Shoot	Control	0.012	0.088	0.027	0.047	0.600
	Treated	0.034	0.323	1.253	0.093	0.850

Table 3.2. *In vivo* analysis of stressed and control soil and water samples through AAS for heavy metals accumulation

Cu concentrations was highest in roots and the presence of Cu in control plants was normal as Cu serve as active constituent of plastocyanins, ascorbic acid oxidase, tyrosinase and phenoloxidase (Odjegba and Fasidi, 2007). The degree of variation of Ni concentration between control and stressed plant were found to be significant in root and bulb but the highest Ni accumulation was observed in stressed shoot. Further, the Pb accumulation in control and stressed root and bulb part were more homogeneous; the only statistically significant difference was that the stressed shoot accumulated this metal to higher extent as compare to control (P<0.01). All the metals studied were accumulated to substantially higher concentration in roots than in shoots. Within bulb, the accumulation of all the HMs was higher in stressed condition than control. As the plant parts metal concentration ratio does not seems to change in an exposure-dependent way, there was significant accumulation of all the HMs in root, shoot and bulb during stressed conditions(P<0.05).

3.3.3 Morphological changes in *Eichhornia* during HMs stress

The growth of the *Eichhornia* plants in dumping site and control areas was monitored and the realtime changes in the various observable parameters have been recorded. The shoots, bulb and root characteristics shows discrete variation between the *Eichhornia* plants grown in stressed and control conditions (Fig 3.4). The *Eichhornia* growing in stress conditions showed chlorosis, leaf rolling and loss of chlorophyll in apical parts whereas underground root and bulb showed increment in length, girth and number as compared to control plants. This findings does corroborate with reported evident symptoms of Cd toxicity as leaf rolling, chlorosis and stomatal closure that are observed in plants (Clemens, 2006).This symptoms may be due to the negative effects of the HMs on mineral nutrition and homeostasis in plant shoot and root growth and development (Metwally et al., 2005; Farinati et al., 2010). There has been report of Cd damaging photosynthetic apparatus and causing a decrease in chlorophyll content in stressed plants (Sanit`a di Toppi and Gabbrieli, 1999). There seems to be clear symptoms of abscission in the root and leaves of stressed *Eichhornia* plants. It has been reported that exposure to HM can induce increase in abscissic acid (ABA) and jasmonate level in plants (reviewed by DalCorso et al., 2008) and this enhanced ABA level may be responsible for the abscission and browning of stressed root and leaves and also for stressed root growth.

Even *E. crassipes* has been reported to substantially accumulate Cu when cultivated at 0.5 mg/L without marked changes in its physiology but above, loss of chlorophyll and increase in protein Cu^{2+} concentration level was reported (Hu et al., 2007). The soil and water Cu concentration has been found out to be 1.078 mg/L and 0.679 mg/L respectively which are in the extreme higher side of contamination level and hence, the appearance of HMs stress symptoms was obvious. The decline in chlorophyll content in plants exposed to HMs stress such us Cu is believed to be due to enzymes inhibition associated with chlorophyll biosynthesis (John et al., 2009). The decrease in chlorophyll content was also reported in sunflower (Zengin and Munzuroglu, 2006) and in almond (Elloumi et al., 2007).

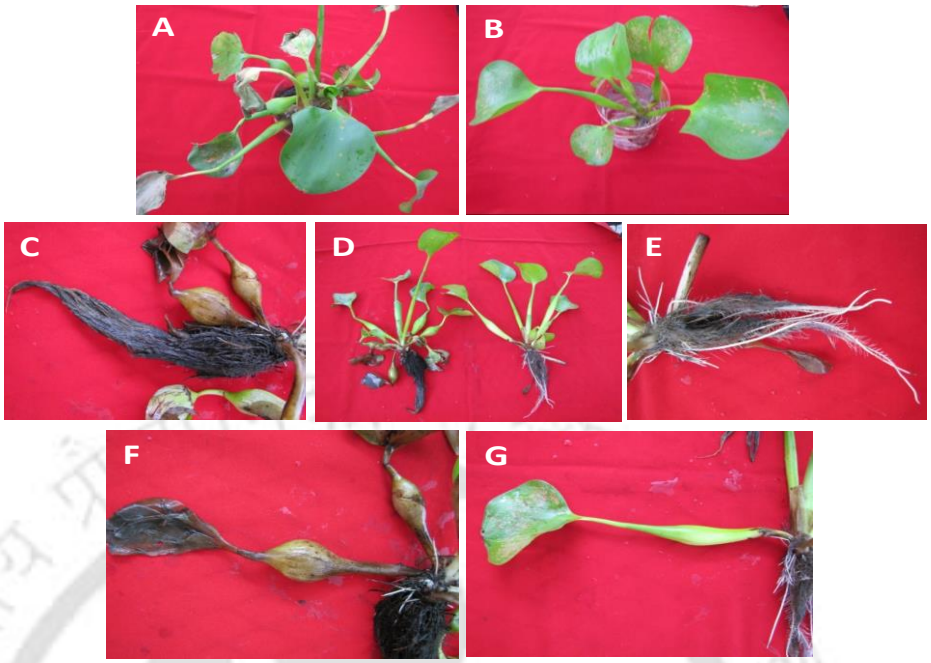

Fig 3.4. Morpho-tissue specific changes of *Eichhornia* plants induced by HMs stress. A. stressed plant, B. Control plant, C. Stressed elongated roots, D. Comparative shoot and root architecture of stressed and control plants, E. Control normal roots, F. stressed enlarged pigmented bulb, G. Control bulb

Eichhornia showed relative increment in growth of the shoot and root in dumping site as compared to the control under *in vivo* condition. Such shoot growth of *Eichhornia* has been previously observed to increase with time and only became constant in the final harvest when cultivated plants are grown in experimental units with continuous water (Freire Martins et al., 2011, Henry-Silva and Camargo 2005). This may also indicates sequestration of HMs to different cellular compartments by increasing the process of growth and development of the plant (Fig 3.5). These observed changes in shoot and root length and bulb girth increment during stress condition may be attributed to alterations in cell cycles and division and chromosomal aberrations due to Cd (Benavides et al., 2005). Furthermore, Cd has been reported to inactivate deoxyribose nucleic acid (DNA) mismatch repair in yeast and human cells (Jin et al., 2003) and the same mechanisms may be applicable in plants leading to abnormal growth. A chromatin remodelling factor OXS3 isolated from *Brassica juncea* was found to protect DNA or alter its transcriptional

selectivity under HM stress (Blanvillain et al., 2008). This postulates that HM stress induces aberrant growth of *Eichhornia*. This may be first general mechanisms of *Eichhornia* plants under stress to accumulate the HMs and sequester it to various organelles.

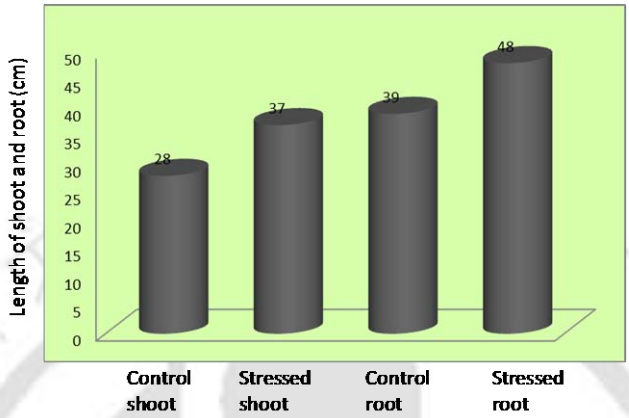

Fig 3.5. Effect of HMs contaminated industrial waste on growth of shoot and root of *Eichhornia* plants

3.3.4 Protein profile changes in *Eichhornia*

When a plant is exposed to any biotic or abiotic stress factor, protein synthesis is one of the most negatively affected anabolic processes along with photosynthesis, transport of metabolites, and uptake and translocation of ions (Bonjoch and Tamayo, 2001). Beside loss of chlorophyll, ribulose-1, 5-bisphosphate carboxylase oxygenase (RubisCO) and other chloroplast proteins are hydrolysed and exported via phloem, followed by hydrolysis of mitochondrial proteins and vascular tissues. Therefore, low and altered protein concentrations may be interpreted as a clear symptom of stress damage in plants (Bonjoch and Tamayo, 2001). There found to be significant difference in the protein profile of the control and stressed shoot of *Eichhornia* (Fig 3.6). The abundant band of 66KD believed to be RubisCO protein in control gel is missing in stressed protein gel, where in fact, a low intensity band of 78 KD is present. The reduction of photosynthetic protein in stressed condition may be due to increase of ABA level which is believed to down regulate enzymes needed for photosynthesis (Chander and Robertson, 1994). Similarly, the intensity of 20.1 KD band in control protein profile seems to be reduced and altered

protein concentration variation in stressed conditions. There seems to be significant variation in other protein bands profile. The difference in protein profile may be due to the negative effect of the HMs such as Cd and Cu on the photosynthetic machinery, chlorophyll content and enzyme inhibition related to carbon assimilation (Sanita di Toppi and Gabbrielli, 1999; Perfus-Barbeoch et al., 2002; John et al., 2009). The decrease in protein band intensity observed in stress condition may be result of the excess Cu that cause cellular damage at the DNA level and organelles such as mitochondria or lysosomes (Lee and Wei, 2001). The observed variation in protein profile may be due to the fact that plants activates their stress coping mechanisms like acclimation of metabolic fluxes, activation of repair processes and long-term metabolic and morphological adaptations, which conform the named general adaptation syndrome (Lichtenthaler, 1996). Such mechanisms include *de novo* synthesis of proteins with specific adaptive functions, osmotic adjustment, antioxidative defence, among others. The altered protein profile in stress may be due synthesis of stress responsive proteins or due to accumulation of free amino acids as histidine, proline and cysteine in tissues, this has been reported in stress metal such as Cd (Nedjim and Daoud, 2009).

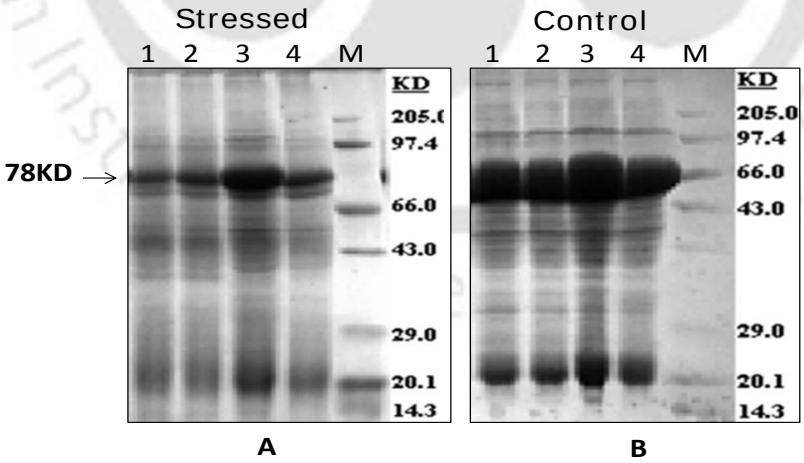

Fig 3.6. SDS-PAGE analysis of total leaf protein from HMs contaminated and control habitat. A: Lane: 1,2,3,4 Proteins from different individual stressed plants; Lane M: Molecular Weight Marker (MWM). B: Lane: 1,2,3,4 Proteins from different individual control plants; Lane M: Molecular Weight Marker (MWM)

3.3.5 Isozymic profile in HMs stress

As mentioned in the introduction, the enzymes evaluated in this study are important in the antioxidant responses of plants to stress. The activity and expression of genes encoding antioxidant enzymes have been shown to change in some plants during environmental stress and the responses of antioxidants to heavy metal induced oxidative stress have provided variable and controversial results (Gomes Junior et al., 2006). ROS may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA injury. The control of oxidant levels is achieved by antioxidative systems. There has been report of high soil Cd concentration drastically altering antioxidant system such as SOD, CAT, POX in wheat seedlings (Lin et al., 2007).The growth of pea plants with 50 µM $CdCl_2$ has affected been shown to have reduction in SOD activity (Rodríguez-Serrano et al 2009). Increasing levels of 0-500 µM Cd_2 alone and/or heat stress has showed increased activities of SOD, guaiacol POX, ascorbate POX and GSH reductase enzymes associated with induced oxidative stress and altered enzyme activities (Nahakpam and Shah, 2011). Plant ADH enzymes were considered to be dimers and the two subunits of ADH are encoded by two unlinked genes (Gottlieb, 1982, Sachs and Ho, 1986). Results concerning ADH, EST and POX activities in this study are based on isozymes gel electrophoresis analysis mobility and intensity. In this study, appearance of comparative high intensity ADH isozymes band during stress than control condition indicates the increase in ADH activity leading to increased enzyme synthesis (Fig 3.7). Increased in ADH enzyme activity has been shown by a change in isozymic profiles of the ADH protein in rice during abiotic stress (Rivoal et al., 1989; Xie and Wu, 1989). The EST isozymes of stress and control conditions were found to be significantly different in term of mobility whereas POX isozymes were variable with respect to intensity. EST and POX pattern were found to be polymorphic and able to differentiate stress and control *Eichhornia* plants under study. Such differences of EST and POX have been previously reported to be a useful induction of abiotic stress tolerance in rice (Zhang et al., 1988; Mandal et al., 2004).

These results showed a strategy of defense of *Eichhornia* against oxidative stress induced by HMs stress leading to variation in the expression of the antioxidant enzymes. Similar results are obtained in plants stressed by Cu (Demirevska-Kepova et al., 2004), or other metals such us Mn, Pb, Ni and Cd (Kopyra and Gwozdz, 2003; Demirevska-Kepova et al., 2004; Sobkowiak et al., 2004; Gomes-Junior et al., 2006). Stress that disrupts the cellular homeostasis, including heavy metal toxicity, can enhance the production of ROS and increase the steady-state level of H_2O_2 up to 30-fold (Mittler, 2002). Although we have not measured H_2O_2 or other ROS in this study, alterations are likely to have occurred based on the responses of the antioxidant enzymes that were measured (Gratao et al., 2008).

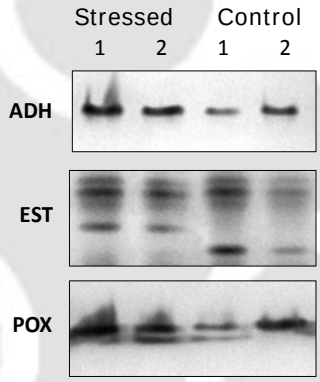

Fig 3.7. Activity staining of isozymes of antioxidant enzymes on anionic PAGE. Alcohol dehydrogenase (ADH), esterase (EST) and peroxidase (POX)

3.4 Summary and conclusions

The mineralogical characterization of soil samples confirmed the contamination of various HMs in the soil of HPLC, Jagir road dumping site. The high level of HMs contamination and bioaccumulation in water and plant parts respectively was revealed by AAS study. The shoot and root showed enhanced growth in *Eichhronia* growing in dumping site than control areas, which indicates higher tolerance and sequestration of HMs for its growth. This may indicates the applicability of *Eichhornia* to be used as agent for phytoremediation of contaminated soil. Significant difference in the protein profile of the *Eichhornia* in shoot and altered expression and

appearance of new forms of EST and POX in root suggests the increase in antioxidant enzymes activities to limit the cellular damage to cope with HMs stress. The various morphological changes along with altered profile of protein and isozymes may be another strategy of *Eichhornia* to cope with the HMs stress by the synthesis of proteins with specific adaptive functions which remain to be identified. The stress adaptive potential of *Eichhornia* may be harness and it can be further tried as model system to study HMs stress tolerance in plants.

4.1 Introduction

Plants constantly struggle throughout their life cycle against adverse environmental conditions to survive on earth. Growth and productivity of most of crops drastically affected due to abiotic stresses, such as salinity, drought, low temperatures, heat shock, HMs, UV radiation and pesticides (Bohnert et al., 1995; Tardieu and Tuberosa, 2010). The presence of toxic compounds, such as HMs, is one important factor that causes damage to plants by altering major plant physiological and metabolic processes (DalCorso et al., 2008; Hossain et al., 2009; Hossain and Fujita, 2010; Rascio and Navari-Izzo, 2011; Villiers et al., 2011). Many plants have capacity to accumulate toxic heavy metals from soil and water. A complete understanding of the molecular mechanisms and genetic basis of phytoremediation can assist in developing plants for enhanced HM stress tolerance.

Plants being sessile have developed an incredible plasticity to adjust to adverse changes in their environment. Plants obtain their essential macronutrient such as K^+, Ca^{2+} along with other toxic ions from soil due to co-transportation and similar ionic radii (Zhao and Eide, 1996; Perfus-Barbeoch et al., 2002). Though plants have a strong preference for macronutrient; by contrast, elevated concentrations of Cd^{2+}, Pb^{2+} and Na^+ are generally toxic to plants. Surprisingly, only few species can withstand HM stress environments and plant researchers have been trying hard to dissect the underlying mechanisms to improve the HM stress tolerance of crops. In contrast to such plants, HM stress tolerant species that grow in high HM conditions have evolved several strategies that enable them to keep the cytosolic concentration of HM low. The detoxification of HM by such plants is mediated through diverse strategies viz., metal uptake, translocation, sequestration into the vacuole (metal transporters); detoxification via buffering the cytosolic metal concentrations (chelators); delivery and trafficking of metal ions(chaperones), antioxidants, GSH or amino acids (Salt et al., 1998; Clemens, 2001; Wei et al., 2009; Sharma and Dietz, 2006; Robinson and Winge, 2010).

Although HM stress response is intensively studied and novel insights by various workers had revealed that GSH plays significant role in HM tolerance (Rauser, 1995, 1999; Hall, 2002). But the depletion of GSH has been a major mechanism in short-term HM toxicity and tolerance index (Metwally et al., 2005). GSH plays key role not only in metal detoxification but also in protecting plant cells from other environmental stresses including intrinsic oxidative stress reactions (Memon and Schroder, 2009; Dubreuil-Maurizi et al., 2011). Since HM hyperaccumulators such as *Eichhornia crassipes, Arabidopsis halleri and Thlaspi caerulescens* are unique plants capable of accumulating high amounts of toxic HMs (Delgado et al., 1993; Maine et al., 2001; Prasad et al., 2001a; Reeves and Baker, 2000). Such natural phenomenon of HM tolerance has motivated the plant scientists to investigate the physiology and genetics of metal tolerance in specialized hyperaccumulator plants (Memon and Schroder, 2009). Therefore, isolation of genes from *Eichhornia* may provide us a good view of naturally selected metal hypertolerance responses and GSH role.

When a gene is cloned and the protein of interest remains elusive from its structure/function, then it hinders the research progress of most molecular and cell biologists. This impediment has been aggravated in recent years due to the ever decreasing percentage of protein sequences in UniProtKB/TrEMBL (The UniProt, 2009) with a deduced protein structure in the PDB library(Berman et al., 2000) to 0.6% by the end of 2009. Recent advances in computer algorithms for predicting protein structure and function have alleviated this problem, and provided biologists with valuable information about their proteins of interest (Zhang, 2003). Here, starting from an amino acid sequence, I-TASSER provides solution that first generates three-dimensional atomic models from multiple threading alignments and iterative structural assembly simulations finally leading to its function and cellular localization predictions (Roy et al., 2010; Zhang, 2008). Though different candidate genes from hyperaccumulators have been identified and analyzed at biochemical and genetic level (Kramer et al., 2007), still lots remain to be known about the molecular regulation of HM stress tolerance. Hence, an effort has been made

to isolate and *in silico* analyze HM stress responsive gene from *Eichhornia*. This work may help to better understand the plant metal homeostasis network, and to genetically engineer crop for enhanced tolerance against environmental HM pollution.

4.2 Materials and methods

4.2.1 *Eichhornia* plant samples, bacterial strains and plasmids.

The *Eichhornia* root and shoot samples were collected on site from the HPLC Paper Mill dumping area, Jagir road, Assam and stored in liquid nitrogen for RNA extraction. Laterally, a control set of *Eichhornia* plant samples were collected and maintained for further analysis. State of the *Eichhornia* plants growth in the contaminated dumping site was as scribed in the legend to Figure 3.1. A preliminary soil analysis reveals the varied composition of HM as demonstrated in chapter 3. *E. coli* strain DH5α was used as host for cloning and plasmid amplification respectively. Cells harbouring recombinant plasmids were grown and maintained on LB medium supplemented with 100 mg/ml ampicillin. TA vector pTZ57R (Fermentas) was used for preliminary cloning and sequencing of *Echmr* gene.

4.2.2 Cloning of the *Echmr* gene from *Eichhornia crassipes*.

Total RNA was extracted from root and shoot with the RNAqueous isolation kit (Ambion). The first-strand cDNA was synthesized from 2 µg of DnaseI (Roche) treated total RNA using oligo(dT)17 primers with First strand cDNA kit (Fermentas). Oligonucleotide degenerate primers (forward primer 5'tggaaagggccttggcgttgg 3' and reverse primer 5'gatgtagcccgcttcaagtat 3') were designed using Primer Express v2.0 (Applied Biosystems) based on the conserved domains of the reported homologues from different organisms. The cDNA was used for PCR amplification with thermalcycler parameter comprising of an initial step for polymerase activation (5 min at 95°C), 35 cycles of amplification (30 s at 94°C, 1 min at 55°C and 2 min at 72°C), and final extension for 10 min at 72°C in 25 µl reaction mixtures. PCR products were then purified with PCR product purification kit (Sigma) and cloned into pTZ57R/T vector (Fermentas). The pTZ57R-*Echmr* recombinant plasmid was confirmed by restriction digestion and then sequenced. Sequence

identity was calculated by the Ident and Sim program in the Sequence Manipulation Suite (http://www.bioinformatics.org/sms/).

4.2.3 Sequence analysis

This analysis was done with a personal computer having internet connection and a web browser. The data input were nucleotide or amino acid sequence of the query protein in FASTA format. Different online and specialized analysis software such as Lasergene (DNASTAR), CloneManager9 and molecular visualizing software, like RASMOL (http://www.openrasmol.org) or PYMOL (http://pymol.sourceforge.net), for viewing the 3D structure of the modeled protein and the predicted functional sites were used.

4.2.3.1 Homology and Phylogenetic analysis

Nucleotide and the deduced amino acid sequences were searched for their homology with the previously existing sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BlastN and BlastP programs (Altschul et al., 1997). The Multi-way alignment of *Echmr* gene with other eukaryotic abiotic stress related genes was done by the exhaustive pairwise alignment of all sequences and progressive assembly of all alignments using Neighbor-joining phylogeny of Clonemanager 9. The phylogenetic analysis was carried out by neighbor joining method (Saitou and Nei 1987).

4.2.3.2 *Cis*-regulatory elements analysis

Search for regulatory elements in the UTRs was performed in PLACE (Plant cis-acting regulatory DNA Elements) database (Higo et al 1999) and SIGNAL SCAN (Prestridge, 1991). For protein domain analysis, sequences were searched against SMART (Simple Modular Architectural Research Tool) database (Schultz et al., 1998; Letunic et al., 2004). Sequences were also searched on rice full-length cDNA consortium database at http://cdna01.dna.affrc.go.jp/cDNA/ (Kikuchi et al., 2003).

4.2.3.3 Protein topology and motifs search analysis

The secondary structure prediction of the EcHMR protein was done by SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) with the use of Self-optimized prediction method (Geourjon and Deleage, 1995). The search for putative functional motifs in EcHMR protein was performed in UniprotKB and UniProtMES which provides an improved SOPM and report prediction of the amino acid sequences of a set of aligned proteins belonging to same protein family.

To find conserved motifs in *Eichhornia* EcHMR family members, Multiple EM for Motif Elicitation (MEME) version 3.5.4 (Bailey and Elkan, 1994) was used. The parameters of this analysis were setup as: number of repetition, any; maximum number of motifs, 10; and optimum width of the motif, $\geq$ 6 and $\leq$ 300.

4.2.3.4 Protein functions and sub-cellular localization prediction

The function and structure prediction of EcHMR was done by I-TASSER: a unified platform for automated protein structure and function prediction (Roy et al., 2010; Zhang 2008). The I-TASSER algorithm consists of three consecutive steps of threading (PPA is a simple sequence Profile-Profile Alignment approach confined with the secondary structure matches.), fragment assembly (Structure assembly simulation), and iteration. The EcHMR protein sequence was compared and aligned with the Protein Database (PDB) library by TM-align (Zhang and Skolnick, 2005). TM-align make use of the using dynamic programming and TM-score rotation matrix for protein structure alignment. The TM-align first finds the best equivalent residues of two proteins based on the structure similarity and then output a TM-score (Zhang and Skolnick, 2004; Xu and Zhang, 2010). This TM-score was then exploited to quantitatively access the quality of protein structure predictions relative to native as TM-score weights the close matches stronger than the distant matches. Then the function prediction was done by COFACTOR, a structure-based method for biological function annotation of protein molecules (Roy et al., 2012; Roy and Zhang, 2012).

The output contains full-length secondary and tertiary structure predictions, and functional annotations on ligand-binding sites, Enzyme Commission numbers and Gene Ontology terms which can be assessed by server analysis run at http://zhang.bioinformatics.ku.edu/I-TASSER. I-TASSER based TM-align were used to predict the sub-cellular localization. Different identified template proteins were scanned for the presence of a transmembrane domain(s) using TM-align which predicts the presence of transmembrane helices in amino acid sequences using TM-score and root mean square deviation(RMSD) based predictions (Zhang and Skolnick, 2004; Xu and Zhang, 2010).

4.3 Results and Discussion

4.3.1 Cloning of the *Echmr* gene from *Eichhornia crassipes*

Eichornnia HM stressed root sample was used to harvest template cDNA. The first PCR gave a smear which was used for the subsequent reaction using the same degenerate primers to amplify three distinct region of variable length (Fig 4.1a). The 1.8 kb amplicon was cloned in pTZ57R/T and confirmed with XbaI/BamHI digestion(Fig 4.1b) and processed for sequencing with universal primers resulting failed sequencing readouts. Hence, a new set of degenerate primers Fwd 5'AACATATGGCGATCGGGWNTTG 3' and Rev'ACTCGACGTNYAGAGRTACKC 3'were used to PCR amplify gene of interest using the (a) stressed cDNA from *Eichhornia* root and (b) the unsequenced 1.8 kb fragment as template, resulting in amplicon of 0.85kb. This 0.85kb fragment was again cloned into pTZ57R/T for automated sequencing. The sequencing of the isolated cDNA 0.85 kb fragment resulted to be novel HM responsive gene *(Echmr)* from *Eichhornia* whose nucleotide analysis has resulted in open reading frame(624 bp, 89 to 712bp), 5' UTR, 88 bp and 3' UTR, 247 bp (fig 4.3).

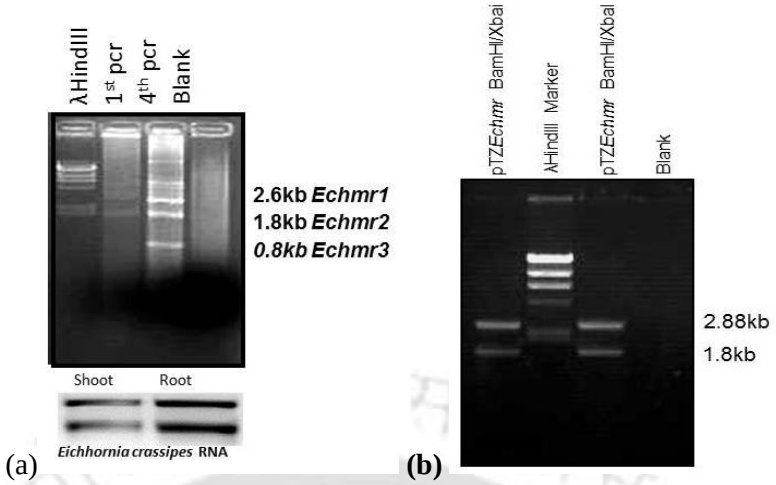

Fig 4.1 (a) Reverse Transcriptase derived cDNA was amplified using degenerate primers. (b) The purified *Echmr2* fragment was cloned in pTZ57R/T for sequencing.

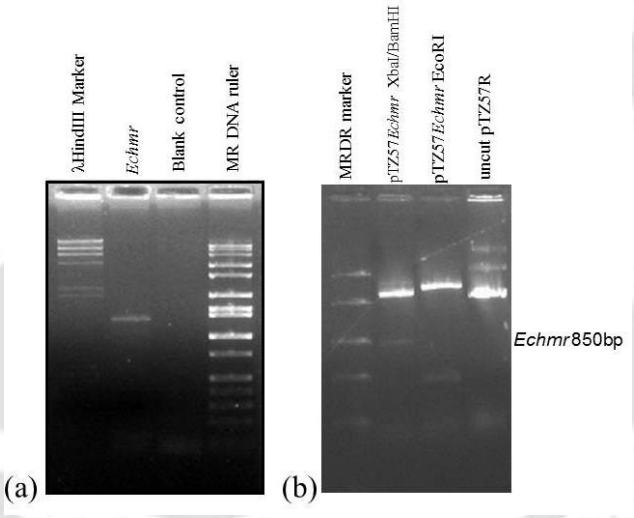

Fig 4.2 The (a) stressed cDNA from *Eichhornia* root and (b) the unsequenced 1.8 kb fragment were used as template for PCR to amplify *Echmr* gene of 0.85 kb amplicon size.

Novel HM stress responsive *Eichhornia* gene (*Echmr*)

CCAGAACGAGCCCGCGTTTTGCGGCGATCCCCCTCCATAGCCACCTGGCCCGCGCACCTCCAGCCGGAGCACTTCCTTG
GAGTTCCACATGTTGAGCACTGCCCATGAGGGCGACGGATCTGTGAGGAAAGTGTCAGCGTCGTCAGCCTCGAGCCAG
CGGTACCCGACGGAGACAGCGAGGAAGGTGTCAAGGGAGAGGGGGTTACGTAGGATGGAGTCGACGTTGCGCGGTTC
AAGTACCCTCCCCACGGGCTCGTTCTGGACGGTTCAAGTACCCTCCCCACAACGTCGCCCGGTTCAAGTACCCTCCCCA
CAACGTCGCCCGGTTCAAGTACCCTCTCCAGAACGAGCCCGCGTTCTGTGGACGTGGGGAGGGTACTTGAACCGTGCA
ACGTCAACATCTGATTTTTGCAGTTCTTTTAAGCGTTACATAGGCTTACTTGCACGGTATAATAGAACACCGTTCATCGG
GCCTAGGGTTTGCTCTTGGCTTTATTTAAATTTCTATACCGAATTCATCCTTAACGCTTGCGTCAAGTGGTATCAGAGCG
AGGAAGAGATCATGACTGATATTCCTACTAGAAAAGAGTTTGAAGGATTAAAAGCAAAGATAGCTCTCCTTACTGCTG
CTATCGAAAAATTGTTTACGAATCAAGGTCCTATTCACACACGTGTCCAACCAGAAGCAAACAACAAAGCCTTATCCCA
CTAGGTGGGGTCGGCTACATGGATCATACGACGCCATTCCGCTCGATGATATTCGTTGATCCATGTTCATGAGATTCGA
CAGGGTTTTACGCTGGCTGTCGGGGCCTAACACAACCCTCATCCTTTATCCGGGACGTGTCCAACCAGAAGCAACAACA
ACAAAGCCTTATCCCACTAGGTGGGGTCGGCTACATGGATCATATTCGTTGATCCATGTTCATGAGATTCGACAGAGTT
TTACGCTGGCTGTC

ORF 89 to 712bp

ATGTTGAGCACTGCCCATGAGGGCGACGGATCTGTGAGGAAAGTGTCAGCGTCGTCAGCCTCGAGCCAGCGGTACCCG
ACGGAGACAGCGAGGAAGGTGTCAAGGGAGAGGGGGTTACGTAGGATGGAGTCGACGTTGCGCGGTTCAAGTACCCT
CCCCACGGGCTCGTTCTGGACGGTTCAAGTACCCTCCCCACAACGTCGCCCGGTTCAAGTACCCTCCCCACAACGTCGC
CCGGTTCAAGTACCCTCTCCAGAACGAGCCCGCGTTCTGTGGACGTGGGGAGGGTACTTGAACCGTGCAACGTCAACA
TCTGATTTTTGCAGTTCTTTTAAGCGTTACATAGGCTTACTTGCACGGTATAATAGAACACCGTTCATCGGGCCTAGGGT
TTGCTCTTGGCTTTATTTAAATTTCTATACCGAATTCATCCTTAACGCTTGCGTCAAGTGGTATCAGAGCGAGGAAGAGA
TCATGACTGATATTCCTACTAGAAAAGAGTTTGAAGGATTAAAAGCAAAGATAGCTCTCCTTACTGCTGCTATCGAAAA
ATTGTTTACGAATCAAGGTCCTATTCACACACGTGTCCAACCAGAAGCAAACAACAAAGCCTTATCCCACTAG

5’ UTR, 88bp

CCAGAACGAGCCCGCGTTTTGCGGCGATCCCCCTCCATAGCCACCTGGCCCGCGCACCTCCAGCCGGAGCACTTCCTTG
GAGTTCCAC

3’ UTR, 247bp

GTGGGGTCGGCTACATGGATCATACGACGCCATTCCGCTCGATGATATTCGTTGATCCATGTTCATGAGATTCGACAGG
GTTTTACGCTGGCTGTCGGGGCCTAACACAACCCTCATCCTTTATCCGGGACGTGTCCAACCAGAAGCAACAACAACAA
AGCCTTATCCCACTAGGTGGGGTCGGCTACATGGATCATATTCGTTGATCCATGTTCATGAGATTCGACAGAGTTTTAC
GCTGGCTGTC

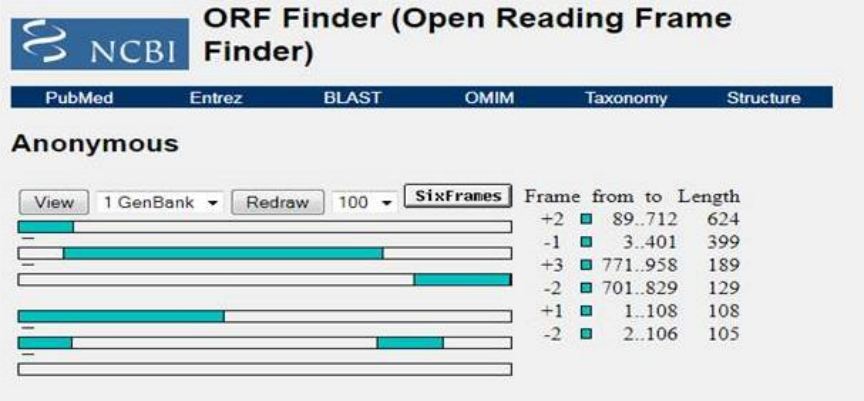

Fig 4.3 Detailed diagrammatic representation of 0.85 kb *Echmr* gene

4.3.2 Homology and phylogenetic analysis

The nucleotide sequences of *Echmr* was analysed by BlastN and BlastP programs (Altschul et al., 1997), MegAlign module of DNASTAR, MultiAlign module of CloneManager 9, which concluded its homology with different salt and water stress unknown ESTs from Glycine max, groundnut, Finger millet and lotus (Fig 4.4). The multi-way alignment of *Echmr* gene was done with other eukaryotic abiotic stress related genes viz, Cd-TDFs SlcDNA: Suillus luteus Cd transcript derived fragments, DhSMacDNA:Mulberry dehydration stress cDNA, DS AhcDNA: Groundnut drought stressed cDNA, EthyR BfcDNA: ethylene treated Botryotinia fuckeliana cDNA, Kudzu cDNA: Kudzu subtraction cDNA, SI GmcDNA:Salt induced D4 GmcDNA, SS EccDNA: Finger millet salt stress cDNA, WSGmcDNA, CX703998: Water stressed 48h Glycine max cDNA, WSGmcDNA, CX708886: *Glycine max* water stressed cDNA, WSGmcDNA, CX709187:*Glycine max* water stressed cDNA (Fig 4.5). The *Echmr* gene was found to be closely related to Cd-TDFs, DhSMacDNA, DS AhcDNA, EthyR BfcDNA, and Kudzu cDNA suggesting that *Echmr* gene might have role in HM tolerance and other abiotic stress responses.

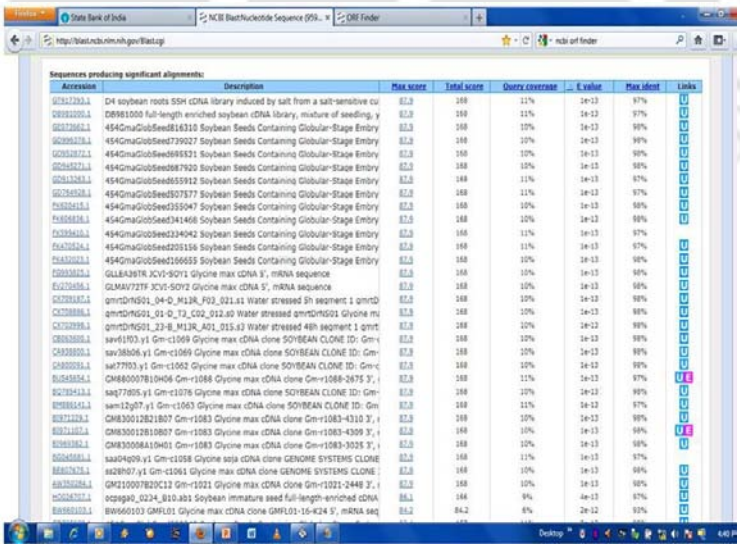

Fig 4.4 Snapshot of BLAST results as displayed on NCBI web server.

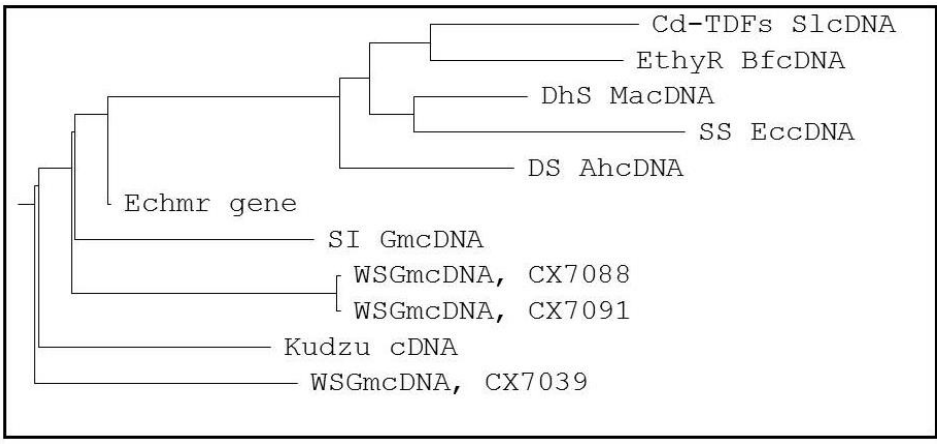

Fig 4.5 Multi-way alignment of *Echmr* gene with other eukaryotic abiotic stress related genes

4.3.3 *Cis*-regulatory elements analysis

The *cis*-regulatory elements present in promoter or UTR regions play a key role in conferring environmental regulation of gene expression. The cis-regulatory element search study was done by using by using in PLACE (Plant *cis*-acting regulatory DNA Elements) database (Higo et al 1999) and SIGNAL SCAN (Prestridge 1991). A list of putative cis-elements i.e. transcription factor binding sites was obtained for the 5' and 3' UTRs sequences of *Echmr* gene and validated using PLACE database (Table 4.1, Table 4.2). All cis-elements responding to abiotic stress were filtered out of the list of binding motifs and which specifically consists of ABRE-like binding site motif, DRE core motif, MYB and bHLH binding site, LTRE, Ca^{2+}/calmoduin binding iste, HSE. One of the prominent motif is ABRE like binding site motif [(C/G/T) ACGTG (G/T)(A/C)] which binds transcription factors involving molecular responses to dehydration and low temperature. However LTRE promoter motif with GCC-box and HSE are involved in response to cold and heat shock stress respectively. The presence of various STREs viz., DRE, ABRE, HSE and Calmudilin binding element in the UTRs of *Echmr* gene gives a hint of its role in abiotic stress responses. This appeal for the design of stress assay experiments subsequebtly to check the functionality of the *Echmr* gene in stress responses.

Factor or Site Name	Loc.(Str.)	Signal Sequence	Role
ABRERATCAL	site 12 (-)	MACGYGB	ABA & water stress regulation
CACTFTPPCA1	site 70 (+)	YACT	Regulatory element of C4 plants
CDA1ATCAB2	site 14 (-)	CAAAACGC	Dark response element
LTRECOREAtCOR15	site 31 (+)	CCGAC	Low temperature responsive element
CGCGBOXAT	site 12 (+)	VCGCGB	Stress related Ca^{++}/calmodulin binding element
CGCGBOXAT	site 50 (+)	VCGCGB	Stress related Ca^{++}/calmodulin binding element
CGCGBOXAT	site 12 (-)	VCGCGB	Stress related Ca^{++}/calmodulin binding element
CGCGBOXAT	site 50 (-)	VCGCGB	Stress related Ca^{++}/calmodulin binding element
EBOXBNNAPA	site 42 (+)	CANNTG	Regulatory element of bHLH TFs
EBOXBNNAPA	site 42 (-)	CANNTG	Regulatory element of bHLH TFs
EECCRCAH1	site 80 (+)	GANTTNC	Binding site of EE ENHANCER & Myb TFs
MYCCONSENSUSAT	site 42 (+)	CANNTG	Dehydration response element
MYCCONSENSUSAT	site 42 (-)	CANNTG	Dehydration response element
RAV1BAT	site 42 (+)	CACCTG	Binding consensus sequence of an AtTF, RAV1
SBOXATRBCS	site 55 (+)	CACCTCCA	ABA & sugar responsive element
SITEIOSPCNA	site 41 (-)	CCAGGTGG	Binding site of Cytc gene promoter
SORLIP1AT	site 40 (+)	GCCAC	phyA-induced motifs
HSE 70A	site 38 (-)	CGAYNRNNNNNNNNNNNNNNHD	Heat shock element

Table 4.1 PLACE analysis of 5' UTRs of *Eichhornia* HM stress responsive gene

Factor or Site Name	Loc.(Str.)	Signal Sequence	ROLE
ABRELATERD1	130 (+)	ACGTG	Early responsive to dehydration element
ACGTABREMOTIFA2OSEM	130 (+)	ACGTGKC	ACGT core of motif in ABRE
ACGTATERD1(2 sites)	130 (+)	ACGT	Early responsive to dehydration element
ARR1AT (2 sites)	68 (+)	NGATT	ARRI response regulator binding element
CACTFTPPCA1	169 (+)	YACT	Regulatory element of C4 plants
CANBNNAPA	102 (+)	CNAACAC	Core of (CA)n element of gene
CBFHV (2 sites)	6 (-)	RYCGAC	Binding site of barley CBFs
CGACGOSAMY3	25 (+)	CGACG	CGACG element of GC rich gene
DOFCOREZM(2 sites)	57(+)	AAAG	Core binding site of Dof protein/Zinc finger TFs
DRECRTCOREAT(2 sites)	6 (-)	RCCGAC	Dehydration response element/C repeat
GADOWNAT	130 (+)	ACGTGTC	ABRE similar motif of GA down regulated genes
GATABOX (3 sites)	44 (+)	GATA	Require for high level light regulated tissue specificity
GT1CONSENSUS	120 (-)	GRWAAW	GT1 binding site for light regulated genes
IBOX	162 (-)	GATAAG	Conserved sequence upstream of light regulated genes
IBOXCORE (2 sites)	121 (-)	GATAA	Conserved sequence upstream of light regulated genes
IBOXCORENT	161 (-)	GATAAGR	Conserved sequence upstream of light regulated genes
LTRECOREATCOR15(3 sites)	6(-)	CCGAC	Core of low temperature responsive element(LTRE)
MYBPZM	136 (+)	CCWACC	Core of maize P homolog binding site
MYBST1(2 sites)	122 (-)	GGATA	Core of maize P homolog binding site
RAV1AAT(3 sites)	147 (+)	CAACA	Binding consensus sequence of an AtTF, RAV1
REBETALGLHCB21	122 (-)	CGGATA	REbeta found in Lhc21 gene promoter
ROOTMOTIFTAPOX1(2 sites)	45 (+)	ATATT	Motifs found in promoter of RolD
SORLIP2AT	98 (+)	GGGCC	phyA-induced motifs
SREATMSD(2 sites)	121 (+)	TTATCC	Sugar repressive element
TAAAGSTKST1	119 (-)	TAAAG	TAAAG motif found in potato KST1 gene

Table 4.2 PLACE analysis of 3' UTRs of *Eichhornia* HM stress responsive gene

4.3.4 Protein topology and motifs search analysis

The *Echmr* gene sequence was translated with the Protein module of the DNASTAR software and then deduced EcHMR protein sequence was analyzed to obtain the detailed information about the general physical, chemical, thermal properties and amino acids composition.

EcHMR protein information

MLSTAHEGDGSVRKVSASSASSQRYPTETARKVSRERGLRRMESTLRGSSTLPTGSFWTVQVPSPQ
RRPVQVPSPQRRPVQVPSPERARVLWTWGGYLNRATSTSDFCSSFKRYIGLLARYNRTPFIGPRVC
SWLYLNFYTEFILNACVKWYQSEEEIMTDIPTRKEFEGLKAKIALLTAAIEKLFTNQGPIHTRVQPE
ANNKALSH.

Molecular Weight **23509.83** Daltons

207 Amino Acids

10.2 Isolectric Point

14.3 Charge at PH 7.0

Melting Temperature: Davis, Botstein, Roth Melting Temp. **84.80˚C**

Amino acids composition

30 Strongly **Basic(+)** Amino Acids (K,R), **16** Strongly **Acidic(-)** Amino Acids (D,E), **64 Hydrophobic** Amino Acids (A,I,L,F,W,V), **65 Polar** Amino Acids (N,C,Q,S,T,Y)

Furthermore, the hydropathy plots of Echmr protein was deduced by the PROTEAN module of the DNASTAR, wherein hydropathy values were assigned for all amino acids and then averaged over a user defined window. Residue hydropathy assignments were derived from water-vapor transfer free energies and the interior–exterior distribution of residue side-chains. This analysis has shown that major part of the EcHMR is hydrophobic (Fig 4.6) and may correspond to transmembrane famiy of proteins or channel/transporter type of proteins.

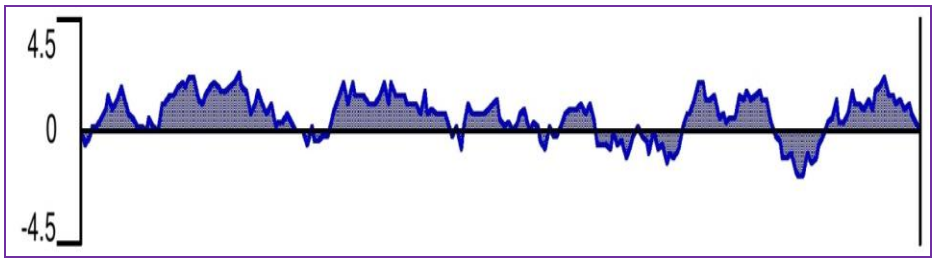

Fig 4.6 Hydropathy plot of Echmr protein

The search for putative functional motifs in EcHMR protein was performed in UnirotKB and UniProtMES which showed its homology to unknown novel proteins in Alga, Zebra fish, Hsps from yeast suggesting the protein being unknown and novel and may have role in stress tolerance (Table 4.3).

Table 4.3 Protein Motif predictions using UniProtKB and UniProt Metagenomic and environmental Sequences (UniMES)

SOPMA correctly predicts 69.5% of amino acids for a three-state description of the secondary structure (alpha-helix, beta-sheet and coil) in a whole database containing 126 chains of non-homologous (less than 25 % identity) proteins (Geourjon and Deleage, 1995). It was used to

predict probable secondary structure of EcHMR amino acid sequences. Little differences in secondary structure patterns were visible only in regions having differences in amino acid sequences. The secondary structure prediction of the EcHMR protein as per SOPMA reveals the alpha helical structure of the protein with predicted structure as 39.61 % alpha-helix, 9.18 % beta-turn and 37.20 % random coil (Fig 4.7) and the details are listed below.

Sequence length: 207

MLSTAHEGDGSVRKVSASSASSQRYPTETARKVSRERGLRRMESTLRGSSTLPTGSFWT
VQVPSPQRRPVeeeecctttccceeeeehccccccccccchhhhhhhhhttthhhhhhhhttttcccccceeeeeccccccccQ
VPSPQRRPVQVPSPERARVLWTWGGYLNRATSTSDFCSSFKRYIGLLARYNRTPFIGPRV
CSWLYLNFYecccccccceeccccccheeeeettchhccccccccchhhhhhhhhhhhhhhhttcccccccccchhhhhhhhTE
FILNACVKWYQSEEEIMTDIPTRKEFEGLKAKIALLTAAIEKLFTNQGPIHTRVQPEANNK
ALSHhhhhhhhhhhhcccthhheecccccchhhhhhhhhhhhhhhhhhhhhcttccceeeecttccttthhhh

Protein Structure of EcHMR:

Alpha helix (Hh) : 82 is 39.61%

3_{10} helix (Gg) : 0 is 0.00%

Pi helix (Ti) : 0 is 0.00%

Beta bridge (Bb) : 0 is 0.00%

Extended strand (Ee) : 29 is 14.01%

Beta turn (Tt) : 19 is 9.18%

Bend region (Ss) : 0 is 0.00%

Random coil (Cc) : 77 is 37.20%

Ambigous states (?) : 0 is 0.00%

Other states : 0 is 0.00%

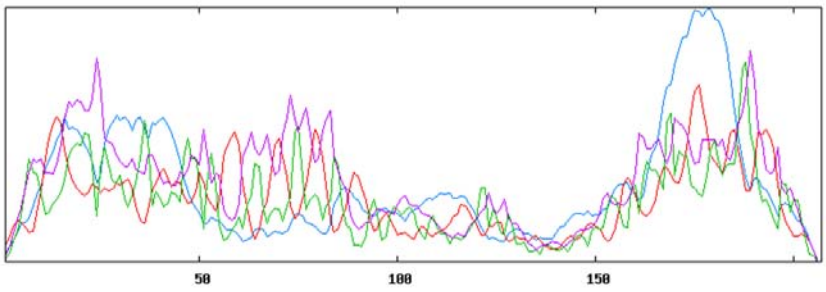

Fig 4.7 Predicted protein structure of EcHMR by SOPMA

Predicted secondary structure for each gene was obtained with regard to its tendency to from beta-strands, alpha helix, coils or turns. The common observation in all HMs stress responsive proteins is abundance of BETA-SHEET at the N-terminal end and alha-helix at the C-terminal end (Vatamaniuk et al., 2000; Cobbett and Goldsbrough, 2002). Thus the catalytic domain containing regulatory active site and activation loop are in N-terminal BETA-sheets. Whereas the sensor and HM binding site are contained in C-terminal end which has characteristic secondary structure of alpha-helix. It has been reported that alpha5/alpha5C sensors *Synechococcus* SmtB and *Mycobacterium tuberculosis* NmtR form metal complexes with biologically required metal ions Zn(II), Co(II) and Ni(II) characterized by four or more coordination bonds to a mixture of histidine and carboxylate ligands derived from the C-terminal alpha5 helices on opposite subunits(Busenlehner et al., 2003). It is hypothesized that distinct allosteric pathways for metal sensing have co-evolved with metal specificities of distinct alpha3N and alpha5 coordination complexes located at C-terminal (Liu et al., 2005). It has beeen reported that the metal-binding loop of HM binding proteins is malleable enough to accommodate modes of coordination other than linear bicoordinate (DeSilva et al., 2002).

To find conserved motifs in EcHMR, Multiple EM for Motif Elicitation (MEME) version 3.5.4 (Bailey and Elkan, 1994) was used. The parameters of this analysis were setup as: number of repetition, any; maximum number of motifs, 10; and optimum width of the motif, $\geq$ 6 and $\leq$ 300. This resulted in the prediction of nine motifs in the protein of which two motifs seems to be metal binding motifs present in the EcHMR protein (Table 4.4). These motifs were further analysed and aligned by MAST tool for motifs similarity and further compared with reported HM binding motifs (Fig 4.8).

Motifs Discovered	E-value & Sites	Sequence logo	Motif location Start and Sites sequence
Motif 1	7.4e-002 3 sites		69 VQVPSPQRRPVQVPSPQR RPVQVPSPER 59 STLPTGSFWTVQVPSPQR RPVQVPSPQR 79 VQVPSPQRRPVQVPSPER ARVLWTWGGY
Motif 2	4.5e+002 3		111 TSTSDFCSSFKRYIGL LARYNRTPFI 165 EEIMTDIPTRKEFEGL KAKIALLTAA 171 IPTRKEFEGLKAKIAL LTAAIEKL
Motif 3	6.7e+001 3		135 FIGPRVCSWLYLNFYT EFILNACVKW 96 RARVLWTWGGYLNRAT STSDFCSSFK 143 WLYLNFYTEFILNACVKWYQSEEEIM
Motif 4	6.0e+002 3		183 KIALLTAAIEKLFTNQGPIH TRVQPEANNK 149 YTEFILNACVKWYQSEEEIM TDIPTRKEFE 0 MLSTAHEGDG SVRKVSASSA
Motif 5	2.6e+003 3		129 RYNRTPFIGPRVCSWL YLNFYTEFIL 105 GYLNRATSTSDFCSSF KRYIGLLARY 40 RKVSRERGLRRMESTL RGSSTLPTGS

Motifs Discovered	E-value & Sites	Sequence logo	Motif location Start and Sites sequence
Motif 6	4.2e+004 3		87 RPVQVPSPERARVLWTWGGYLNRATS 53 STLRGSSTLPTGSFWTVQVPSPQRRP 118 SSFKRYIGLLARYNRTPFIGPRVCSW
Motif 7	4.4e+004 3		12 STAHEGDGSVRKVSASSASSQRYPTE 30 SSQRYPTETARKVSRERGLRRMESTL 201 IHTRVQPEANNKALSH
Motif 8	1.3e+005 3		22 RKVSASSASSQRYPTETARKVSRERG 159 KWYQSEEEIMTDIPTRKEFEGLKAKI 193 KLFTNQGPIHTRVQPEANNKALSH
Motif 9	1.1e+006 3		177 FEGLKAKIALLTAAIE KLFTNQGPIH 201 IHTRVQPEANNKALSH 47 GLRRMESTLRGSSTLPTGSFWTVQVP

Table 4.4 Putative Motifs of EcHMR protein by MEME (Multiple EM for Motif Elicitation) analysis

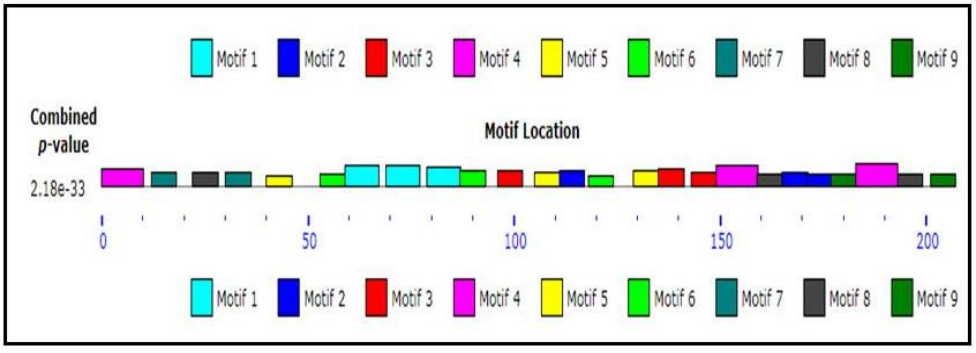

Fig 4.8 Motifs location of Echmr protein as per MAST (Motifs alignment and search tool) analysis

The metal binding domains have homologous sequences containing the GMTCXXC, GMTCNSC, MXCXXC motif found in many proteins involved in detoxification systems in yeast and bacteria (DeSilva et al., 2002). This sequence is able to bind and transport a host of different HMs in various environments (Vatamaniuk et al., 2000; Cobbett and Goldsbrough, 2002). It has been found that MNK1 shows differential specificity with change in CXXC motifs to bind various metals besides copper, such as Hg(II) and Ag(I) which prefer a linear bicoordinate geometry, as well as Cd(II) and Ni(II) which tend to bind proteins in an octahedral fashion (DeSilva, 2000). It was the change in register of the cysteines CAAC, CACA, and CCAA which governs the HM selectivity. Comparing with the previous metal binding motifs and reports, the motif3 (YLNBFAACTV) of EcHMR is found to be metal binding motif, which may have role to play in HM stress responses. Understanding metal specificity may provide insight into new methods for environmental as well as human HM detoxification.

4.3.5 Protein functions and sub-cellular localization prediction

The I-TASSER server provides an integrated platform for automated protein structure and function prediction based on the sequence-to-structure-to-function paradigm (Roy et al., 2010; Zhang, 2008). The I-TASSER has been used to predict the function and subcellular localization of EcHMR following three-dimensional (3D) atomic models generation by multiple threading alignments and iterative structural assembly simulations. Then the function of the EcHMR protein was inferred by structurally matching the 3D models with other known proteins. The output of EcHMR typical server run containing full-length secondary and tertiary structure predictions, and functional annotations on ligand-binding sites, Gene Ontology terms and its localization has been listed as follows.

Predicted secondary structures of EcHMR protein in the I-TASSER platform

SequenceMLSTAHEGDGSVRKVSASSASSQRYPTETARKVSRERGLRRMESTLRGSSTLPTGSFWT
VQVPSPQRRPVQVPSPQRRPVQVPSPERARVLWTWGGYLNRATSTSDFCSSFKRYIGLLARYNRTP
FIGPRVCSWLYLNFYTEFILNACVKWYQSEEEIMTDIPTRKEFEGLKAKIALLTAAIEKLFTNQGPIH
TRVQPEANNKALSH

PredictionCCCCCCCCCCCCSSSSCCCCCCCCCCCCCHHHHHCCCCHHHHHHHHHHHCCCCCCCCCCCSSSSCCCCCCCCCCCCCCCCCCCCCCCCCCCCCSSSSCCCSCCCCCCCHHHHHHHHHHHHHHHHHHCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHCHHHHHHCCCCHHHHHHHHHHHHHHHHHHHHHHCCCCCCCSCCCCCCCCCCCCC

Conf.Score9653445887325650243344557606665415602247889887436656777732445789889998898999999879898889778850361034433047899999999999873478876742435578888999999999998730888863476055441178999999999999863588740202644454211 49

One of the most important highlights of the I-TASSER simulations is the ability of template refinement and the search for most closely EcHMR related full-length atomic models along with the estimated accuracy (TM-score and RMSD to the native) of the models(Fig 4.9). C-score is typically in the range of (-5, 2), where a higher C-score value signifies a model with a high confidence and vice-versa. TM-score and RMSD are known standards for measuring structural similarity between two structures which are usually used to measure the accuracy of structure modelling when the native structure is known. In case where the native structure is not known, it becomes necessary to predict the quality of the modelling prediction, i.e. what is the distance between the predicted model and the native structures? Then it became imperative to correlate the TM-score and RMSD of the predicted models with the native structures based on the C-score. It has been found that model 1 correlation coefficient of C-score with TM-score to the native structure is 0.91, while the coefficient of C-score with RMSD to the native structure is 0.75. These data actually lay the base for the reliable prediction of the TM-score and RMSD using C-score. Hence among all the predicted models, the best predicted model of EcHMR protein is model 1 with estimated accuracy of 0.33± 0.11 (TM-score), 13.8±3.9Å (RMSD).

Model 1, C-score=-3.53 Model 2, C-score=-4.12 Model 3, C-score=-4.17

Model 4, C-score=-4.36 Model 5, C-score=-4.43

Fig 4.9 Top five models of EcHMR proteins predicted by I-TASSER

The comparison of the EcHMR to find out the highly similar structure in PDB by TM-align found out yeast Mg^{2+} Channel protein Mrs2, to be the best identified structural analogs in PDB with TM score of 0.879 and coverage 0.927(Khan et al unpublished, DOI:10.2210/pdb3rkg/pdb, UniProtKB:Q01926). Among the identified stuctural analogs in PDB, the EcHMR was also found to have similarity with CorA Mg^{2+} transmembrane transporter homologue from *Thermotoga maritima* involved in Mg^{2+} homeostasis with TM-score of 0.612 and coverage 0.768 (Payandeh and Pai, 2006) and ZntB cytoplasmic domain from *Vibrio parahaemolyticus* RIMD 2210633 with TM-score 0.529 and coverage 0.792 (Tan et al., 2009) (Table 4.5).

Rank	CscoreGO	Tmscore	RMSDa	IDENa	Cov.	PDB Hit(Functional protein)
1	0.09	0.6092	3.38	0.08	0.78	2iubF(divalent metal ion transporter CorA at 2.9 angstrom resolution)
2	0.08	0.5261	4.47	0.07	0.78	3nvoA(CorA Mg^{2+} transporter homologue from *T. maritima*)
3	0.08	0.5202	4.30	0.05	0.77	3ck6E(Domain Structure of the ZntB Zn^{2+} Efflux System)
4	0.08	0.5348	4.67	0.08	0.80	2bbhA(Crystal structure of the CorA Mg^{2+} transporter)
5	0.07	0.5109	4.19	0.02	0.70	2hn1A(ZntB cytoplasmic domain from *V. parahaemolyticus*)
6	0.07	0.4478	5.54	0.04	0.75	2nuuD(CorA soluble domain from *A. fulgidus* in complex with Co^{2+})
7	0.07	0.4520	5.39	0.06	0.75	2b2fA(membrane-embedded H^{+-} translocating pyrophosphatase)
8	0.06	0.4579	5.55	0.06	0.79	3hd6A(*T. Maritima* Na^{+} pumping membrane integral pyrophosphatase)
9	0.06	0.4414	5.67	0.04	0.75	2wp8J(Human *Rhesus* Glycoprotein RhCG)
10	0.06	0.3700	5.86	0.04	0.66	2vnuD(*E. coli* ammonia channel AmtB protein)

Table 4.5 Top ten structural analogs which are structurally closest to the predicted 3D model

Furthermore, when the EcHMR protein was compared with with the structurally most similar 3D models with other known functional proteins, it has been inferred that EcHMR protein may have transition metal ion transmembrane transporter activity (GO:0072509) or divalent inorganic cation transmembrane transporter activity (GO:0072509) with GO score of 0.30 as molecular function. Consensus predictions regarding the gene ontology (GO) has revealed the biological function of EcHMR as transition metal ion transport (GO:0000041) or transmembrane transport (GO:0055085) with GO score of 0.34 and 0.30 respectively(Table 4.6). Functional predictions for the EcHMR protein in terms of gene ontology terms and ligand-binding sites have also revealed that the EcHMR protein may have probable biological function in transmembrane transport, transition metal ion transport, divalent metal ion transport.

Molecular Function		Biological Process		Cellular Location	
GO term	GO-Score	GO term	GO-Score	GO term	GO-Score
GO:0046915	0.30	GO:0055085	0.34	GO:0071944	0.31
GO:0072509	0.30	GO:0000041	0.30	GO:0031224	0.31

Table 4.6 Consensus Prediction of Gene Ontology terms

I-TASSER based TM-align module was used to predict the most probable cellular location of the EcHMR protein where the accumulation occurs in large amount when upregulated by stress stimuli to execute its function. The predicted binding site of EcHMR has revealed its closest similarity with crystal structure of the CorA Mg^{2+} transporter (PDB hit 2bbhA, Lunin et al 2006) and with Ca^{2+} ATPase pump crystal structure (PDB hit 2agvA, Obara et al 2005)(Table 4.7).

Rank	C_{score}^{LB}	PDB Hit	TM-score	RMSD[a]	IDEN[a]	Cov.	Predicted binding site residues in the model
1	0.01	2bbhA	0.535	4.67	0.077	0.802	84,137,141,144,174
2	0.01	2bbhA	0.535	4.67	0.077	0.802	84,181,184
3	0.01	2agvA	0.425	5.13	0.047	0.681	53,89,99
4	0.01	3ar3A	0.423	5.00	0.042	0.667	52,84,85

Table 4.7 Predicted binding site (Template proteins with similar binding site)

The processing of EcHMR protein in the I-TASSER platform has revealed that the most probable cellular location of EcHMR protein was at cell periphery (GO:0071944, GO score 0.31) or intrinsic to membrane (GO:0031224, GO score 0.31). Most of the ionic transporter/antiporter get accumulated in the cytoplasm surrounding cell periphery or intrinsic to membrane as would be predicted for these transporter to be involved in influx/efflux of HM cations to maintain the homeostatic balance of cell in hostile environment. However, loss of function mutants of *Arabidopsis thaliana* has helped to characterize cellular localization of many membrane transporters *viz, AtHKT1:1* involved in long distance Na^+ transport (Rus et al., 2004), the plasma membrane Na^+/H^+ antiporter *SOS1* (Zhu, 2000) and rice OsHKT2;1 (Horie et al., 2007), and the

vacuolar pyrophosphatase *AtVP1* (Flowers and Colmer, 2008; Gaxiola et al., 2001). The preferable site of reported ionic transporters shows corroboration with EcHMR predicted celluar location and hence, it may possibly confers the same predicted function. It is well established that uptake, efflux, translocation, and compartmentalization of toxic ions serve as basis for HM tolerance in plants and potential avenues to improve crops (Brini and Masmoudi 2012). Whatsoever, a better understanding of the predicted membrane transport EcHMR protein may turn out to be a way to progress in HM responses.

4.4 Summary and Conclusions

In summary, the present study reports the isolation of putative HM stress responsive (*Echmr*) gene from HMs hyperaccumulator *Eichhornia crassipes* roots that may have a role to play in HM stress response. Moreover, our bioinformatic findings provide evidence that the presence of various STREs viz., DRE, ABRE, HSE and Calmudilin binding element , and HM binding motifs in EcHMR, which suggests its possible role in abiotic stress tolerance. The hydrophobic nature of EcHMR along with diverse alpha sheets in C terminal shows similarity with known HM binding proteins. The analysis also reveal that EcHMR protein has predicted molecular and biological function of metal ion transmembrane transporter activity with predicted binding site of EcHMR closest to crystal structure of the CorA Mg^{2+} transporter and Ca^{2+} ATPase pump crystal structure. The EcHMR protein cellular location was predicted at cell periphery or intrinsic to membrane. Compiling all outcomes though EcHMR seems to possess putative HM stress response function but a complete validation of EcHMR functions will be an important subject for further HM tolerance mechanisms.

5.1 Introduction

Plants exposed to abiotic stresses such as HMs, heat, cold and UV-B, have been found to induce oxidative stress due to excess accumulation of reactive oxygen species (ROS) and cellular ionic imbalance (Bowler et al., 1992). During HM stress, the plants may generate ROS directly through Haber-Weiss reactions or indirectly by interacting with the antioxidant system (Wojtaszek, 1997; Mithofer et al., 2004; Srivastava et al., 2004), disrupting the electron transport chain (Qadir et al., 2004) or disturbing the metabolism of essential elements (Dong et al., 2006) or through lipid peroxidation (Demiral and Türkan, 2005). An important approach of sustenance under stress condition may be the development of multiple stress tolerance. However, plants could survive in adverse heavy metal toxic environment by the production of low molecular weight thiols viz., GSH and cysteine that detoxify HMs (Bricker et al., 2001). GSH (γ-glutamate-cysteine-glycine) is a sulfur-containing tri-peptide thiol synthesized by two ATP dependent enzymes γ-glutamylcysteine synthetase (GSH1) and GSH synthetase (GSH2) (Noctor et al., 1998, 2002; Sugiyama et al., 2004). Out of which, GSH1 being the regulatory enzyme of GSH synthesis (Rüegsegger and Brunold, 1992; Rennenberg et al., 2007). In addition, it is involved in the transfer and storage of sulfur (Herschbach and Rennenberg, 2001) and in the detoxification of HMs, which form complexes with GSH-derived phytochelatins (Blum et a.l 2007; Freeman et al., 2004; Grill et al., 1989). The protective and regulatory roles of GSH are based on its redox state which is defined by the reducing capacity of GSH (GSH concentration) and the half-cell reduction potential of the GSH/GSSG couple ($E_{GSSG/2GSH}$). It differs in various organs, tissues, cells, and compartments and also changes with growth and development of the plants (Szalai et al., 2009). Generally during stresses, the decrease in the GSH/GSSG ratio could be due to the removal of ROS in the form of GSH conjugates (GS conjugates) or to GSH degradation (Kellos et al., 2008; Kranner et al., 2006). Till date many genes such as GSH1, GSH2, cystathionine synthase (CTS), ATP sulfurylase (APS), serine acetyltransferase (SAT), GSH reductase (GR), phytochelatin synthase (PCS) and glyoxalases have been widely studied in relation with HM

stress tolerance by regulating GSH and PCs levels (Yadav, 2010). Even overexpression of these genes in various plants has contributed to enhance tolerance against HMs.

There have been several reports of overexpression and insertion of foreign genes coding membrane-modifying enzymes, radical-scavenging enzymes, and stress-induced proteins to enhance stress tolerance potential in organisms. Nevertheless, extensive work has been done to ascertain the role of genes in offering tolerance to various metals but there is limited studies regarding its involvement in multiple stress tolerance. Transgenic tobacco expressing GSH1, PCS and SAT (either separately or in combination) have increased Cd concentration in roots suggesting their role in HM stress tolerance in plants (Wawrzyński et al., 2006). It is also reported that a modified bacterial GSH1 gene (S1ptTECS) upon expression in GSH1-deficient, HM sensitive *cad2-1* mutant of *A. thaliana* restored full Hg tolerance and partial Cd tolerance to the mutant (Li et al., 2006). Further there has been a report of differential effects of HMs, as As treatment was most effective in increasing the levels of γ-EC, GSH and PCs than Cd and Hg treatments. Interestingly, Cd exposure also induced 3–5 fold increase in *γ-EC* related peptides in the transgenics but could not provide tolerance to Cd stress (Li et al., 2005). Similarly, overexpression of a bacterial GSH1 gene in the cytosol or chloroplast of *Populus canescens* elevated the GSH level and provided tolerance to HMs (Bittsánszkya et al., 2005).. Keeping these facts into account, we hypothesized that enhanced expression of *Eichhornia crassipes Echmr* gene in Cd sensitive *E. coli Δgsh* mutants might complement and provide tolerance against HMs and other abiotic stresses like heat, cold, UV-B because all these stresses leads to ROS generation in the organisms.

5.2 Materials and methods

5.2.1 Bacterial strains and plasmids

E. coli strain DH5α and BL 21 (DE3) (Novagen) were stored as 20 % (v/v) glycerol stocks at -80°C and streaked on LB plates at 37°C. Cells harbouring recombinant plasmids pTZ57R:*Echmr* were grown and maintained on LB medium supplemented with 100 mg/ml ampicillin (Sambrook

and Russell, 2001). *E coli* wild-type strain W3110 and its Cd sensitive *ΔgshA* and *ΔgshB* mutant derivatives (Helbig et al., 2008a) were grown at 37°C in LB medium or in tris-buffered mineral salts medium (Mergeay et al., 1985) containing 0.2 % glycerol and 0.3 % casamino acids (TMM) with appropriate addition of antibiotics and metals (Helbig et al 2008b). Plasmids pTZ57R (Fermentas) and high expression vector pGEX-3X (GE Healthcare, USA) were used as a molecular tool for cloning.

5.2.2 Construction of expression vector

The *Echmr* cDNA fragment from pTZ57R:*Echmr* was prepared out with *BamHI* and *SmaI* ends taking pTZ57R-*Echmr* as template, eluted and purified. The resultant cDNA fragment was cloned into pGEX-3X with T_4 DNA ligase (NEB) and the recombinant plasmid pGEX-3X-*Echmr* was transformed into *E. coli* BL 21 and W3110, Δ*gshA*, Δ*gshB* strains and then grown in LB and TMM medium respectively. An empty vector pGEX-3X was also simultaneously introduced into the same *E. coli* strains to act as vector control. The recombinant plasmid was then isolated and restriction digested to confirm the presence of *Echmr* cDNA fragment in all the transformed recombinant *E. coli* strains and northern assay was also done to confirm its expression.

5.2.3 Complex thiol assay

The protein extract for enzyme assay was prepared by harvesting recombinant BL 21 (DE3) cells at 5000 rpm for 10 min and washed with extraction buffer (50mM Tris–HCl, 10 mM $MgCl_2$, 20mM KCl, pH 7.5). The pellet was resuspended in 5ml extraction buffer and subjected to grinding under liquid nitrogen to break the cells. The extract was centrifuged at 10,000 rpm for 15 min. The supernatant was acetone/TCA precipitated and centrifuged at 10,000 rpm, 15 min. The pellet was resuspended in 400µl Tris buffer (10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, pH 7.5). The samples were aliquoted and stored for further characterization. Amount of proteins in different extracts were estimated by Bradford method (1976). Non-protein total thiols (NP-SH) were measured following the Ellman method (1959) using GSH as standard and reduced GSH

content by the method of Anderson (1985) respectively. Then, the activity of EcHMR was determined by the level of complex thiol inside the cells by using the formula: total thiol - GSH.

5.2.4 Mode of stress application

1mM stock solutions of $CdCl_2.2H_2O$, $PbSO_4$ were prepared and filter sterilized by using a Millipore membrane filter (0.22 µm) and was added to sterilized medium as per concentration and incubated. *E. coli* cultures were exposed to cold and heat shock at 4^0C, 180 min and 50^0C, 20 min in cold room and temperature regulated incubator respectively. Two milliliters bacterial culture suspensions (OD 0.5) were transferred into quartz cuvettes (PyeUnicamB538751 A, thickness 1 mm, capacity 4 ml) exposed to artificial UV-B radiation in laminar hood for 15 min.

To measure the survival of *E. coli* cells transformed with pGEX-3X (vector control) against, heat, cold, HMs such as, cadmium and lead; cells were treated with different concentrations $CdCl_2$ and $PbSO_4$ (0, 50, 100, 150, 200 µM) in TMM medium respectively. The LC50 for cadmium ($CdCl_2$), temperature and UV-B were determined by the plate colony count method. Approximately 50% survival of *E. coli* was observed at $CdCl_2$ (100 µM), temperature 47^0C, and UV-B (10 min), respectively.

5.2.5 Stress growth assays

The 1:100 or 1:1000 diluted overnight primary cultures of *E. coli* strains in fresh LB or TMM media were used to seed secondary culture. After 2h, the secondary cultures were diluted 1:100 in fresh LB or TMM media with or without metal and incubated with shaking at 37°C. The turbidity at 600nm over 10 to 14h was monitored by using a SmartSpec3000 photometer (Bio-Rad, Munich, Germany). Specific growth rate (SGR) was calculated by using the equation: $SGR= [\ln(n_2/n_1)]/[t_2-t_1]$ where n_1, n_2 stands for absorbance of culture suspension at the beginning (t_1) and end (t_2) of selected time interval. Three independent measurements were carried out and the average value was used for making the final data.

5.2.5.1 Functional stress assay of Cd sensitive *Δgsh* mutant cells

In order to confirm the role of EcHMR, the effects of above abiotic stresses on the recovery of transformed *E. coli* cells with pGEX-3X (empty vector) and the recombinant plasmid pGEX-3X-*Echmr* were examined. For HM stress, the seeded secondary culture of the recombinant *Δgsh* mutants, W3110 and vector control were diluted serially to 10^{-6} and 5 µl of it were dotted on TMM plates supplemented with different concentrations of $CdCl_2$ and $PbSO_4$ (0, 50, 100, 150, 200 µM) respectively and incubated at 37˚C for overnight and analyzed. Whereas for other abiotic stress analysis, the 4 hr secondary culture of the above strains were given heat shock (50^0C, 20min), cold stress (4^0C, 30 min) and UV treatment (15min under laminar hood) respectively and then streaked on LB plates supplemented with IPTG. The plates were incubated at 37˚C overnight and analyzed. All these assays were done in duplicates and were repeated thrice for confirmation of results and consistency; then the best plate was considered for representation.

5.2.5.2 Expression analysis of *Echmr* in different abiotic stresses

RNA was isolated using TRIzol reagent for transcriptional regulation of the *Echmr* gene using northern assay and subjected to different abiotic stresses such as Cd, Pb, heat shock, cold shock and UV-B respectively. Recombinant *E. coli* BL21 (DE3) harboring pGEX3X-*Echmr* plasmid was grown in LB medium supplemented with 100µg/ml ampicillin and incubated at 37°C, 180 rpm. At OD 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM and then incubated for next 6 hrs. RNA was isolated from mid-exponential phase cells harboring the pGEX-3X and pGEX3X-*Echmr* plasmid before and after IPTG induction using the TRIzol reagent as per the instructions given in the manufacturer's protocol and all RNA samples were quantified (Sambrook and Russell 2001). To ascertain the equal concentration of RNA in the different samples rRNA were loaded and used as an internal control. After resolving RNA, RNA samples were capillary-blotted to positively-charged nylon membranes (Genescreen plus, NEN, USA) using standard protocols (Sambrook and Russell 2001). The blots were hybridized with denatured radiolabeled probe pTZ57:*Echmr* prepared by

using "Megaprime Labeling Kit" (Amersham, UK) at 42°C. The blots were then washed and exposed to films in hyper cassettes at -80°C (KODAK, USA) and finally developed.

5.3 Results and Discussion

GSH (*L-γ-glutamyl-L-cysteinyl-Gly*), a low-M_r thiol tripeptide is a product of two sequential ATP-dependent enzymatic reactions catalyzed by *Glu-Cys* ligase (GshA) and GSH synthetase (GshB; Fig. 5.1) (Cameron and Pakrasi, 2010). GshA catalyzes the ligation of Cys with the γ-carboxyl group of Glu to form γ-glutamyl-Cys (γ-EC). This first step has been documented as major control point under conditions of increasing demand for GSH (Noctor et al 1998). GshB ligates Gly to the Cys residue of γ-EC to form GSH. GSH accumulates to millimolar levels within the cell, primarily in the reduced form (GSH). GSH can undergo intermolecular oxidation to form GSH disulfide (GSSG), a process that can be reversed enzymatically by GSH reductase through NADPH dependent reduction of the disulfide (Serrano et al., 1984). The reduction potential of the 2GSH/GSSG redox couple is dependent on the absolute concentration and the ratio of reduced to oxidized GSH, and it has been proposed that this redox couple contributes significantly to the cellular redox environment (Schafer and Buettner 2001). Changes to the GSH redox state have been proposed to be involved in cellular signalling pathways in plants (Meyer 2008). However, analysis of the cellular redox environment is complicated by the fact that even within a single cell; different cellular organelles may maintain the GSH couple at different reducing potentials (Meyer et al., 2007; Wolf et al., 2008).

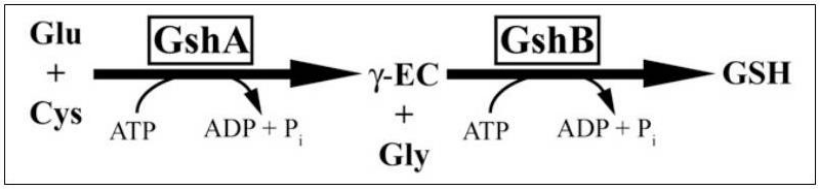

Fig 5.1 Schematic of GSH biosynthetic pathway (Cameroon and Prakashi 2010)

In plants, GSH is critical for many cellular functions specially tolerance to HMs and abiotic stresses (Mullineaux and Rausch, 2005; Rouhier et al., 2008; Foyer and Noctor, 2009). Even in *E.coli* and *Rhizobium* sp, GSH has been found to play important role in HM detoxification and tolerance (Helbig, 2008a; Latinwo et al., 1998; Figueira et al., 2005; Lima et al., 2006). Further the comparative simplicity of the *E.coli* cell makes biochemical interpretations more clear, potentially providing insight into the functions of GSH regulated HM tolerance potential of the plant gene *Echmr*. Hence, the *Δgsh* mutants of the *E.coli* have been preferred for functional validation of *Echmr* for abiotic stress tolerance.

5.3.1 Molecular cloning of *Echmr* gene

State of the *Echmr* gene in cloned in the expression vector pGEX3X with *BamH1* and *Sma1* was done as scribed in the construct linear map (Fig 5.2A). The *Echmr* cDNA was of about 850 bp containing the theoretical length of the a *Echmr* gene ORF was ligated to pGEX-3X *BamHI* and *SmaI* digested backbone fragment and transformed into *E. coli* strain BL21, W3110, *Δgsh* mutants. The construct was verified by double digestion with *BamH1* and *Sma1* Showing a release of 0.85 kb band (Fig. 5.2B). A 3–4 h exposure of cells transformed with recombinant plasmid to 0.5 mM IPTG was found to produce induced transcript of *Echmr* as analysed by northern assay.

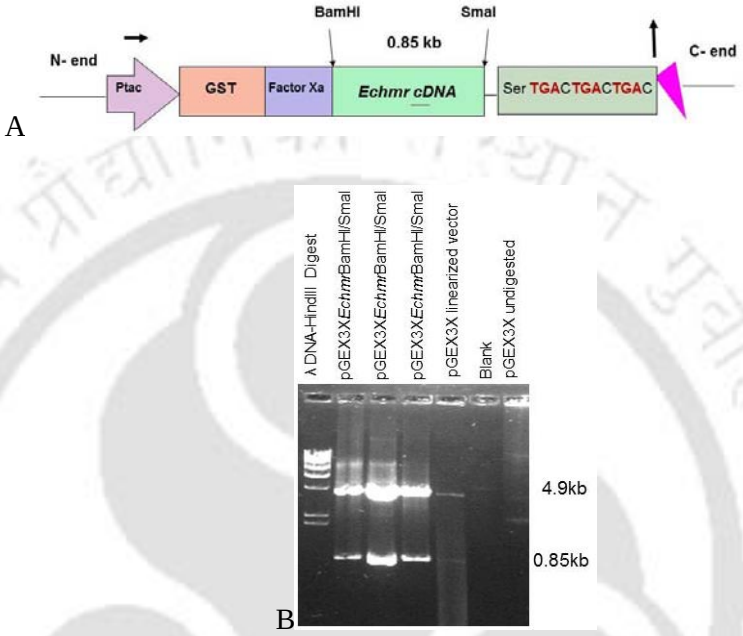

Fig 5.2 (A) GST fusion cassette of pGEX3X expression vector with *Echmr* cDNA cloned (B) Confirmation of recombinant clones with *BamHI* and *SmaI* double digestion showing the presence of 850 bp fragment and 4.9 kb pGEX-3X vector.

5.3.2 Thiol analysis of *E. coli*

It has been well established that the deletion of the *gshA* gene (encoding the γ-Glu-Cys synthetase), the *gshB* gene (encoding the GSH synthetase), or both genes from the genome of the *E. coli* wild-type strain W3110 has resulted in a decrease of the cellular GSH concentrations in cells grown in TMM and in LB medium-grown cells (Helbig et al., 2008a). So, in order to know the effect of the *Echmr* in the complex thiol concentration, the BL 21 (DE3) complemented with pGEX3X:*Echmr* was compared with wild type in induced and uninduced conditions.

Analysis of the complex thiol level in the complemented BL 21 strain showed that the intracellular concentration of complex thiol in recombinant BL 21 (induced) reaches the

maximum of 6 nmol/mg protein as compared to uninduced (4 nmol/mg protein). Whereas, the control BL21 cells shows the thiol level below the limit of detection to 0.2 nmol/mg protein (Fig 5.3). The considerable level of complex thiol even in uninduced recombinant BL 21 may be possibly due to the fact that GSH accumulates in the absence of IPTG because multiple copies of the *lac* promoter titrate out endogenous *lac* repression (Zagursky and Haus, 1983).

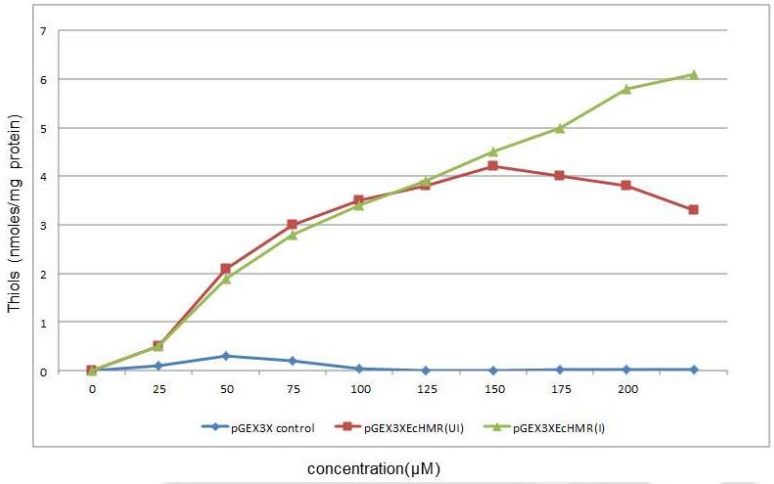

Fig 5.3 Thiol compound analysis of stressed recombinant *E.coli* strains

It has been reported that attempts to raise the level of GSH in the complemented mutant by provision of cysteine and glutamate in the growth medium were without effect (May and Leaver 1994). Furthermore, there is report that Cd stress elevated the γ-EC and thiol level of Δ*gshB* cell (Helbig et al 2008b). It is possible that the cloned *Echmr* when expressed; happens to help in the formation of complex thiols, which may lead to achieve a structure which facilitates optimal activity within the respective organisms. This is perhaps reflected in the low thiol levels measured in *E. coli* control extracts even when substrates were not limiting. Alternatively, the Echmr protein may have other transporter like activity which demands the formation of GSH complexed compounds for its functionality. Otherwise, the low level of complex thiol in control may be because the intracellular conditions within *E. coli* are not optimal for plant enzyme activity or that

the presence of a putative membrane transit peptide or improper folding results in only a small proportion of the expressed protein being active.

5.3.3 Measurement of growth

The cells transformed with recombinant plasmid (pGEX-3X:*Echmr*) showed better growth (approximately 30-40% more) than those transformed with empty vector (Fig.5.4). During the lower dosed of Cd stress, the growth of *E. coli* cells transformed with pGEX-3X (empty vector) was not inhibited in liquid culture(data not shown). In the initial phase of the Cd stress, the pGEX-3X:*Echmr* transformed cells showed only marginal difference in the specific growth rate as compared to control. The pGEX-3X:*Echmr* transformed *ΔgshA* cells showed significant difference in growth with the progress of time as compared to *ΔgshB* and vector control. The *E coli* cells synthesizing GSH (GSH; wild type), γ-glutamylcysteine (Δ*gshB* mutant), or neither of the two cellular thiols (Δ*gshA* mutant) were reported to respond to cadmium shock similarly, with the up-regulation of genes involved in protein, disulphide bond, and oxidative damage repair; cysteine and iron-sulfur cluster biosynthesis; the production of proteins containing sensitive iron-sulfur clusters; the storage of iron; and the detoxification of Cd^{2+} by efflux (Helbig et al., 2008b).

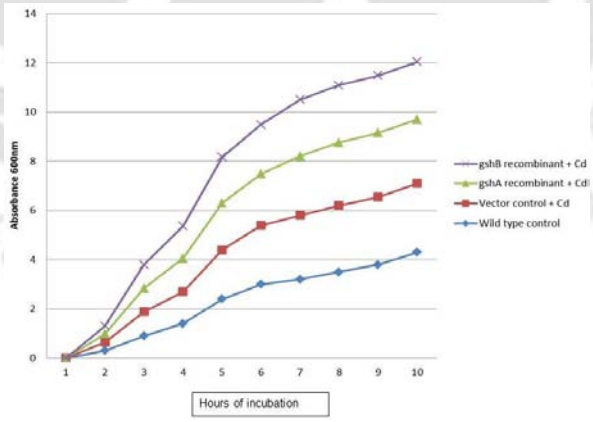

Fig 5.4 Growth assay of *E.coli* strains in 100 µM Cd in minimal medium

Even it has been reported that the loss of the ability to synthesize GSH influenced metal tolerance in wild-type cells only slightly, GSH was important for residual metal resistance in cells without

metal efflux systems. But *ΔgshB* cells containing γ-EC have shown less Cu and Cd tolerance than cells that contained neither γ-EC nor GSH (Helbig et al., 2008a). Despite its role as the sole thiol in halobacteria (Fahey, 2001), γ-EC is not able to fulfill the function of GSH in transition-metal resistance by *E coli* (Helbig et al., 2008a). In contrary to this, we investigated the impact of Cd on the growth of *E. coli* recombinant Δ*gshB* mutant cells to be lesser as compared to the wild-type, vector control and recombinant Δ*gshA* mutant cells. This was in contrary to the previous reports suggesting that *ΔgshA* mutant cells behaves well in Cd stress as compare to *ΔgshB* and wild type cells. The presence of *Echmr* gene in the mutant background and the elevated cellular complex thiols were the only difference between this study and previous reports. Since it has been proved that Cd toxicity is mainly due to the binding of Cd^{2+} to sulfide, thiol groups, and sulfur-rich complex compounds rather than from Cd^{2+} replacement of transition-metal cations from nitrogen or oxygen-rich biological compounds, so the formation of the complex thiol in Cd stressed *E coli* cells seem to be obvious. The high preference of the bacteria to detoxify HMs mainly by efflux than to sequester metals to the phytochelatin educt GSH may be crucial in this result (Nies, 1999, 2003). The presence of two cysteine residues and four carboxyl groups in GSH that leads to the formation of stable octahedral bisglutathionato complexes during Cd stress (Vatamaniuk et al., 2000) and may be the high growth rate of recombinant *Δgsh* mutants is due to the efflux function of Echmr protein to reduce the cellular Cd toxicity.

5.3.4 Expression and Functional HM stress assay of *Δgsh* mutant

To assess the functionality of the *Echmr* gene in HM stress, the *E coli* strains *viz*, recombinant *ΔgshA, ΔgshB* mutant cells containing pGEX-3X:*Echmr*, W3110 containing empty pGEX-3X and its control wild types were grown and exposed to Cd and Pb stresses. The growth of the serially diluted and dotted *E coli* cells in the Cd and Pb supplemented plates were monitored and the northern analysis of the recombinant stressed and unstressed cells was also performed to examine the level of *Echmr* expression. The Cd and Pb stresses triggered significant and discrete difference in the increase of the transcript level of *Echmr* gene than unstressed condition (Fig.

5.5). Whereas, the level of *Echmr* expression in Cd and Pb stresses of control pGEX-3X cells were comparable with no observable difference (data not shown). The level of transcript expression in Cd stress was comparatively 0.25 % lesser as compared to Pb stress.

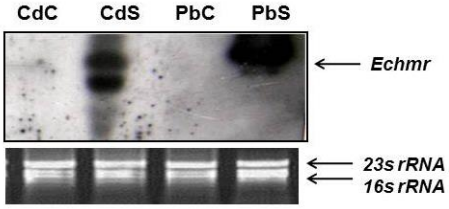

Fig 5.5 Expression of *Echmr* in recombinant *E.coli* strains in control and stress condition

The stress assay of the recombinant *ΔgshA, ΔgshB* mutant cells containing pGEX-3X:*Echmr*, W3110 containing empty pGEX-3X and its control wild types grown in excess of Cd and Pb have shown differential pattern of survival with varying concentration of Cd and Pb. In the lower doses of Pb concentration, wild type W3110 and *ΔgshB* cells could grow considerable well upto 100µM. The recombinant *ΔgshB* mutant cells were found to survive well even in the increasing concentration of Pb upto 200 µM (Fig 5.6).

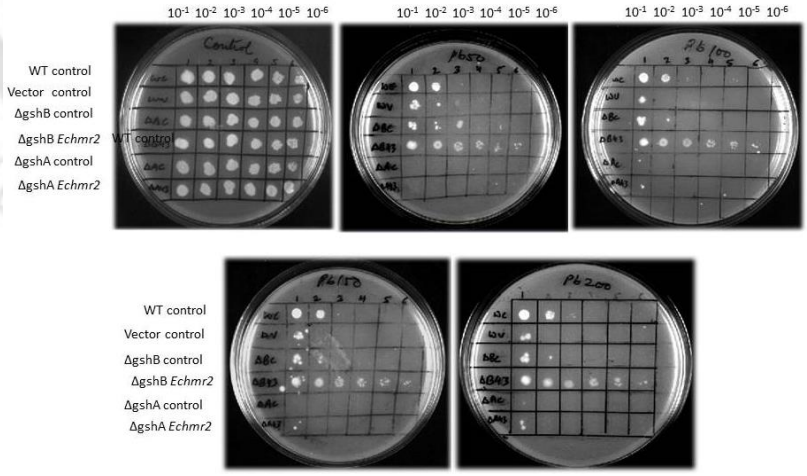

Fig 5.6 *Echmr* functional complementation in *ΔgshA* & *ΔgshB* mutants *of E.coli* in TMM media under Pb stress

It seems interesting that even the wild type W3110 could survive in 200 µM upto 10^{-2} dilution only, which may be due to upregulation of tryptophan biosynthesis in wild type cells upon HM stress (Helbig et al., 2008b). In the absence of GSH in TMM containing Pb media plates, the cytoplasmic concentration of GSH and the affinity of most transition metals for sulfur may help Pb^{2+} entering *E. coli* cell to form a bis-glutathionato complex (Nies, 2007). The variable complex such as Hg^{2+}-bis-glutathionato complex (Oram et al., 1996) and the Cu^{2+}-monoglutathionato complex (Osterberg et al., 1979) were found to regulate the sensitivity of *E coli* cells to such transition metals. Similarly the high level of Pb^{2+} bis-glutathionato complex may have formed in *ΔgshB* recombinant cells which may be due to high cellular complex thiol and the possible transporter activity of Echmr protein might have provided a mechanism of tolerance to the cells during Pb stress.

Similar to the findings for Pb stress, GSH-free cells (Δ*gshA* cells) and wild type W3110 suffered increased toxicity of Cd in TMM plates. The deletion of the gene for the *Glu-Cys* ligase were seems to have much stronger effect of cadmium toxicity in Δ*gshA* cells than the presence of γ-EC (*ΔgshB* cells). Thus, the recombinant Δ*gshB* mutant cells were shown to have better protection in Cd exposure in TMM plates than other *E coli* cells (Fig 5.7). The growth of the wild type, vector control and *ΔgshA* recombinant cells were seems to be totally inhibited even in 50µM concentration of Cd suggesting the highly diverse toxic effects of Cd^{2+} on these cells.

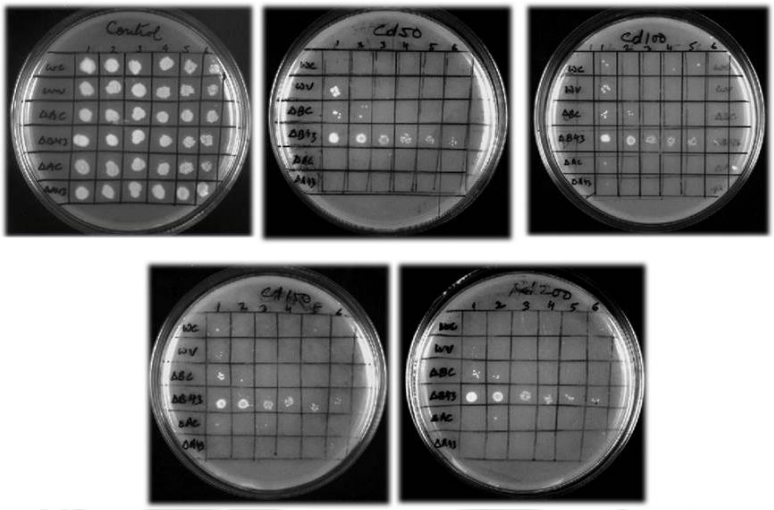

Fig 5.7 *Echmr* functional complementation in *ΔgshA* & *ΔgshB* mutants *of E.coli* in TMM media under Cd stress

When the Cd stress assay was performed with same cells in LB plates containing Cd, the wild type W3110, vector control, *ΔgshB* cells were found to grow normally up to 450 µM Cd concentration. Thereafter, the gradual inhibition of the growth was seen at 650 µM and the recombinant *ΔgshB* cells could survive upto 1000 µM Cd concentration (Fig 5.8). Even the Δ*gshA* recombinant cells were found to grow well upto 10^{-2} dilution in 450 µM Cd concentration with gradual reduction in cell survival at 650 µM and 1000 µM Cd LB plates. The high level of tolerance of *E coli* cells in LB medium against Cd may be due to the fact that LB medium contains yeast extract and, therefore, contains approximately 0.9 % of GSH in it. So, may be the wild type and *ΔgshA* cells could uptake external GSH by the GsiABCD uptake system (Suzuki et al 2005). It may possibly increase the Cd^{2+}-bis-glutathionato complex formation and may be the Echmr transporter functions helping to extend the survival limit in increasing Cd concentration. Even though LB medium-grown cells were not entirely GSH free, hence *E. coli Δgsh* mutant strains with multiple deletions in *gsh* genes plus *Echmr* gene probably having metal efflux function were tested in LB plates. As the minor residual amount of the tripeptide should not be able to affect toxic metal concentrations and that this results lends support to the functionality of *Echmr* in Cd tolerance to Δ*gsh* mutant cells.

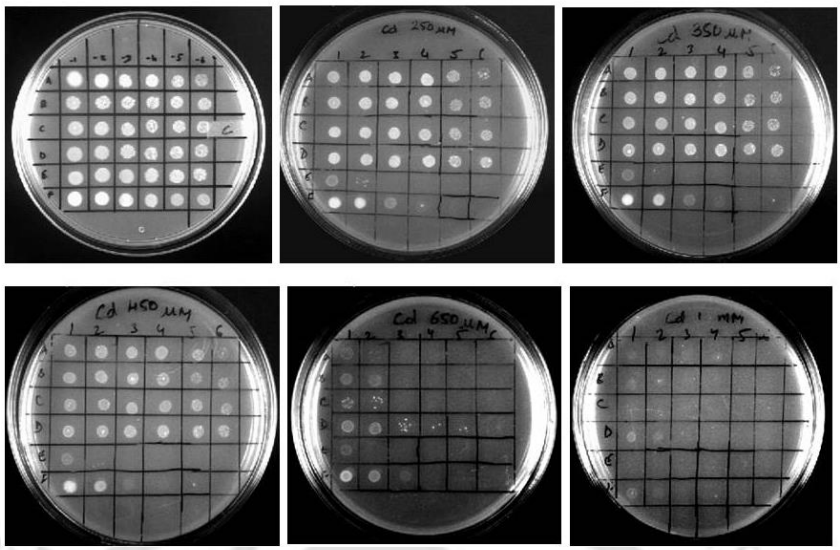

Fig 5.8 *Echmr* functional complementation in *ΔgshA* & *ΔgshB* mutants *of E.coli* in LB media under Cd stress

There is dearth of information about the interplay of HMs and GSH in bacteria, except that GSH influences resistance to arsenite and mercury in *E. coli* (Latinwo et al., 1998) and cadmium tolerance in *Rhizobium leguminosarum* bv. *viciae* (Figueira et al., 2005; Lima et al., 2006). On the other hand, GSH is heavily involved in transition-metal homeostasis in eukaryotes. In *Saccharomyces cerevisiae,* GSH is essential for full cadmium resistance (Gharieb and Gadd 2004). Furthermore, there has been reports of formation of the γ-glutamyl Cu-binding peptide in *Schizosaccharomyces pombe* (Reese et al., 1988) and accumulation of non-protein metal-binding polypeptides (γ-glutamyl-cysteinyl)n-glycine in selected cadmium-resistant tomato cells (Steffens et al., 1986). Laterally, these reports extend support to the formation of Cd/Pb-bis-glutathionato or γ-EC complexes in *E coli* cells also upon HM stress. Recently, there has been report that increasing Cd stress induces decrease in the level of the GSH and γ-EC level in wild-type and Δ*gshB* cells (Helbig 2008b). This may be due to the fact that Cd-bis-glutathionato or γ-EC complexes may be exported by a yet-unknown detoxification system. Hence, may be Echmr protein is possibly offering the efflux function here and lending support to its hypothesized transporter function.

Expression and functional analysis of *Echmr* in different abiotic stresses

Northern blot analysis of total RNA isolated from *E. coli* control and *Echmr* complemented stressed *Δgsh* mutant strains under different abiotic stresses *viz*, heat, cold and UV treatment were used to determine the pattern of expression of the gene corresponding to the *Echmr* cDNA cloned. An radiolabelled pTZ57:*Echmr* probe fragment containing the entire cDNA hybridized to the blotted abundant mRNA of -0.85 kb;distinct varied levels of expression were seen in the stressed and control cells complemented with *Echmr* gene (Fig. 5.9). All the administered stresses seem to induced significant discrete increase in the transcript level of *Echmr* gene as compared to controls. The comparative induction of the transcript level among all the stresses registered an increase of approximately 1.1-fold in heat shock and 1.8 fold in cold shock compared to UV stress. The highest level of *Echmr* expression was triggered in cold stress followed by heat shock and the least in UV stress in the *E.coli* cells.

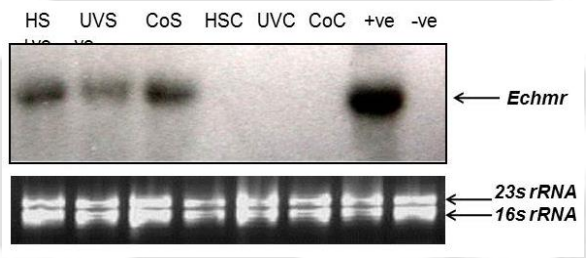

Fig 5.9 Expression profile of *Echmr* in recombinant *E.coli* strains during various abiotic stresses (HS:heat shock treated, UVS:UV stress, CoS:cold shock treated cells. HSC, UVC and CoC refers to control of respective stresses)

The pGEX3X:*Echmr* transformed wild type W3110 and Cd sensitive mutants Δ*gshA* and Δ*gshB* were used to analyze functional complementation potential against different abiotic stresses *viz.*, heat shock, cold shock and UV stress. Functional assay on LB media supplemented with IPTG reveals that *Echmr* confers heat stress tolerance recovery to Δ*gshA* and Δ*gshB* cells (Fig 5.10). The recombinant *ΔgshA* and wild type cells showed better recovery and growth after heat shock compared to *ΔgshB* cells in LB plate. Though the control *ΔgshB* and recombinant *ΔgshB* were comparable in term of recovery after heat shock and seems unaffected by heat shock. Whereas

wild type and *ΔgshA* control cells after heat shock were found to be inhibited by heat shock and shown poor recovery.

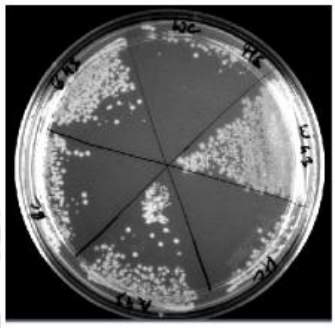

Fig 5.10 *Echmr* confers heat shock tolerance to recombinant *E.coli* mutant strains at 50ºC, 30 mins

The functional complementation assay of *Echmr* in W3110 and Cd sensitive mutants strains *ΔgshA* & *ΔgshB* of *E.coli* under UV stress reveals that Echmr protein also confers UV-B stress tolerance and recovery. The transformed *ΔgshB* and *ΔgshA* cells with recombinant plasmid (pGEX-3X:*Echmr*) showed better growth than those transformed with empty vector and its respective control cells (Fig. 5.11). Although all the recombinant *ΔgshA, ΔgshB* and wild type cells were found to have better recovery after UV treated but plain *ΔgshB* cells were seems to highly affected by UV stress than *ΔgshA* and wild type cells.

Fig 5.11 *Echmr* complemented recombinant *E.coli* mutant strains survives UV-B stress for 20 mins

In case of the cold stress assay, all the recombinant *ΔgshA, ΔgshB* and wild type cells showed distinct and comparable recovery and the Echmr expression in these cells seems to complement the cell to survive after cold shock (Fig 5.12). The high level of cold stress recovery to these cells may be due to elevated expression level of *Echmr* gene during cold stress as observed in northern assay. All the respective control cells of these strains have showed similar post cold recovery but *ΔgshB* cells were found to survive better comparatively. Additionally, it has been well known that GSH plays fundamental role in many cellular detoxification processes of HMs and ROS. There are reports of GSH acting as an antioxidant and involve directly in the reduction of most ROS generated during stress (Millar et al 2003, Foyer and Noctor 2005, Shao et al 2008). The high level of complex thiols in *ΔgshB* cells during abiotic stresses supported by the probable efflux function of Echmr protein may have provided complimentary effect to the *Δgsh* mutants to survive well post stresses.

Fig 5.12 *Echmr* confers cold stress tolerance to recombinant *E.coli* mutant strains at 4ºC, 30 mins.

In view of the functional assay of Echmr (Fig. 5.10, 5.11, 5.12) tolerance and recovery against different abiotic stress appears justified. Similar functional assay results had been previously reported in *E.coli* where *E. coli* transformed with stress responsive genes such as *pcs* and *ahpC* genes from *Anabaena* PCC 7120 were found to offer tolerance against multiple abiotic stresses, respectively (Chaurasia et al., 2008; Mishra et al., 2009). Similar report of heterologous

expression and functional validation of *Dps* gene from *Anabaena* has been published recently which also seems to deliver multiple abiotic stress tolerance to *E. coli* cells (Narayan et al., 2010). To discover the relationship between environmental stresses and *Echmr* induction, the expression of *Echmr* gene was validated by northern analysis (Fig. 5.9). A significant up-regulation of *Echmr* transcript as observed under different abiotic stresses suggested a stress dependent regulation of *Echmr* gene. Our results can be explained in the light of the several reports where *E. coli* Δ*gsh* mutants were sensitive to metal stress, oxidative and osmotic stress respectively (Helbig et al., 2008a; Kuanyu et al., 2004; McLaggan et al., 1990). In accordance of the report where GSH has been described as stress response protein and HM-stress induced transition metal binding protein in *E. coli* (Helbig et al., 2008b) and *Saccharomyces cerevisiae*, respectively (Glaeser et al., 2004). Our hypothesis that Echmr protein may have stress responsive functions seems rational. These outcomes brightly established that enhanced expression of *Echmr* gene from *Eichhornia crassipes*, a heterologous source, can offer protection in non-photosynthetic *Δgsh* mutant *E. coli* cells against HM and abiotic stresses.

5.4 Summary and Conclusions

Several physiological studies have indicated the role of GSH in heavy metal detoxification as well as in the maintenance of ionic homeostasis (Zenk, 1996; Hirata et al., 2005). This suggests that GSH should not be limiting. Hence, the functionality of the HM stress responsive gene were tested in GSH deficient *E. coli* cells to get a hint of its role in HM or other abiotic stress. In this study, we have utilized *E.coli ΔgshA* and *ΔgshB* deletion mutants to investigate the functions of *Echmr* and its associated GSH effects during various stresses. Furthermore, we analysed thiol compounds in the stressed recombinant *Δgsh* strains during exposure to environmental perturbations commonly utilized to study bacterial physiology. Our findings demonstrate that complex thiols metabolism and transcript level of *Echmr* gene was invoked under multiple abiotic stress conditions, suggesting a role in the acclimation response to many diverse alarms. Our endeavour has revealed that *Echmr* gene from *Eichhornia* offers multiple abiotic stress tolerance

to Cd sensitive *E.coli* Δ*gsh* mutants. So, this *Echmr* gene may be used as candidate gene towards engineering of crop plants for enhanced HM stress tolerance. Knowledge gained through use of heterologous expression and model plant systems provides an extremely useful starting point for the development of HM-tolerant crop plants. However, before any claims of HM tolerance can be authenticated; robust data on yield measurements is required preferably from field-based trials.

6.1 Introduction

Cd is considered to be one of the most perilous HM in the environment and poses a significant risk to human health (Jarup et al. 1998). Cd induces oxidative stress, which arbitrates cellular damage in many plants and animals (Hart et al. 1999; Sandalio et al. 2001). This in turn leads to the disruption of the permeability mechanism of the plasma membrane, causing ionic homeostatic imbalance and subsequent cell death (Koizumi et al. 1996). Humans and livestock can be under the threat of Cd via the intake of agricultural products grown in Cd-contaminated soils. Apart from farm products, Cd exists everywhere in the daily used materials including paint and batteries. It has been well established from earlier studies that certain plant species possess inherent potential to tolerate Cd than other plants (Boominathan et al. 2003; Kupper et al. 2000). Cd-tolerant plants have been found to either prevent the absorption of Cd or to sequester and detoxify Cd after it has been absorbed.

Plants provide first line of defence against excess Cd absorption by secreting organic acids, such as oxalate, that precipitate Cd in rhizospheres (Nigam et al. 2001). This is followed by the plant Casparian strip of the endodermal layer which repressed the translocation of Cd from roots to shoots (Lux et al. 2004). Once the HMs enters into cell, then the plants employ diverse strategies of Cd detoxification. One strategy may be the synthesis of metal-chelating peptides, such as GSH and phytochelatins, or Cys-rich proteins such as metallothioneins, that bind to Cd(II) and reduce its toxicity (Palmiter, 1998; Ranieri et al. 2005). Another mechanism of detoxification involves efflux of Cd or Cd(II)-conjugated molecules across the plasma membrane and tonoplast. In animals as well as in plants, excretion of conjugates with GSH, and glucuronide is driven directly by ATP hydrolysis (Endicott and Ling, 1989; Gottesman and Pastan, 1993; Higgins, 1992; Klein et al.1996; Martinoia et al. 1993) and the transporters proteins are involved in efflux of GSH-Cd or PC-Cd conjugate transport (Muller et al. 1994; Tommasini et al. 1996; Kim et al. 2006; Kim et al. 2007). For instance, Cd(II)-GSH complexes are compartmentalized into vacuoles by transporters localized in the tonoplast (Petrovic et al. 2000). Likewise AtMRP3,

an ABC transporter, has been implicated in the sequestration of Cd(II)-GSH complexes in vacuoles (Rea et al. 1998; Tommasini et al. 1998), and AtCAX2, an H^+/Cd^{2+} exchanger (Hirschi et al. 2000), mediates Cd(II) accumulation in vacuoles. Another ABC transporter, AtPDR8 has also been found to be involved in Cd(II) efflux at the plasma membrane (Kim et al. 2007) and so is the plasma membrane multidrug efflux carrier AtDTX1 (Li et al. 2002). Antioxidants and antioxidant-synthesizing enzymes may be another approach in enhancing plant tolerance to HMs, particularly Cd(II) and nickel. HMs induce ROS generation, ultimately leading to the up regulation of genes that encode the antioxidant-synthesizing enzymes (Dixit et al. 2001; Hsu and Kao, 2004; Vitoria et al. 2001). For example, overexpression of *Arabidopsis* aldehyde dehydrogenase, a scavenger of lipid peroxidation products, enhances tolerance to ROS-inducing stimuli, such as Cd(II), salt, and drought (Sunkar et al. 2003). In support of this mechanism of tolerance to HMs, many plants that are hyperaccumulators of Ni/Cd contain high antioxidant levels and high expression levels of the antioxidant synthesizing enzymes (Freeman et al. 2004). Thus, the transmembrane type of transporter protein is one of the crucial components of plant strategy and hence, EcHMR may have instrumental role in delivering HMs stress tolerance which need to be validated.

The yeast Cd factor (YCF1) is the product of a yeast gene that confers Cd tolerance (Szczypka et al. 1994) and is also responsible for the GSH-conjugate transport activity in yeast (Li et al. 1996; Tommasini et al. 1996). There have been reports of the yeast *ΔYcf1* mutant being used for the functional validation studies of homologous HMs transporter type genes. The yeast *ΔYcf1* mutant has been complemented with human MRP1, resulting in both restored GS-conjugate transport activity and Cd^{2+} tolerance (Tommasini et al. 1996). Furthermore, it has been shown that the mechanism responsible for YCF1-mediated Cd tolerance involves the transport of (GSH)-Cd^{2+}complexes, while phytochelatins ([γ-glu-cys] n-gly) are not accepted (Li et al. 1997). A database search for plant genes homologous to MRP1 and YCF1 revealed that at least four genes of *Arabidopsis thaliana* are highly similar to MRP1 and YCF1 and must therefore be

considered putative GSH-conjugate transporters (Tommasini et al. 1997). Hence, in this study, we made an endeavour to analyse and express the full length cDNA of a novel *Echmr* gene isolated from *Eichhornia* root and used the *Δycf1* yeast mutant for functional analysis of the gene product. The *Echmr* gene encodes a protein with a structure indicative of a transmembrane cation transporter molecule and was found to be involved in Cd(II) detoxification. It was aimed to evaluate the functionality of the *Echmr* gene in yeast cells against Cd and dehydration stress in suitable *Saccharomyces cerevisiae* strain *viz*., yeast Cd factor *(*Δ*Ycf1*) mutant (Falcon-Perez et al. 1999) or FY3 (Winston et al. 1995). EcHMR was found to conferred Cd(II) tolerance to Cd sensitive *ΔYcf1* mutant yeast strain and desiccation tolerance to FY3 strain respectively. These results suggest a potential role for this protein in Cd(II) tolerance in *planta.*

6.2 Material and methods

6.2.1 Cloning of plant *Echmr* gene in yeast expression vector

For over-expression in yeast, a yeast centromeric plasmid having strong and constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene called pGV8 was used (Agarwal et al. 2003). *Echmr* cDNA was cloned in MCS of pGV8 *BglII/HindIII* sites of pGV8. The required clones were confirmed by restriction enzyme analysis. Recombinant plasmids and vector were linearized within the *ura3* gene with *ApaI* or *EcoRV* to ensure targeted insertion of these cassettes at the ura3 region in the yeast genome. Recombinant plasmids were used for transformation of *Δycf1(*A Δ*ycf1* derivative of *S. cerevisiae* strain W303–1A(Ronne and Rothstein 1988) (*MATa, ycf1*Δ*::URA3, ade2–1, his3–11, 15, leu2–3,112, trp1–1, ura3–1*) and FY3(*MATa ura3-52*) yeast cells, and the transformants were selected on minimal media plates lacking uracil.

6.2.2 Yeast transformation

6.2.2.1 Yeast competency treatment

Overnight culture of yeast cells was started in 3ml of YPD broth (1 % yeast extract, 2 % peptone, 2% dextrose) at 28°C, 200 rpm. Next morning, secondary culture was started in 10 ml of YPD

with an initial OD_{600} of 0.1-0.2. After 3-4 h of culturing, 2 ml of the secondary culture was spun down by pelleting the cells (6,500 rpm, 5 min, RT). The pellet was resuspended in 400 µl of 100 mM lithium acetate. The cells were rinsed at least twice in 100 mM lithium acetate. The resuspended cells were finally placed at room temperature.

6.2.2.2 Preparation of DNA solution

Approximately 5 µg of plasmid DNA was linearized by appropriate restriction enzyme. The linearized DNA was straightaway used. Yeast transformation was performed by standard lithium acetate method (Gietz et al. 1995). For yeast transformation, transformation mix was prepared which included DNA (linearized), 240 µl 50 % PEG, 36µl 1M lithium acetate and herring sperm DNA (1mg/ml, as carrier). Final volume of the solution was made up to 500-600 µl with SDW. The competent yeast cells were spun down (6500 rpm, RT, 5 min). The yeast pellet was resuspended in the transformation mix. The mixture was incubated at 42°C for 2 h. The cells were again spun down, supernatant was discarded and the pellet was dissolved in 1 ml of YPD. Yeast cells were placed for recovery at 28°C (200 rpm, 2 h). Finally, the cells were centrifuged (6500 rpm, 5 min, RT). The pellet was dissolved in minimum volume of SDW and plated on SD (synthetic defined medium containing 0.17 % yeast nitrogen base, 0.5 % ammonium sulphate and 1.8 % agar all w/v) minimal plates for selection. The plates were incubated at 28-30°C for 2 days till yeast colonies were seen on the plates.

Recombinant pGV8-*Echmr* plasmids were used for transformation of *Δycf1* and FY3 yeast cells, and the transformants were selected on minimal media plates lacking uracil. The transformed yeast cells were checked by PCR and northern analysis for the integrity and expression of genes cloned. Mutant cells transformed with pGV8 vector were used as a negative control. The wild-type strain W303 [*Mata WT his3-Δ200,leu2-3, 2-112, lys2-801, trp1-1(am), ura3-52*] (details available at http://www.yeastgenome.org/straintable.shtml#W303) and FY3 were used as a control.

6.2.3 PCR screening of the recombinant yeast colonies

Two ml of overnight grown primary yeast culture was spun down (6,000 rpm, 4°C). Pellet was resuspended in 200 µl of "Yeast Breaking Buffer" (2 % Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA) and 200 µl TE buffer (pH 8). Cells were lysed with the help of 200 µl equivalent glass beads (2 mm, MERCK) by vigorous vortexing. 400 µl of P:C:I (25:24:1) was added and the solution was mixed-thoroughly. The tubes were centrifuged at 12,000 rpm for 10 min. DNA in the aqueous phase was carefully taken in a fresh tube and precipitated with two volume of chilled ethanol. Tubes were incubated at -20°C for 30 min after which the contents were pelleted in microcentrifuge (12,000 rpm, 30 min). DNA was washed with 70 % ethanol and air-dried. Finally, DNA was resuspended in appropriate volume of TE buffer (pH 8). 200 ng of total genomic DNA was used for PCR screening of the recombinant yeast colonies or wild type yeast cells.

6.2.4 Northern analysis

For electrophoresis of RNA, samples were denatured in 4 volume of sample buffer [25 % (v/v) formaldehyde, 60 % (v/v) formamide, 12.5 % (v/v) 10X morpholinopropane sulphonic acid (MOPS) with 250 µg ml^{-1} ethidium bromide] by incubating at 65°C for 10 min followed by snap-cooling in ice for 5min. For application on gel, 0.1 volume of loading dye [10 % (v/v) 10X MOPS, 10 % (v/v) glycerol, 0.25 % bromophenol-blue] was added to each sample. The samples were resolved by electrophoresis in denaturing gel casted by dissolving 1 % agarose in 1X MOPS buffer [200 mM MOPS, 50 mM Na acetate and 10 mM EDTA (pH 7)]. After resolving RNA, denaturing gel was washed with SDW for 30 min following which RNA was transferred to positively-charged nylon membrane (Genescreen plus, NEN, USA) and was UV cross-linked as described by Sambrook and Russell (2001).

For hybridization, northern blots were incubated in prehybridization buffer O/N at 42°C followed by addition of denatured radiolabeled probe. Radiolabeled probe was made by using

Megaprime Labeling Kit (Amersham, UK) with αP^{32}-dCTP (BRIT, India) as a radio-labeling molecule. The blots were washed once with hot Solution 1 (5X SSC, 0.1 % SDS). After Solution 1, the blots were rinsed for ~10 min with Solution 2 (5X SSC, 0.1 % SDS). Similarly, washes were given with Solution 3 (2X SSC, 0.1 % SDS) and Solution 4 (0.1X SSC, 0.5% SDS). Depending on the stringency of washing required, Solution 3 was either warmed or the time of washing was increased. Blots were repeatedly checked for differential counts. After a final wash with Solution 4, blots were carefully wrapped in Cling film (Kling wrap) and excess of the solution was removed with the help of a glass rod. The blots were exposed for different time intervals depending on the counts, in hyper cassettes at -80°C (KODAK, USA).

6.2.5 Cd sensitivity assay

For the metal tolerance experiment, yeast strains were grown overnight (25ºC, 200 rpm) in YPD medium after which secondary culture was seeded and the cell density was adjusted to 1×108 cells/ml. A 1000 µM concentration of $CdCl_2.2H_2O$ were added and the cultures were incubated (25ºC, 200 rpm) for 2 hrs. Subsequently, cells were pelleted, washed and resuspended in sterile distilled water. Tenfold serial dilutions of the cells were made and 5 µl of 10 fold dilution was dotted on YPD plates (25ºC, 2 days), in duplicate. Unstressed controls lacking $CdCl_2.2H_2O$ were also included in all the experiments.

6.2.6 Yeast desiccation tolerance assay

The *Saccharomyces cerevisiae* strain FY3 (*MATa ura3-52*) was used for the desiccation tolerance assay (Winston et al. 1995). Different yeast strains [namely FY3 (wild type), FY3-pGV8 and FY3-*Echmr*] grown in rich YPD medium (overnight, 25ºC), were used to initiate a fresh culture in YPD. A secondary culture of yeast cells (initial OD_{600} 0.05) was started and incubated (25°C, 200 rpm) till OD_{600} reaches 0.15 (~3 h). OD_{600} of the secondary culture was also measured and if there was slight variation, it was normalized. Aliquots of 200µl of the yeast cells from all the strains were made in MCTs and were pre-incubated at 28ºC for 1h. Then the MCTs were

immediately plunged into ice till the time of spotting. For spotting, tenfold serial dilutions (till 10^{-4}) of the cells were prepared in sterile water and 5µl of each was dotted on YPD-agar plates (YPD containing 1.8% w/v agar plus 4 % PEG). The plates were dried in the laminar flow and finally incubated at 28°C for 2 days. For control, samples were cooled just after pre-incubation at 37ºC and were further processed in the same way.

6.3 Results and discussion

In this study, the *Echmr* cDNA was cloned with *BglII/HindIII* restriction sites in yeast centromeric plasmid pGV8 and then transformed into Δ*ycf1* mutants and FY3 yeast cells. The schematic representation of the *Echmr* cloning in the vector pGV8 has been illustrated in (Fig 6.1A). the recombinant pGV8:*Echmr* clones were first linearized with *EcoRV* and then compared with *ApaI* linearized empty PGV8 to screening the cones. Then the pGV8:Echmr plasmids were isolated and validated by *BglII/HindIII* digestion which clearly revealed the release of 0.85kb Echmr cDNA band(Fig 6.1 B) Then the confirmed recombinant pGV8:Echmr construct was used to transformed Cd sensitive *Δycf1* mutants and FY3 cells, which were further used to assessed the stress tolerance level under 1000 µM Cd concentration of EcHMR complemented Δ*ycf1* mutant cells. This was also followed by the validation of the role of EcHMR protein against dehydration in FY3 cells under 4 % PEG concentration.

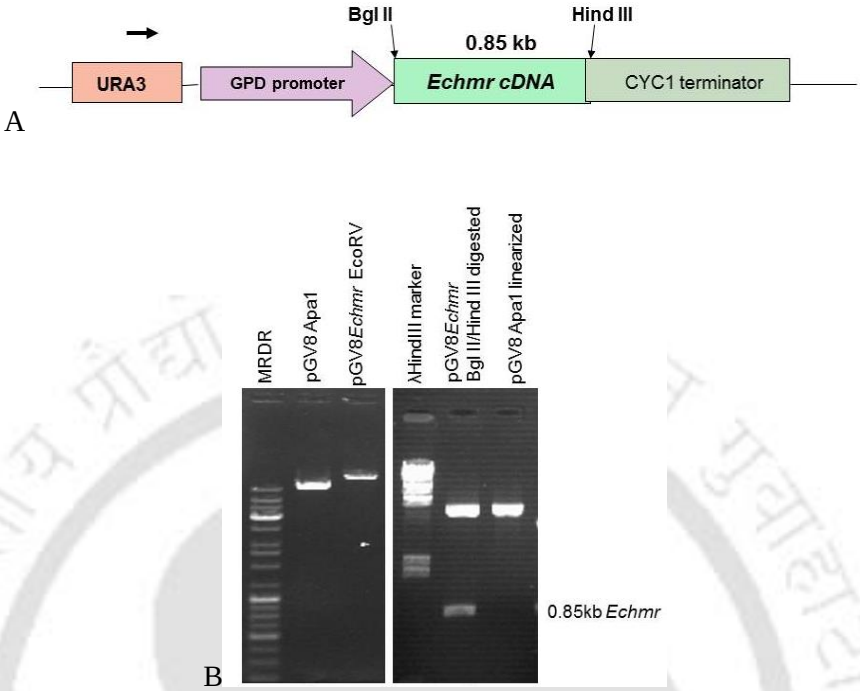

Fig 6.1 (A) Linear map of pGV8-*Echmr* gene construct. (B) Cloning of *Echmr* CDS into *Bgl II/Hind III* restriction sites in yeast centromeric plasmid pGV8

Following transformation of the *Δycf1* cells with pGV8:*Echmr* construct, the clones were screened by PCR taking the genomic DNA of the yeast clones as template. The genomic DNA PCR of the *Δycf1* mutant cells transformed with pGV8:*Echmr* gene has revealed a distinct band of 0.85kb amplicons of *Echmr* gene (Fig 6.2).

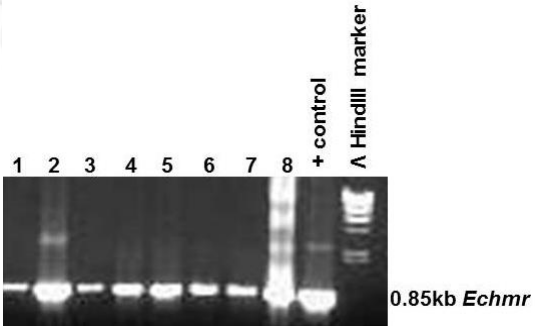

Fig 6.2 Genomic DNA PCR of recombinant *Δycf1* cells bearing pGV8:*Echmr*

To further check the transcriptional expression level of the *Echmr* gene during Cd condition, RNA was isolated from wild type, Δ*ycf1* mutant, Δ*ycf1* mutant+pGV8:*Echmr* and Δ*ycf1* mutant +pGV8 cells exposed to Cd stress. The isolated total RNA from yeast cells during Cd stress was blotted for northern analysis and radiolabelled linearized *Echmr* was used for hybridizing. The northern analysis revealed the enhanced expression of the *Echmr* in the Cd stressed *Δycf1* cells complemented with pGV8:*Echmr* cells (Fig 6.3). These results confirmed the transformation and expression of *Echmr* into the *Δycf1* mutant cells which may be further used to validate the functionality of *Echmr* gene in Cd stress and desiccation tolerance.

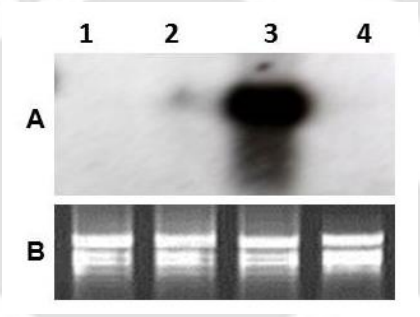

Fig 6.3 Expression profile of *Echmr* in wild type and Cd sensitive Δ*ycf1* mutant yeast cells. A. Northern analysis of yeast cells of wild type(lane 1), Δ*ycf1* mutant (lane 2), Δ*ycf1* mutant +pGV8:*Echmr* (lanes 3) and Δ*ycf1* mutant+pGV8 (lane 4) cells. B. Ethidium bromide stained RNA gel to ensure equal loading

To confirm the role of *Echmr* in Cd(II) tolerance, *Δycf1* cells were transformed with pGV8:*Echmr* and grown in suspension cultures with $CdCl_2.2H_2O$ for 2hrs and then the cells were washed, diluted and spotted on YPD agar medium separately control and stressed cells for comparative qualitative and quantitative assessment. *Δycf1* cells that expressed EcHMR grew much better than those transformed with the control empty vector (VC) (Fig. 6.4). They also grew better than wild type (WT) yeast control cells. To characterize the Cd(II) tolerance of EcHMR-expressing yeast, the number of colonies was counted to calculate survival percentage considering control of each type separately as 100 %. The WC, wild-type W303-1A control; WS, wild type W303-1A after $CdCl_2$ stress; Δ*ycf1* mutant yeast cells in control medium were used as control for calculation.

In control condition, the growth of EcHMR-transformed *Δycf1* cells was similar to WC cells or empty vector-transformed *Δycf1* cells (VC) (Fig. 6.4). But in the $CdCl_2H_2O$ stressed yeast cells, the growth and cell density decreased in a visually distinctive manner. EcHMR-expressing *Δycf1* cells grew better than WC, VC and *Δycf1* mutants yeast cells post $CdCl_2H_2O$ stress when grown in YPD medium, whereas empty vector-transformed *Δycf1* (VC) cells did not grow as well as wild type stressed (WS) cells (Fig. 6.5).

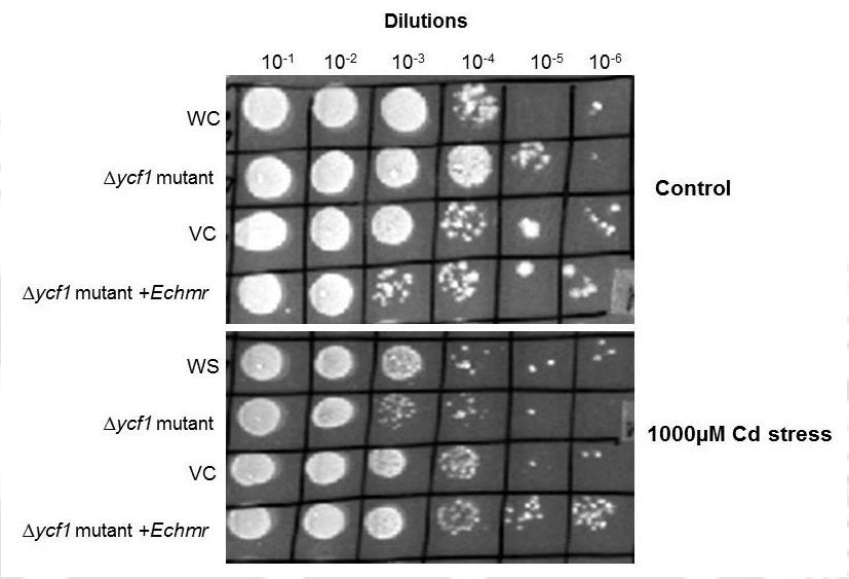

Fig 6.4 Resistance profile of Δ*ycf1* mutants in Cd stress. WC, wild-type W303-1A control; WS, wild type W303-1A after $CdCl_2$ stress; Δ*ycf1* mutant yeast cells; Δ*ycf1* mutant yeast cells complemented with *Echmr* cDNA; VC, Δ*ycf1* mutant harboring vector alone

In the stressed condition, the final cell density of EcHMR-expressing *Δycf1* cells was twice as high as *Δycf1* cells transformed with empty vector (VC) (Fig. 6.5). This may be due to the fact that in the presence of Cd(II) stressed, EcHMR-expressing *Δycf1* cells grew more rapidly than either WC or empty vector transformed *Δycf1* (VC) cells during the exponential growth phase, resulting in a much higher cell density in the stationary phase (Fig. 6.5) and resulting in better HMs stress recovery. To date, the best characterized of the known vacuolar transporters and channel involved in metal tolerance is YCF1 from *Saccharomyces cerevisiae*. YCF1 is a MgATP energized GSH S-conjugate transporter responsible for vacuolar sequestration of organic

compounds after their S-conjugation with GSH, as well as GSH–metal complexes. It catalyses the transport of bis(glutathionato) Cd (Cd-GS2) into vacuoles (Li et al 1997), as well as As-GS3 (Ghosh et al 1999) and Hg-GS2 (Gueldry et al 2003). In a recent report, Song et al. (2003) have successfully overexpressed YCF1 in *Arabidopsis thaliana* to full functional activity. YCF1 proteins in cells of YCF1-transgenic plants were found to be associated with the tonoplast and the plasma membrane. It has been reported that the vacuoles of YCF1-transgenic plants had a fourfold higher uptake of Cd-GS2 and GSH–Pb complex than those of wild-type plants suggesting a role towards Pb and Cd tolerance. The stress tolerance level under 1000µM Cd concentration and high post stress recovery reveals higher survival percentage of EcHMR complemented Δ*ycf1* mutant cells. These demonstrated that EcHMR confers yeast cells tolerance to Cd(II).These results probably suggest that EcHMR may enhance export of Cd(II) from the yeast cell complementing the knockout Δ*ycf1* gene, thereby decreasing Cd content and mediating Cd(II) tolerance in Δ*ycf1* mutant cells.

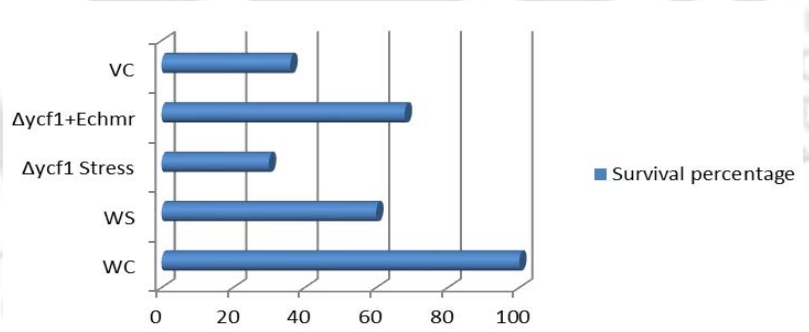

Fig 6.5.Survival percentage of *Δycf1* mutants during Cd stress. WC, wild-type W303-1A control; WS, wild type W303-1A after $CdCl_2$ stress; Δ*ycf1* stressed, Δ*ycf1* mutant yeast cells after stress; Δ*ycf1+Echmr,* Δ*ycf1* mutant yeast cells complemented with *Echmr* cDNA; VC, Δ*ycf1* mutant harboring vector alone.

The mechanisms of HM tolerance have been studied primarily in *Arabidopsis* and in HM hyperaccumulators, such as *Thlaspi caerulescens* and *Arabidopsis halleri*. To date, there have been several reports of toxic HM tolerance in plants which includes studies on organic acid secretion and consequent immobilization of Pb(II) by tolerant rice varieties (Yang et al 2000),

isolation of the phytochelatin synthase gene from wheat (Clemens et al. 1999), induction of OsPDR9 expression by Cd(II) and Zn(II) in the rice root (Moons, 2003), and hypersensitivity to Cu(II), Zn(II), and Pb(II) of rice mutant plants that are deficient in expression ofOsHMA9(Lee et al. 2007). EcHMR is a new transporter protein of *Eichhornia* that can contribute to toxic HM tolerance. In conclusion, we have shown that EcHMR is a novel gene that contributes to Cd(II) tolerance when overexpressed in *Δycf1* mutant yeast. Among all the cellular mechanisms for metal tolerance, one strategy is to keep the concentration of toxic metal ions in the cytoplasm low by preventing the metal from being transported across the plasma membrane, either by reduced uptake through modified ion channels, or by pumping the metal out of the cell with active efflux pumps (Tong et al. 2004). The vacuole is generally considered to be the main storage site for metals in yeast and plant cells and there is evidence that phytochelatin–metal complexes are pumped into the vacuole in fission yeast (*Schizosaccharomyces pombe*) (Oritz et al. 1995) and in plants (Salt and Rauser, 1995). Compartmentalization of metals in the vacuole is also part of the tolerance mechanism of some metal hyperaccumulators. The Ni hyperaccumulator *Thlaspi goesingense* enhances its Ni tolerance by compartmentalizing most of the intracellular leaf Ni into the vacuole (Krämer et al. 2000). High-level expression of a vacuolar metal ion transporter TgMTP1 in *T. goesingense* was proposed to account for the enhanced ability to accumulate metal ions within shoot vacuoles (Persans et al. 2001). May be EcHMR functions in the same way as transporter and may have other important functions in plants.

To investigate the role of *Echmr* against desiccation stress, the pGV8:*Echmr* was transformed into FY3 cells and the wild type FY3, empty vector transformed FY3 and FY3 complemented *Echmr* cells were spotted on YPD supplemented with 4% PEG and incubated for 2 days at 28ºC. In the control condition, all the cells *viz*., wild type FY3, vector control and FY3 complemented with *Echmr* grew equally (Fig 6.6). But in the presence of 4% PEG, FY3 cells complemented with *Echmr* gene have shown better growth as compare to wild type FY3 and

vector control cells (Fig 6.6). This result suggests that *Echmr* may have a role to play in desiccation tolerance also.

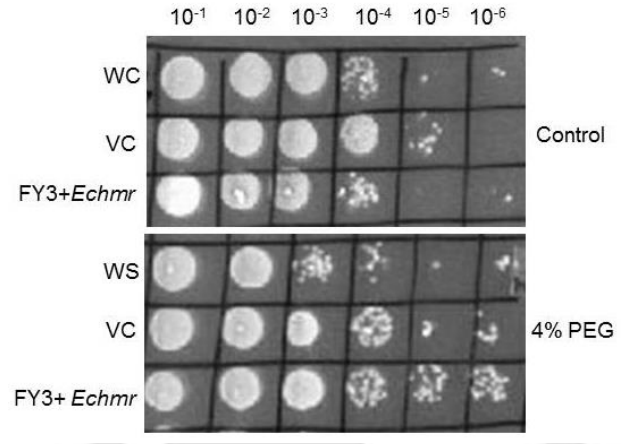

Fig 6.6 Tolerance profile of FY3 yeast cells in dehydration stress. WC, wild-type FY3 control; WS, wild type FY3 in 4% PEG stress; FY3 yeast cells complemented with *Echmr* cDNA; VC, FY3 harboring vector alone.

However, the mechanism and regulation of this phenomenon are poorly understood. Correlations between desiccation tolerance and potential effectors have been reported in many species, but their physiological significance has not been established *in vivo*. Although the budding yeast *Saccharomyces cerevisiae* exhibits extreme desiccation tolerance, its usefulness is hampered by an inability to reduce tolerance more than a few fold by physiological or genetic perturbations. It has been reported that fewer than one in a million yeast cells from low-density logarithmic cultures survive desiccation, while 2–4% of cells from saturated cultures survive and it has been found that mutants defective in trehalose biosynthesis, hydrophilins, responses to hyperosmolarity, and hypersalinity, ROS scavenging and DNA damage repair nevertheless retain wild-type levels of desiccation tolerance, suggesting that this trait involves a unique constellation of stress factors (Calahan et al. 2011). Anyhow the ability to confer better growth to FY3 cells by *Echmr* during desiccation stress may be fruitful in further study. Hence extended study of other

related regulators to *Echmr* and their targets should provide important clues to the desiccation tolerance in long run.

6.4 Summary and conclusions

Heterologous expression of *Echmr* gene in yeast mutant defective for YCF1 transporter and FY3 strain have shown role of *Echmr* gene in tolerance responses specially to Cd and desiccation stress respectively. We demonstrated that a *Eichhornia* HMs stress responsive gene (*Echmr*) confers strong Cd and desiccation tolerance in yeast and this tolerance is mediated at least in part by its divalent cation transporter activity. The yeast ortholog *Scycf1* confers similar Cd tolerance to yeast, while its knockout Cd hypersensitive mutants were successfully complemented by *Echmr*, suggesting its role in Cd transport and tolerance. In conclusion, we have shown that *Echmr* is a novel gene that contributes to Cd(II) tolerance when overexpressed in Δ*ycf1* mutant yeast and it may suggest that *Echmr* functions in Cd(II) detoxification *in planta*. Real breakthroughs on the way to engineering the ideal phytoremediator will therefore require a thorough understanding of metal uptake and translocation processes in higher plants on a molecular basis, particularly in natural metal hyperaccumulators. Until then, trial and error is the only tool available to advance research and understanding.

7.1 Introduction

The metal cations constitutes crucial ingredient of nutrition in plants for sustenance. Several metals *viz*, Cu, Fe, Zn and Mn, act as important cofactors for many enzymes in required amount. However, when supplied in excess, these essential cations can become toxic, like HMs such as Cd, Pb, or Hg. To maintain micronutrient metal homeostasis and to cope with excess of nonessential HMs, plants have evolved a complex network of metal uptake, chelation, trafficking, and storage processes. Metal transporters are required to maintain metal homeostasis and thus constitute important components of this network (Clemens, 2001; Hall and Williams 2003). Furthermore, the thiol tripeptide glutathione (GSH; γ-Glu-Cys,Gly), which occurs in abundance in plants forms a basis of important functions ranging from scavenging of reactive oxygen species to heavy metal detoxification (Hausladen and Alscher, 1993; Rennenberg, 1997; May et al., 1998). In the leaves the synthesis of GSH is thought to take place in the chloroplasts and cytosol (Hausladen and Alscher, 1993; Rennenberg, 1997; Noctor and Foyer, 1998). Further, the subcellular localization studies of GSH has shown that GSH synthesis is possible in the chloroplasts and cytosol and that the degradation of GSH and GS conjugates occurs in the vacuoles and perhaps in the apoplast (Foyer et al., 2001). Hence, the varied subcellular abundance of GSH and associated functions of the protein may govern the HM stress responses and localization of protein.

In recent years, a number of membrane transport protein families have been reported in plants. Membrane proteins are a key set of proteins as these form part of the boundary of the organelles and represent many important functions such as transporters, receptors, and trafficking (Sadowoski et al., 2008). These include the cation diffusion facilitators (CDF), the Zrt-, Irt-like proteins (ZIP), the cation exchangers (CAX), the copper transporters (COPT), the heavy-metal P-type ATPases (HMA), the natural resistance-associated macrophage proteins (NRAMP), and the ATP-binding cassette (ABC) transporters (Williams et al., 2000; Maser et al., 2001; Cobbett et al., 2003; Hall and Williams, 2003). However, the transport specificities, patterns of expression,

or subcellular localizations of metal transport proteins are still largely unknown. To further understand plant metal homeostasis, it will be necessary to elucidate the contribution of each of these HM stress responsive proteins to the uptake, trafficking, and storage of essential metals, detoxification of toxic heavy metals and subcellular existence of the protein to deliver its functions.

Assigning the sub-cellular location of a protein is of utmost importance to biologists in the elucidation of its role and in the refinement of cellular processes intricacies by tracing certain activities to specific organelles (Sadowski et al., 2008). Common approaches of subcellular localization involve tagging with GFP or its variants, or raising a specific antibody and carrying out analysis by microscopy. To date, the largest study investigating protein location has been conducted in *Saccharomyces cerevisiae* by O'Shea and coworkers (Huh et al., 2003). Of which, 75 % of *S. cerevisiae* proteins were fused at the C-terminal end with GFP and studied by the use of fluorescence microscopy. Furthermore, more than 1300 *Arabidopsis* protein localization results in the SUBA database were outcomes of the GFP tagged localization studies (Heazlewood et al.,2007). In this article, we present an efforts to follow the same GFP tagged approach to study the subcellular localization of GSH related HM stress responsive EcHMR protein isolated from water hyacinth. Our results indicate that the ectopic expression of a foreign EcHMR gene along with GFP in the protoplast of *Arabidopsis* discretely revealed its localization in the plasma membrane or cytosol and this may implies that the EcHMR may have suspected function in the HM stress tolerance.

7.2 Materials and Methods

The *Eichhornia Echmr* gene was PCR amplified from cDNA taking into account that it did not contained any introns in these region. To generate 35SCaMVP:*Echmr*:GFP, *Echmr* ORF was amplified by PCR using *Echmr* XbaI forward and *Echmr* BamH1 reverse primers. The amplicons were digested with *Xba1* and *BamHI* and were cloned in the vector p326-GFP to generate a fusion with GFP (Fig. 7.1). It was then cloned in MCS between the 35S CaMV promoter and nos-

terminator of the p326-GFP vector, which were later digested with *XbaI* and *BamHI* to conform the cloning of p326-*Echmr*-GFP construct.

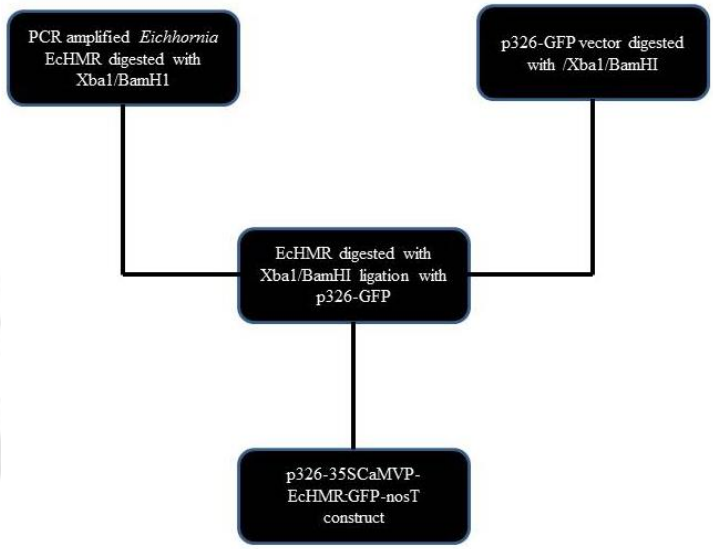

Fig. 7.1. Strategy employed for cloning of *Echmr* gene in fusion with GFP reporter gene

7.2.2 Subcellular localization of *Echmr*

7.2.2.1 Growth of Plants

Arabidopsis thaliana (ecotype Columbia) was grown either on Murashige and Skoog (1962) plates at 22°C in a culture room or in a greenhouse under conditions of 70 % RH and a 16 hr-light/8 hr-dark cycle.

7.2.2.2 Transient expression of *Echmr* in protoplast

The fusion construct (CaMV35SP:EcHMR:GFP) was introduced into *Arabidopsis* protoplasts prepared from whole seedlings by polyethylene glycol–mediated transformation (Kang et al., 1998). Briefly, leaf tissues (5 g) of 3 to 4 week-old *Arabidopsis* plants grown on soil in a greenhouse were cut into small squares (5 to 10 mm^2) with a new razor blade and incubated with 50ml of enzyme solution (0.25 % Macerozyme) (Yakult Honsha Co., Ltd., Tokyo, Japan) R-10, 1.0% Cellulase (Yakult Honsha Co., Ltd.) R-10, (400 mM mannitol, 8 mM $CaCl_2$, and 5 mM Mes-KOH, pH 5.6) at 22°C for 5 hr with gentle agitation (50 to 75 rpm). After incubation, the

protoplast suspension was filtered through 100 µm mesh and protoplasts were collected by centrifugation at 46 g for 5 min. The pelleted protoplasts were resuspended in 5 to 10 ml of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes-KOH, pH 5.6), overlaid on top of 20 mL of 21% sucrose, and centrifuged for 10 min at 78g. The intact protoplasts at the interface were transferred to a new falcon tube containing 20 ml of W5 solution. The protoplasts were pelleted again by centrifugation at 55 g for 5 min and resuspended in 20 ml of W5 solution. The protoplasts were incubated on ice for 30 min.

To transform DNA into protoplasts, protoplasts were pelleted again at 46g for 5 min and resuspended in MaMg solution (400 mM mannitol, 15 mM $MgCl_2$, and 5 mM Mes-KOH, pH 5.6) at a density of $5x10^6$ protoplasts/ml. Plasmid DNA (20 to 50µg total at a concentration of 2 mg/mL) was added to 300µl of protoplast suspension followed by 325 µl of PEG solution [400 mM mannitol, 100 mM $Ca(NO_3)_2$, and 40 % polyethylene glycol 4000]. The mixture was mixed gently and incubated for 30 min at room temperature. After incubation, the mixture was diluted with 10 ml of W5 solution. Protoplasts were recovered by centrifugation at 50 g for 5 min, resuspended in 3 ml of W5 solution, and incubated at 22°C in the dark. Expression of protein was monitored at various times after transformation and images captured.

7.2.2.3 Microscopy

All the samples were viewed and images were captured with a cooled charge-coupled device camera using a Zeiss Axioplan fluorescence microscope. The wavelength characteristics used for filter sets were XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) and (exciter, 540AF30; dichroic, 570DRLP; emitter, 585ALP) (Omega, Inc., Brattleboro, VT) for green fluorescent protein and auto fluorescence of chlorophyll, respectively. Data were then processed using Adobe (Mountain View, CA) Photoshop software, and the images were rendered in pseudocolor.

7.3 Results and Discussion

7.3.1 Cloning of p326-EcHMR-GFP expression cassette

The p326-EcHMR-GFP expression construct is designed for the constitutive expression of the EcHMR gene by the enhancer-equipped CaMV35S promoter, which is active in most cells of the plant. The amplified EcHMR ORF was digested with *XbaI/BamHI* and ligated to MCS between the 35SCaMV promoter and nos-terminator of the p326-GFP vector to yield 35sCaMVP:*Echmr*:GFP fusion construct (Fig 7.2). The 35SCaMVP:EcHMR:GFP fusion construct was later digested with *XbaI/BamHI* to confirmed the cloning by release of EcHMR ORF fragment of 0.65 kb (Fig. 7.3).

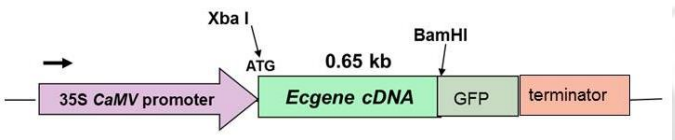

Fig 7.2 Linear map of 35SCaMV promoter:*Echmr*:GFP construct

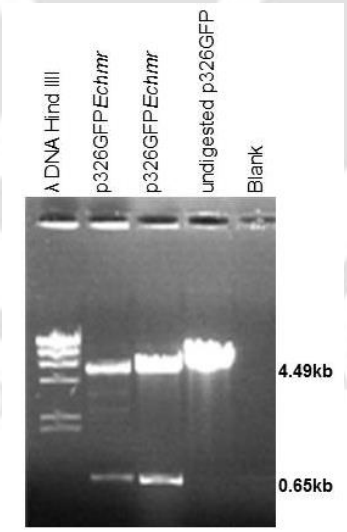

Fig 7.3 p326*Echmr*GFP construct for subcellular localization study in *Arabidopsis* protoplast

7.3.2 Subcellular localization of the EcHMR protein in *Arabidopsis* protoplast

The aim of the present work was to determine the subcellular distribution of EcHMR in the protoplast of *Arabidopsis*. To this end the *Arabidopsis* (ecotype Columbia) plants was used to derive protoplast and the fusion construct (CaMV35SP:EcHMR:GFP) was introduced into *Arabidopsis* protoplasts by polyethylene glycol–mediated transformation (Kang et al., 1998). The eexpression of protein was monitored at various time intervals after transformation and images captured with characteristic wavelength of filters set at XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) and (exciter, 540AF30; dichroic, 570DRLP; emitter, 585ALP) (Omega, Inc., Brattleboro, VT) for green fluorescent protein and auto fluorescence of chlorophyll, respectively.

After demonstrating that EcHMR is a complex transmembrane HM stress responsive protein, we investigated its subcellular localization in transformed *Arabidopsis* protoplast stably expressing EcHMR:GFP proteins. Stably expressed EcHMR:GFP fusion protein localized predominantly in the plasma membrane or cytosol (Fig. 7.4). Whereas, the protoplast transformed with 35SCaMVP:GFP act as control showed localization in the whole cytosol which may be due to constitutive expression. However, for EcHMR not even the most basic information about its membrane orientation and its subcellular localization was available. Hence, we performed a comprehensive nucleotide and protein sequence bioinformatic analysis of this uncharacterized protein to accumulate primary information. Then we first demonstrated that EcHMR is an integral GSH related HM stress responsive transmembrane protein using heterologous expressions in *E.coli Δgsh* and yeast *Δycf1* mutants. The localization of EcHMR in plasma membrane or cytosol may provide an unexpected link to GSH abundance or its transient cation transporter activity.

(exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23)

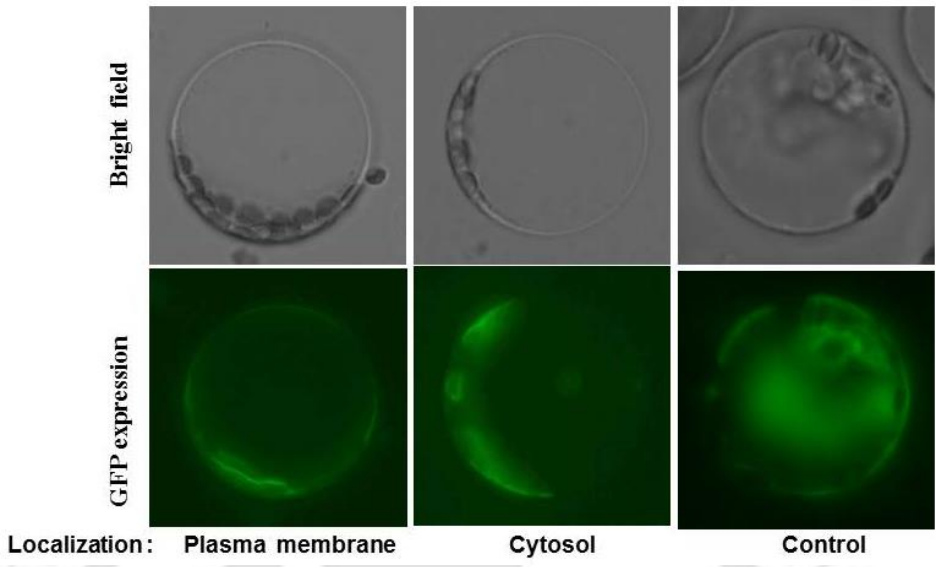

Fig 7.4 Expression of EcHMR:GFP fusion protein in protoplasts. The green color represents the localization of EcHMR in the transiently transformed protoplast with 35SCaMVP:*Echmr*:GFP construct. Green fluorescence of EcHMR:GFP was viewed by confocal laser scanning microscopy (lower panel). The same protoplasts were viewed by differential interference contrast (upper panel).

Since *Echmr* gene has been found to be related to GSH and shown functions governing HM and abiotic stress tolerance, the subcellular localization of EcHMR may be attributed to GSH abundance and its functionality. There has been report of spatial changes in GSH content in maize and wheat at low temperatures in shoots and roots (Kocsy et al., 2001). However, the changes in the size and redox state of the GSH pool at the cellular level could be even more important for the proteins to respond to abiotic stress in various organs or tissues (Meyer, 2008). Studies on the subcellular localization of GSH showed that GSH synthesis is possible in the chloroplasts and cytosol and that the degradation of GSH and GS conjugates occurs in the vacuoles and perhaps in the apoplast (Kopriva, 2006; Sugiyama et al., 2004). But recently, the subcellular immunocytochemical analysis has detected the highest concentrations of glutathione in mitochondria and not in plastids (Zechmann et al., 2008). Therefore, large GSH redox gradients

may exist between the various subcellular compartments (Foyer et al., 2001). For example, cECS is localized exclusively in the cytosol (Pasternak et al., 2008) and is active in the oxidized state (Jez et al., 2004), whereas the active form of the nonexpressor of pathogen related genes (NPR1) is reduced and localized in the nucleus (Mou et al., 2003). If proteins differing in their redox activation mechanism need to be activated successively during the stress response, their induction could be ensured by changes in the redox state of the relevant compartment. Taken together, it may be possible that the GSH abundance or changes in the redox state of GSH at the cytosol or plasma membrane respectively promotes the localization of EcHMR in the cell to execute its exact function.

In addition to the transporters which predominantly localized in plasma membrane or cytosol, there are reports of several genes related to excess HM stress (Uraguchi and Fujiwara, 2012). Several ATP-binding cassette (ABC) proteins mediates vacuolar compartmentalization of Cd glutathione and/or phytochelatin (PC) conjugates in baker's yeasts (Li et al., 1997), fission yeasts (Prévéral et al., 2009), the worm *Caenorhabditis elegans* (Vatamaniuk et al., 2005) and *A. thaliana* (Park et al., 2012). Recently apart from these genes, a novel rice gene low cadmium (LCD) has been reported to involve in Cd accumulation and tolerance (Shimo et al.,2011) but the LCDGFP was found to localize in the cytoplasm and nucleus, suggesting that LCD is not a membrane-transporter. Even LCD is not homologous to any other genes, and the authors concluded that LCD is a novel protein related to Cd homeostasis. Similarly, a novel cysteine-rich peptides encoded by OsCDT1 has been reported to possibly involved in rice Cd tolerance (Kuramata et al., 2009). Furthermore, the overexpression of OsCDT1 in *A. thaliana* increased the growth of plants under Cd exposure (Kuramata et al., 2009). These reports indicate that novel mechanisms mediated by unknown molecules functions in HM tolerance in plants and also indicate that EcHMR may be such unknown potential genetic molecule that has functions related to HM stress and/or accumulation with localization at plasma membrane or cytosol.

7.4 Summary and conclusions

The subcellular localization study of EcHMR gene isolated from water hyacinth shows that the EcHMR is localized predominantly in the plasma membrane or cytosol. The transformed *Arabidopsis* protoplast using CaMV35SP:EcHMR:GFP fusion construct showed GFP expression at plasma membrane. The localization pattern of the EcHMR consistently at plasma membrane is reported although, it was also found often in the whole cytoplasm. However, this localization pattern changed to whole cytosol in case of CaMV35SP:GFP gene construct transformed protoplast which may be due to constitutive expression. Furthermore, there are reports that transporters can localize randomly in different sub-cellular organelles according to its varied requirement of functions. Hence, the EcHMR which was reported to be localized at plasma membrane or cytosol, could be a potential transmembrane protein with varied sub-cellular function.

REFERENCES

Abe H, Urao T, Ito T, Sekic M, Shinozakic K, Yamaguchi-Shinozaki K (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Pl Cell 15:63–78

Adejumo SA, Togun AO, Adediran JA, Ogundiran MB (2011) *In-Situ* remediation of heavy metal contaminated soil using Mexican sunflower (*Tithonia diversifolia*) and cassava waste composts. World J Agric Sci 7: 224-233

Agarwal M, Sahi C, Katiyar-Agarwal S, Agarwal S, Young T, Gallie DR, Sharma VM, Ganesan K, Grover A (2003) Molecular characterization of rice hsp101: complementation of yeast hsp104 mutation by disaggregation of protein granules and differential expression in indica and japonica rice types. Pl Mol Biol 51(4):543-53

Aina R, Labra M, Fumagalli P, Vannini C, Marsoni M, Cucchi U, Bracale M, Sgorbati S, Citterro S (2007) Thiol-peptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots. Environ Exp Bot 59:381–92

Alexandersson E, Fraysse L, Sjo¨vall-Larsen S, Gustavsson S, Fellert M, Karlsson M, Johanson U, Kjellbom P (2005) Whole gene family expression and drought stress regulation of aquaporins. Pl Mol Biol 59:469–484

Alfenito MR, Souer E, Goodman CD, Buell R, Mol J, Koes R, Walbot V(1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione-S-transferases. Pl Cell 10:1135–1149

Altschul S, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402

Anderson ME (1985) Determination of GSH and GSH disulphide in biological samples. Meth Enzymol 113:548–55

Andruini I, Godbold DL, Onnis A (1999) Cadmium and copper uptake in Mediterranean tree seedlings. Pl Physiol 97:111-117

Angelone, M. and Bini, C. 1992. Trace elements concentrations in soils and plants of Western Europe. p. 19-60. *In*: D.C. Adriano (ed.), Biogeochemistry of trace metals. FL: Lewis Publishers, Boca Raton.

Arazi T, Kaplan B, Sunkar R, Fromm H (2000) Cyclic nucleotide and Ca^{2+}/calmodulin regulated channels in plants: targets for manipulating heavy metal tolerance, and possible physiological roles. Biochem Soc Trans 28:471-475

Arnaud N, Murgia I, Boucherez J, Briat J, Celler F, Gaymard F (2006) An iron-induced nitric oxide burst precedes ubiquitin-dependent protein degradation for *Arabidopsis* AtFer1 ferritin gene expression. J Biol Chem 281:23579–23588

Asavapisit S, Fowler G, Cheeseman CR (1997) Solution chemistry during cement hydration in the presence of metal hydroxide waste. Cement Concr Res 27: 1249-1260

Bailey TL, Elkan C (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In:Proc Sec Intern Conf Intelligent Syst Mol Biol, 28–36

Baker AJM, Brooks RR (1989) Terrestrial higher plants which accumulate metallic elements: A review of their distribution, ecology and phytochemistry. Biorecovery 1:81–126

Balestrazzi A, Botti S, Zelasco S, Biondi S, Franchin C, Calligari P, Racchi M, Turchi A, Lingua G, Berta G, Carbonera D (2009) Exprepression of the PsMTA1 gene in white poplar engineered with the MAT system is associated with heavy metal tolerance and protection against 8-hydroxy-2-deoxyguanosine mediated-DNA damage. Pl Cell Rep 28:1179-1192

Bañuelos G, LeDuc DL, Pilon-Smits EAH, Terry N (2007) Transgenic indian mustard overexpressing Selenocysteine lyase or Selenocysteine methyltransferase exhibit enhanced potential for Selenium phytoremediation under field conditions. Environ Sci Technol 41 (2): 599–605

Barman SC, Kisku GC, Salve PR, Misra D, Sahu RK, Ramteke PW, Bhargava SK (2001) Assessment of industrial effluents and its effects on soil and plants. J Environ Biol 22:251-256

Barman SC, Sahu RK, Bhargava SK, Chatterjee C(2000) Distribution of heavy metals in wheat, mustard and weed grains irrigated with industrial effluents. Bull Environ Conta Toxicol 64:489-496

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Pl Sci 24:23–58

Baxter I, Tchieu J, Sussman MR, Boutry M, Palmgren MG, Gribskov M, Harper JF, Axelsen KB. (2003) Genomic comparison of P-type ATPase ion pumps in *Arabidopsis* and rice. Pl Physiol 132:618–628

Benavides MP, Gallego SM, Tomaro M (2005) Cadmium toxicity in plants. Braz J Pl Physiol 17: 21-34

Bennett LE, Burkhead JL, Hale KL, Pinger LM (2003) Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. J Environ Qual 32:432–440

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE (2000)The Protein Data Bank. Nucleic Acids Res 28:235–42

Beyersmann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 82:493-512

Bittsánszkya A, Kfmives T, Gullner G, Gyulai G, Kiss J, Heszky L, Radimszky L, Rennenberg H (2005) Ability of transgenic poplars with elevated glutathione content to tolerate zinc($^{2+}$) stress. Environ Interact 31:251–254

Blanvillain R, Kim JH, Wu S, Lima A, Ow DW (2008) OXIDATIVE STRESS3 is a chromatin associated factor involved in tolerance to heavy metals and oxidative stress. Pl J 57: 657-665

Blinda A, Koch B, Ramanjalu S, Dietz KJ (1997) *De novo* synthesis and accumulation of apoplastic proteins in leaves of heavy metals barley seedlings. Cell Environ 20:961-981

Blum R, Beck A, Korte Al, Stengel A, Letzel T, Lendzian K, Grill E (2007) Function of phytochelatin synthase in catabolism of glutathione-conjugates. Pl J 49(4):740–749.

Bohnert HJ, Nelson DF, Jenson RG (1995) Adaptation to environmental stresses. Pl Cell 7: 1099-1111

Bonjoch NP, Tamayo PR (2001) Protein content quantification by Bradford method. Kluwer Academic Publishers.283-295

Boominathan R, Doran PM (2003) Cd tolerance and antioxidative defenses in hairy roots of the Cd hyperaccumulator, *Thlaspi caerulescens*. Biotechnol Bioeng 83:158–167

Borsani O, Zhu J, Verslues P L, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulates salt tolerance in *Arabidopsis*. Cell 123:1279-1291

Boudsocq M, Barbier-Brygoo H, Lauriere C (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. J Biol Chem 279:41758–41766

Boursiac Y, Chen S, Luu DT, Sorieul M, van den Dries N, Maurel C (2005) Early effects of salinity on water transport in *Arabidopsis* roots. Molecular and cellular features of aquaporin expression. Pl Physiol 139:790–805

Bowler C, Van Montagu M, Inze D (1992) Superoxide dismutase and stress tolerance, Annu Rev Pl Physiol Pl Mol Biol 43:83–116

Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem72: 248-254

Brewer GJ, Sing EF (1970) An Introduction to Isozyme Techniques. Academic Press, New York, 182

Bricker TJ, Pichtel J, Brown HJ, Simmons M (2001) Phytoextraction of Pb and Cd from superficial soil: effects of amendments and croppings. J Environ Sci Health, Part A Toxic/Hazardous Substances and Environmental Engineering 36:597–1610

Brini F, Masmoudi K (2012) Ion transporters and abiotic stress tolerance in plants. ISRN Molecular Biology Volume 2012, Article ID 927436, 13

Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. Trends Genet 22:268–280

Buchet JP, Lauwerys R, Roels H, Bernard A, Bruaux P, Claeys F, et al.(1990) Renal effects of cadmium body burden of the general population. Lancet 336:699-702

Burnett EC, Desikan R, Moser RC, Neill SJ (2000) ABA activation of an MBP kinase in *Pisum sativum* epidermal peels correlates with stomatal responses to ABA. J Expt Bot 51:197-205

Busenlehner LS, Pennella MA, Giedroc DP (2003) The SmtB/ArsR family of metalloregulatory transcriptional repressors: Structural insights into prokaryotic metal resistance. FEMS Microbiol Rev 27(2-3):131-43

Ca´novas D, Vooijs R, Schat H, Lorenzo VD (2004) The role of thiol species in the hypertolerance of *Aspergillus sp*. P37 to arsenic. J Biol Chem 279:51234–51240

Cailliatte R, Lapeyre B, Briat J.F, Mari S, Curie C (2009) The NRAMP6 metal transporter contributes to cadmium toxicity. Biochem J 422:217-228

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ (2006) Maturation of *Arabidopsis* seeds is dependent on glutathione biosynthesis within the embryo. Pl Physiol 141: 446–455

Calahan D, Dunham M, DeSevo C, Koshland DE (2011) Genetic analysis of desiccation tolerance in *Saccharomyces cerevisiae*. Genetics 189:507–519

Cameron JC, Pakrasi HB (2010) Essential role of GSH in acclimation to environmental and redox perturbations in the *Cyanobacterium Synechocystis* sp. PCC 68031. Pl Physiol 154:1672–1685

Chandler PM, Robertson M (1994) "Gene expression regulated by abscisic acid and its relation to stress tolerance". Annu Rev Pl Physiol Pl Mol Biol 45: 113–141

Chaurasia V, Mishra Y, Rai LC (2008) Cloning expression and analysis of phytochelatin synthase *(pcs*) gene from *Anabaena* sp. PCC 7120 offering multiple stress tolerance in *E. coli*. Biochem Biophys Res Commun 376:225–230

Chinnusamy V, Ohta M, Kanrar S, Lee B, Hong X, Agarwal M, Zhu J (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes Dev 17:1043–1054

Chinnusamy V, Stevenson B, Lee B, Zhu JK (2002) Screening for gene regulation mutants by bioluminescence imaging. Sci STKE 140:l10

Cho UH. Park JO (2001) Mercury-induced oxidative stress in tomato seedlings. Pl Sci 156:1-9

Choi H, Hong J, Ha J, Kang J, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem 275:1723–1730

Choi HI, Park HJ, Park JH, Kim S, Iim MY, Seo HH, Kim YW, Hwang I, Kim SY (2005) *Arabidopsis* calcium-dependent protein kinase AtCPK32 interacts with ABF4, a transcriptional regulator of abscisic acid-responsive gene expression, and modulates its activity. Pl Physiol 139:1750–1761

Ciais PH, Reichstein M, Viovy N et al. (2005) Europe-wide reduction in primary productivity caused by the heat and drought in 2003. Nature 437:529–533

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212: 475–486

Clemens S (2006) Evolution and function of phytochelatin synthases. J Pl Physiol 163: 319-322.

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 15:3325–3333

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:156-182

Cobbett CS (2000a) Phytochelatin biosynthesis and function in heavy metal detoxification. Curr Opin Plant Biol 3:211–216

Cobbett CS (2000b) Phytochelatins and their roles in heavy metal detoxification. Pl Physiol 123:825–832

Cobbett CS, Meagher RB (2002) *Arabidopsis* and the genetic potential for the phytoremediation of toxic elemental and organic pollutants. The *Arabidopsis* book. American Society of Plant Biologists. e0032. doi: 10.1199/tab.0032

Cobbett CS, Hussain D, Haydon MJ (2003) Structural and functional relationships between type 1B heavy metal-transporting P-type ATPases in *Arabidopsis*. New Phytol 159: 315–321

Cohen CK, Garvin DF, Kochian LV (1998) Characterization of an iron-regulated micronutrient transporter from *Pisum sativum* L. Pl Physiol Abstract no. 646. http://www.rycomusa.com/aspp.1998/45/0488.shtml.

Connolly EL, Guerinot ML (2002) Iron stress in plants. Genome Biol 3(8):1024.1–1024.4

Cotter-Howells J (1996) Lead phosphate formation in soils. Environ Poll 93:9–16

Courbot M, Willems G, Motte P, Arvidsson S (2007) A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase. Pl Physiol 144:1052–1065

Cunningham SD, Berti WR, Huang JW (1995) Phytoremediation of contaminated soils. Trends Biotech 13:393–397

Curie C, Briat JF. (2003) Iron transport and signaling in plants. Ann Rev Plant Biol 54:183–206.

Cuypers A, Vangronsveld J, Clijsters H (1999) The chemical behavior of heavy metals plays a prominent role in the induction of oxidative stress. Free Rad Res 31:839–843

DalCarso G, Fariniti S, Maistri S, Furini A (2008). How plants cope with cadmium staking all on metabolism and gene expression. J Integr Pl Biol 10:1268-1280

DalCorso G, Farinati S, Furini A (2010) Regulatory networks of cadmium stress in plants. Plant Signal Behaviour 5(6):663-667

Das S, Jana BB (1999) Dose-dependent uptake and *Eichhornia*-induced elimination of cadmium in various organs of freshwater mussel, *Lamellidens marginalis* (Linn.). Ecol Engg 12:207–229

Davis BJ (1964) Disc electrophoresis II. Methods and application to human serum proteins. Ann NY Acad Sci 121: 404–427

De Knecht JA, Van Dillen M, Koevoets PLM, Schat H, Verkleij JAC, Ernst WHO (1994) Phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*: chain length distribution and sulfide incorporation. Pl Physiol 104:255–261

De Miranda JR, Thomas MA, Thurman DA, Tomsett AB (1990) Metalliothionein genes from the flowering plant Mimulus guttatus. FEBS Letters 260:277–280

De Vos CHR, Schat H (1991) Free radicals and heavy metal tolerance. *In*: J. Rozema and J A C. Verleij (ed.), Ecological responses to environmental stress. Kluwer, Dordrecht, 22-30.

Deckert J (2008) Modulation of gene expression in plants exposed to heavy metals. In: Khan NA, Singh S (eds) Abiotic stress and plant responses. I.K. International Publishing House Pvt. Ltd., New Delhi, 125–138

Delgado M, Bigeriego M, Guardiola E (1993) Uptake of Zn, Cr and Cd by water hyacinths.Water Res 27:269-272.

Delhaize EP, Ryan R (1995) Aluminum toxicity and tolerance in plants. Pl Physiol 107:315–321

Demiral T, Türkan I (2005) Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environ Expt Bot 53:247–257

Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Hölzer R, Feller U (2004) Biochemical changes in barley plants after excessive supply of copper and manganese. Environ Exp Bot 52: 253-266

DeSilva TM (2000) "Structural biology of heavy metal binding proteins". *Dissertations available from ProQuest.* Paper AAI9976412. http://repository.upenn.edu/dissertations/AAI9976412

DeSilva TM, Porcelli GVF, Prantner AM, Opella S J (2002) Selectivity in heavy metal-binding to peptides and proteins. Biopolymers 64(4):189–197

Dietz KJ, Baier M, Kramer U (1999) Free radicals and the reactive oxygen species as mediators of heavy metal toxicity in plants, 73-97. *In*: M. N. V. Prasad, and J. Hagemeyer (eds.), Heavy metal stress in plants; from molecules to ecosystems. Springer-Verlag, Berlin.

Ding Y, Chen Z, Zhu C (2011) Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*). J Exp Bot doi: 10.1093/jxb/err046

Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN (2008) Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. Science 320: 942–945.

Dixit V, Pandey V, Shyam R (2001) Differential antioxidative responses to Cd in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J Expt Bot. 52:1101–1109

Doherty CJ, Van Buskirk HA, Myers SJ, Thomashow MF (2009) Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. Pl Cell 21:972–984

Dong C, Agarwal M, Zhang Y, Xie Q, Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. PNAS USA 103:8281–8286

Dong J, Wu FB, Zhang GP (2006) Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). Chemosphere 64:1659–1666

Dongre AR (1988) Emerging tendem mass spectroscopy techniques for rapid identification of proteins. Trends Biotechnol 15:418-425

Dormer UH, Westwater J, McLaren NF, Kent NA, Mellor J, Jamieson DJ (2000) Cadmium-inducible Expression of the Yeast GSH1 Gene Requires a Functional Sulfur-Amino Acid Regulatory Network. J Biol Chem 275:32611-32616

Doty SL (2008) Enhancing phytoremediation through the use of transgenics and endophytes. New Phytol 179:318–333

Du J, Huang YP, Xi J, Cao MJ, Ni WS, Chen X, Zhu JK, Oliver DJ, Xiang CB (2008) Functional gene-mining for salt-tolerance genes with the power of *Arabidopsis*. Pl J 56:653–664

Dubey S, Misra P, Dwivedi S, Chatterjee S, Bag SK, Mantri S, Asif MH, Rai A, Kumar S, Shri Tripathi MP, Tripathi RD, Trivedi PK, Chakrabarty D, Tuli R (2010) Transcriptomic and metabolomic shifts in rice roots in response to Cr (VI) stress. BMC Genomics 11:648-667

Dubreuil-Maurizi C, Vitecek J, Marty L, Branciard L, Frettinger P, Wendehenne D, Meyer A.J, Mauch F, Poinssot B (2011) Gutathione deficiency of the *Arabidopsis* mutant *pad2-1* affects oxidative stress-related events, defense gene expression, and the hypersensitive response. Pl Physiol 157:2000–2012

Dykema PE, Sipes PR, BrendaMA, Biermann J, Crowell DN, Randall SK(1999) A new class of proteins capable of binding transition metals. Pl Mol Biol 41:139–150

Eapen S, D'Souza SF (2005) Prospects of genetic engineering of plants for phytoremediation of toxic metals. Biotechnol Adv 23:97–114

Edwards R, Dixon D (2005) Plant glutathione transferases. Meth Enzymol 401:169–186

Edwards R, Dixon DP, Walbot V (2000) Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Pl Sci 5:193–198

Eide D, Broderius M, Fett J, Guerinot ML (1996) PNAS USA 93:5624-5628

Elia G, DeMarco A, Rossi A, Santoro MG (1996) Inhibition of HSP70 expression by calcium ionophore A23187 in human cells: an effect independent of the acquisition of DNA-binding activity by the heat shock transcription factor. J Biol Chem 271:16111–16118

Ellman GL(1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–7

Elloumi N, Ben F, Rhouma A, Ben B, Mezghani I, Boukhris M (2007) Cadmium induced growth inhibition and alteration of biochemical parameters in almond seedlings grown in solution culture. Acta Physiol Plant 29:57-62

Endicott JA, Ling V (1989) The biochemistry of P-glycoprotein mediated multidrug resistance. Annu Rev Biochem 58:137-171

Fahey RC (2001) Novel thiols of prokaryotes. Annu Rev Microbiol 55:333–356

Falcón-Pérez JM, Mazón MJ, Molano J, Eraso P (1999) Functional domain analysis of the yeast ABC transporter Ycf1p by site-directed mutagenesis. J Biol Chem 274(33):23584-90

Faller P, Kienzler K, Liszlay AK (2005) Mechanism of Cd^{2+} toxicity: Cd^{2+} inhibits photoactivation of photosystem II by competitive binding to the essential Ca^{2+} site. Biochim Biophys Acta 1706:158–164

Farinati S, Dol Corso G, Varotto S, Furini A (2010) The *Brassica juncea* BjCdR15, an ortholog of *Arabidopsis* TGA3, is a regulator of cadmium uptake, transport and accumulation in shoots and confers cadmium tolerance in transgenic plants. New Phytol 185: 964-978

Fedoroff NV (2002) RNA-binding proteins in plants: the tip of an iceberg? Curr Opin Plant Biol 5:452–459

Figueira EI, Lima G, Pereira SIA (2005) Cadmium tolerance plasticity in *Rhizobium leguminosarum* bv. *viciae*: GSH as a detoxifying agent. Can J Microbiol 51:7–14

Flowers TJ, Colmer TD (2008) "Salinity tolerance in halophytes," New Phytol 179(4):945–963

Fox TC, Guerinot ML (1998) Molecular biology of cation transport in plants. Annu Rev Plant Physiol Pl Mol Biol 49: 669–696

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Pl Cell 17:1866–1875

Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid Rad Signal 11:861–905

Foyer CH, Theodoulou FL, Delrot S (2001) The functions of inter- and intracellular glutathione transport systems in plants. Trends Pl Sci 6:486–492

Freeman JL, Persans MW, Nieman K, Albrecht C, Peer W, Pickering IJ, Salt DE (2004) Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. Pl Cell 16:2176–2191

Freire Martins DF, Vitoria de Moura MF, Bezerra Loiola MI (2011).Temporal and physiological influence of the absorption of nutrients and toxic elements by *Eichhornia crassipes*. J Environ Monit 13: 274–279

Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2006) ABA-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. PNAS USA 103: 1988–1993

Fusco N, Micheletto L, Dal Corso G, Borgato L, Furini A (2005) Identification of cadmium-regulated genes by cDNA-AFLP in the heavy metal accumulator *Brassica juncea* L. J Exp Bot 6: 3017–3027

Galloway JN, Thornton JD, Norton SA, Volcho HL, McLean RA (1982) Trace metals in atmospheric deposition: a review and assessment. Atmos Environ 16: 1677

Gasic K, Korban SS (2007) Expression of *Arabidopsis* phytochelatin synthase from Indian mustard (*Brassica juncea*) plants enhances tolerance for Cd and Zn. Planta 25:1277–1285

Gaxiola RA, Li J, Undurraga S, Dang LM, AllenGJ, AlperSL, Fink GR (2001) "Drought- and salt tolerant plants result from overexpression of the AVP1 H^+-pump," PNAS USA 98(20):11444–11449

Geourjon C, Deléage G (1995) SOPMA: Significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments. CABIOS 11:681-684

Ghabbour EA, Davies G, Lam YY, Vozzella ME (2004) "Metal binding by humic acids isolated from water hyacinth plants (*Eichhornia crassipes*)" [Mart.] SolmLaubach: Pontedericeae) in the Nile Delta, Egypt. J Environ Poll 131:445451

Gharieb MM, Gadd GM (2004) Role of GSH in detoxification of metal(loid)s by *Saccharomyces cerevisiae.* Biometals 17:183–188

Ghosh M, Shen J, Rosen BP (1999) Pathways of As(III) detoxification in *Saccharomyces cerevisiae.* PNAS USA 96:5001–5006

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAC/SS–DNA/PEG procedure. Yeast 11:355–360

Gill SS, Khan NA, Anjum NA, Tuteja N (2011) Amelioration of cadmium stress in crop plants by nutrient management: morphological, physiological and biochemical aspects. Pl Stress 5(1):1-23

Glaeser H, Coblenz A, Kruczek R, Ruttke I, Ebert-Jung A, Wolf K (2004) GSH metabolism and heavy metal detoxification in *Schizosaccharomyces pombe*. Curr Genet 19(3):207-213

Gleba D, Borirjuk NV, Borisjuk LG, Gonden P (1999) Use of plant roots for phytoremediation and molecular farming. PNAS USA 96:5973–5977

Gomes-Junior RA, Moldes CA, Delite FS, Pompeu GB, Gratao PL, Mazzafera P, Lea PJ, Azevedo RA (2006) Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. Chemosphere 65: 1330-1337

Gong Q, Li P, Ma S, Rupassara SI, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile Thellungiella halophila in comparison with its relative *Arabidopsis thaliana*. Pl J 44:826–839

Gottesman M, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62:385-427

Gottlieb LP (1982) Conservation and duplication of isozymes in plants. Science 216: 373-380

Goyer RA (1997) Toxic and essential metal interactions. Annu Rev Nutr 17:37–50

Grably MR, Stanhill A, Tell O, Engelberg D (2002) HSF and Msn2/4p can exclusively or cooperatively activate the yeast HSP104 gene. Mol Microbiol 44: 21-35

Gratao PL, Monteiro CC, Antunes AM, Peres LEP. Azevedo RA (2008) Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. Annu Appl Biol 153: 321-333

Grill E, Loffler S, Winnacker EL, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific c-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). PNAS USA 86:6838–6842

Groppa MD, Rosales EP, Iannone MF, Benavides MP (2008) Nitric oxide, polyamines and Cd-induced phytotoxicity in wheat roots. Phytochem 69:2609–2615

Grün S, Lindermayr C, Sell S, Dumer (2006) Nitric oxide and gene regulation in plants. J Exp Bot 57:507–516

Gueldry O, Lazard M, Delort F, Dauplais M, Grigoras I, Blanquet S, Plateau P (2003) Ycf1p-dependent Hg(II) detoxification in *Saccharomyces cerevisiae*. Eur J Biochem 270:2486–2496

Guerinot ML (2000) The ZIP family of metal transporters. Biochimicaet Biophysica Acta 1465:190-198

Gupta M, Cuypers A, Vangronsveld J, Clijster H (1999) Copper affects the enzymes of the ascorbate-glutathione cycle and its related metabolites in the roots of *Phaseolus vulgaris*. Physiol Plant 106: 262-267

Haag-Kerwer A, Schäfer HJ, Heiss S, Walter C, Rausch T (1999) Cadmium exposure in *Brassica juncea* causes a decline in transpiration rate and leaf expansion without effect on photosynthesis. J Expt Bot 50:1827–1835

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1–11

Hall JL, Williams LE (2003) Transition metal transporters in plants. J. Exp. Bot. 54: 2601–2613

Hamer DH (1986) Metallothionein. Annu Rev Biochem 55:913–951

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Kramer U (2008) Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. Nature 453:391–395

Harper J, Breton G, Harmon A (2004) Decoding Ca2+ signals through plant protein kinases. Annu Rev Pl Biol 55:263–288

Hart B, Lee CH, Shukla GS, Shukla A, Osier M, Eneman JD, Chiu JF (1999) Characterization of Cd-induced apoptosis in rat lung epithelial cells: evidence for the participation of oxidant stress. Toxicol 133:43–58

Hartley-Whitaker J, Ainsworth G, Vooijs R, Ten Bookum W, Schat H, Meharg AA (2001) Phytochelatins are involved in differential arsenate tolerance in *Hokus lanatus*. Pl Physiol 126:299-306

Hausladen A, Alscher RG (1993) Glutathione. *In* RG Alscher, JL Hess, eds, Antioxidants in Higher Plants. CRC Press, Boca Raton, FL, 1–30

Haydon MJ, Cobbett CS. (2007) Transporters of ligands for essential metal ions in plants. New Phytol 174:449–506

Heazlewood JL, Verboom R E, Tonti-Filippini J, Small I, Millar A H (2007) SUBA, The*Arabidopsis* subcellular database. Nucleic Acids Res 35: D213–D218

Helbig K, Bleuel C, Krauss GJ, Nies DH (2008a) GSH and transition-metal homeostasis in *Escherichia coli*. J Bacteriol 190:5431–5438

Helbig K, Grosse C, Nies DH (2008b) Cadmium Toxicity in GSH Mutants of *Escherichia coli*. J Bacteriol 190(15):5439–5454

Henry-Silva GG, Camargo AFM (2005), Interaco˜es ecologicas entre as macrofitas aquaticas flutuantes *Eichhornia crassipesPistia stratiotes*, Hoenea 32(3): 445–452

Herbette S, Taconnat L, Hugouvieux V, Piette L, Magniette ML, Cuine S, Auroy P, Richaud P, Forestier C, Bourguignon J, Renou JP, Vavasseur A, Leonhardt N (2006) Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. Biochimie 88:1751–1765

Herschbach C, Rennenberg H (2001) Significance of phloem-translocated organic sulfur compounds for the regulation of sulfur nutrition. Prog Bot 62:177–192

Higgins CF (1992) ABC-transporters: from microorganisms to man. Annu Rev Cell Biol 8:67-113

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database:1999. Nucleic Acids Res 27(1):297-300

Hirata K, Tsuji N, Miyamoto K (2005) Biosynthetic regulation of phytochelatins, heavy metal-binding peptides. J Biosci Bioengg 100:593–599

Hirayama T, Shinozaki K (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. Trends Pl Sci 12:343–351

Hirotaka Y, Hiroyuki F, Tomohito A, Akio O, Tsukasa N, Koji M, Satomi N (2010) Gene expression analysis in cadmium-stressed roots of a low cadmium-accumulating solanaceous plant, *Solanum torvum*. J Exp Bot 61 (2):423-437

Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ (2000) Expression of *Arabidopsis* CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Pl Physiol 124:125–133

Horie T, Costa A, Kim TH, Han MJ, Horie R, Leung HY, Miyao A, Hirochika H, An G, Schroeder JI. (2007) "Rice *OsHKT2;1* transporter mediates large Na^+ influx component into K^+-starved roots for growth" EMBO J 26(12):3003–3014

Hossain MA, Hossain MZ, Fujita M (2009) Stress-induced changes of m ethy lg lyoxal le ve l and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene. Aus J Crop Sci 3(2):53–64

Hossain MA, Fujita M (2010) Evidence for a role of exogenous glycinebetaine and proline in antioxidant defense and methylglyoxal detoxification systems in mungbean seedlings under salt stress. Physiol Mol Biol Pl 16(1):19–29

Houseley J, Tollervey D (2009) The many pathways of RNA degradation. Cell 136:763–776

Hsu YT, Kao CH (2003) Role of abscisic acid in cadmium tolerance of rice (*Oryza sativa* L.) seedlings. Pl Cell Environ 26:867-874

Hsu YT, Kao CH (2004) Cd toxicity is reduced by nitric oxide in rice leaves. Pl Growth Regul 42:227–238

Hu C, Zhang EL, Hamilton ED, Zhou W, Yang T, Zhu D (2007) Physiological responses induced by copper bioaccumulation in *Eichhornia crassipes* (Mart.). Hydrobiologia 579: 211-218

Huh W K, Falvo J V, Gerke L C, Carroll A. S. *et al.* (2003) Global analysis of protein localization in budding yeast. Nature 425: 686–691

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. Pl Cell 16:1327–1339

Imlay JA, Chin SM, Linn S (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*. Science 240: 640-642

Inan G, Zhang Q, Li P, et al. (2004) Salt cress. A halophyte and cryophyte *Arabidopsis* relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. Pl Physiol 135:1718–1737

Ismail AS, Abael-Sabour RM (1996) Water hyacinth as an indicator for heavy metal pollution in different selected sites and water bodies around greater Cairo. Egypt J Soil Sci 36:343-354

IUPAC (1997) Compendium of Chemical Terminology, 2nd ed. (the "Gold Book").Online corrected version: (2006) "Transition element".

Jarup L, Berglund M, Elinder CG, Nordberg G, Vahter M (1998) Health effects of Cd exposure: a review of the literature and a risk estimate. Scand J Work Environ Health 124:1–51

Jayaweera MW, Kasturiarachchi JC (2004) Removal of nitrogen and phosphorus from industrial waste waters by phytoremediation using water hyacinth (*Eichhornia crassipes* (Mart.) Solms. Water Sci Technol 50:217225

Jensen WB (2003) The Place of Zinc, Cadmium, and Mercury in the Periodic Table. J Chem Edu 80(8):952–961

Jez JM, Cahoon RE, Chen S (2004) *Arabidopsis thaliana* glutamate-cysteine ligase: Functional properties, kinetic mechanism, and regulation of activity. J Biol Chem 279:33463–33470

Jin YH, Clark AB, Slebos RJ, AI-Refai H, Taylor JA, Kunkel TA, Resnick MA, Gordenin DA (2003). Cadmium is a mutagen that acts by inhibiting mismatch repair. Nat Genet 34:326-329

John R, Ahmad P, Gadgil K, Sharma S (2009) Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation by *Brassica juncea* L. Int J Pl Prod.3: 65-76

Jonak C, Nakagami H, Hirt H (2004) Heavy metal stress. Activation of distinct mitogen activated protein kinases pathways by copper and cadmium. Pl Physiol 136:3276-3283

Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Pl Biol 57:19–53

Kaldenhoff R, Fischer M (2006) Functional aquaporin diversity in plants. Biochimicaet Biophysica Acta 1758:1134–1141

Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, Zhu JK, Toriiad KU (2008) SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to *Arabidopsis* stomatal differentiation. Pl Cell 20:1775–1785

Kang SG, Jin,JB, Piao HL, Pih KT, Jang HJ, Lim JH, Hwang I (1998) Molecular cloning of an *Arabidopsis* cDNA encoding a dynamin-like protein that is localized to plastids. Pl Mol Biol 38:437–447

Kaplan B, Davydov O, Knight H, Galon Y, Knight MR, Fluhr R, Fromm H (2006) Rapid transcriptome changes induced by cytosolic Ca^{2+} transients reveal ABRE-related sequences as Ca^{2+}-responsive *cis*-elements in *Arabidopsis*. Pl Cell 18:2733–2748

Kay SH, Haller WT, Garrard LA (1984) Effect of heavy metals on water hyacinths [*Eichhornia crassipes* (Mart.) Solms]. Aquatic Toxicol 5: 117-128.

Kellos T, Timar V, Szilagyi G, Szalai G, Galiba G, Kocsy G (2008) Stress hormones and abiotic stresses have different effects on antioxidants in maize lines with different sensitivity. Pl Biol 10:63–572

Khan MB, Sponder G, Sjoeblom B, Svidova S, Schweyen RJ, Carugo O, Djinovic-Carugo, K. Structural and functional characterization of the N-terminal moiety of Mrs2 the Mg^{2+} transporter from yeast inner mitochondrial membrane. Journal: To be Published DOI:10.2210/pdb3rkg/pdb, (UniProtKB:Q01926)

Kieffer P, Dommes J, Hoffmann L, Hausman JF, Renaut J (2008) Quantitative changes in protein expression of cadmium-exposed poplar plants. Proteomics 8:2514–30

Kieffer P, Planchon S, Oufir M, Ziebel J, Dommes J, Hoffmann L, et al. (2009) Combining proteomics and metabolite analyses to unravel cadmium stress-response in poplar leaves. J Proteome Res 9:400–17

Kikuchi S, Satoh K, Nagata T et al (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science 301:376-379

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. Pl J 50:347–363

Kim DY, Bovet L, Kushnir S, Noh EW, Martinoia E, Lee Y (2006) AtATM3 is involved in heavy metal resistance in *Arabidopsis.* Pl Physiol 140:922-932

Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Pl J 50(2):207–218

Klein M, Burla B, Martinoia E (2006) The multidrug resistance-associatedproteins (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants. FEBS Letters 580:1112–1122

Klein M, Weissenbock G, Dufaud A, Gaillard C, Kreuz K, Martinoia E (1996) Different energization mechanisms drive the vacuolar uptake of a flavonoid glucoside and a herbicide glucoside. J Biol Chem 271:29666-29671

Knetsch MLW, Wang M, Snaar-Jagalska E, Heimovaara-Dijkstra S (1996) Abscisic acid induces mitogen-activated protein kinase activation in barley aleurone protoplasts. Pl Cell 8:1061-1067

Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, Hobo T, Yamamoto A, Hattori T (2005) Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. Pl J 44: 939–949

Kocsy G, Galiba G, Brunold C (2001) Role of glutathione in adaptation and signalling during chilling and cold acclimation in plants. Physiol Plant 113:158–164

Kohler A, Blaudez D, Chalot M, Martin F (2004) Cloning and expression of multiple metallothioneins from hybrid poplar. New Phytol 164: 83-93

Koizumi T, Shirakura H, Kumagai H, Tatsumoto H, Suzuki KT (1996) Mechanism of cadmium induced cytotoxicity in rat hepatocytes-cadmium induced active oxygen-related permeability changes of the plasma-membrane. Toxicol 114:125–134

Kopriva S (2006) Regulation of sulfate assimilation in *Arabidopsis* and beyond. Ann Bot 97:479–495

Kopyra M, Gwozdz EA (2003) Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. Pl Physiol Biochem 41: 1011-1017

Kopyra M, Stachoń-Wilk M, Gwóźdź EA (2006) Effects of exogenous nitric oxide on the antioxidant capacity of cadmium-treated soybean cell suspension. Acta Physiol Plant 28: 525–536

Kovalchuk I, Titov V, Hohn B, Kovalchuk O (2005) Transcriptome profiling reveals similarities and differences in plant responses to cadmium and lead. Mutat Res 570:149–161

Kramer U (2010) Metal hyperaccumulation in plants. Annu Rev Pl Biol 61: 517–534

Krämer U, Clemens S (2005) Functions and homeostasis of zinc, copper and nickel in plants. *In* M Tamás, E Martinoia, eds, Topics in Current Genetics 14. Springer, Heidelberg, 215–271

Kramer U, Talke IN, Hanikenne M (2007) Transition metal transport. FEBS Lett 581:2263–2272

Kranner J, Harel S, Granit R (2006) Betalains - A new class of dietary cationized antioxidants. J Agric Food Chem 49:5178-5185

Kuanyu L, Hein S, Zou W, Klug G (2004) The GSH-glutaredoxin system in *Rhodobacter capsulatus*: part of a complex regulatory network controlling defense against oxidative stress. J Bacteriol 186:6800–6808

Kuhn JM, Schroeder JI (2003) Impacts of altered RNA metabolism on abscisic acid signaling. Curr Opin Pl Biol 6:463–469

Kupper H, Lombi E, Zhao FJ, McGrath SP (2000) Cellular compartmentalization of Cd and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212:75–84

Kuramata M, Masuya S, Takahashi Y, Kitagawa E, Inoue C, Ishikawa S, Youssefian S. Kusano T (2009) Novel cysteine-rich peptides from *Digitaria ciliaris* and *Oryza sativa* enhance tolerance to cadmium by limiting its cellular accumulation. Pl Cell Physiol 50:106–117

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lantzy RJ, Mackenzie FT (1979) Atmospheric trace metals: global cycles and assessment of man's impact. Geochim Cosmochim Acta 43: 511

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Pl Physiol 138:882–897

Latinwo LM, Donald C, Ikediobi C, Silver S (1998) Effects of intracellular GSH on sensitivity of *Escherichia coli* to mercury and arsenite. Biochem Biophys Res Commun 242:67–70

LeDuc DL, AbdelSamie M, Montes-Bayon M, Wu CP, Reisinger SJ, Terry N (2006) Overexpressing both ATP sulfurylase and selenocysteine methyltransferase enhances selenium phytoremediation traits in Indian mustard. Environ Poll 144:70–76

Lee HC, Wei YH (2001) Mitochondrial alterations, cellular response to oxidative stress and defective degradation of proteins in aging. Biogerontology 2: 231-244

Lee S, Kim YY, Lee Y, An G (2007) Rice P_{1B}-type heavy-metal ATPase, OsHMA9, is a metal efflux protein. Pl Physiol 145:831–842

Lee S, Moon JS, Ko T, Petros D, Goldsbrough PB, Korban SS (2003) Overexpression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmiumstress. Pl Physiol 131:656–663

Lemen RA, Lee JS, Wagoner JK, Bleier HP (1976) Cancer mortality among cadmium production workers. Ann NY Acad Sci 271: 273–279

Letunic I, Copley RR, Schmidt S, Ciccarelli FD, Doerks T, Schultz J, Ponting CP, Bork P (2004) SMART 4.0: towards genomic data integration. Nucleic Acids Res 32(1): D142-4

Li L, He Z, Pandey GK, Tsuchiya T, Luan S (2002) Functional cloning and characterization of a plant efflux carrier for multidrug and HM detoxification. J Biol Chem. 277:5360–5368

Li Y, Dankher OP, Carreira L, Jiang LX. (2006) The shoot-specific expression of gamma-glutamylcysteine synthetase directs the long-distance transport of thiol-peptides to roots conferring tolerance to mercury and arsenic. Pl Physiol 141:288–298

Li Y, Dhankher O, Carreira L, Balish R, Meagher R (2005) Engineered overexpression of γ-glutamylcysteine synthetase in plants confers high level arsenic and mercury tolerance. Environ Toxicol Chem 24:1376–1386

Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, Rea PA (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*:YCF1-catalyzed transport of bis (glutathionato) cadmium. PNAS USA 94:42–47

Li ZS, Szczypka M, Lu YP, Thiele DJ, Rea PA (1996) The yeast Cd factor protein (YCF1) is a vacuolar GSH S-conjugate pump. J Biol Chem 271:6509-6517

Lichtenthaler HK (1996) Vegetation stress: An introduction to the stress concept in plants. J Pl Physiol 148: 4-14

Lida K, Seki M, Sakurai T, Satou M, Akiyama K, Toyoda T, Konagaya A, Shinozaki K(2004) Genome wide analysis of ore-mRNA splicing in *Arabidopsis thaliana* based on full length cDNA sequences. Nucl Acids Res 32:5096-5103

Lide DR (1992) CRC Handbook of Chemistry and Physics, A ready-reference book of chemical and physical data. 73rd edition. D.R. Lide, Editor in chief (CRC Press, Boca Raton)

Lima, AIG, Corticeiro SC, Figueira E (2006) GSH-mediated cadmium sequestration in *Rhizobium leguminosarum*. Enzy Microb Technol 39:763–769

Lin R, Wang X, Luo Y, Du W, Guo H, Yin D (2007) Effects of soil cadmium on growth, oxidative stress and antioxidant system in wheat seedlings.(*Triticum aestivum* L.). Chemosphere 69: 89-98

Liu T, Golden JW, Giedroc DP (2005) A zinc(II)/lead(II)/cadmium(II)-inducible operon from the Cyanobacterium anabaena is regulated by AztR, an alpha3N ArsR/SmtB metalloregulator. Biochem 44(24):8673-83

Loeffler S, Hochberger A, Grill E, Winnacker EL, Zenk MH (1989) Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. FEBS Lett 258:42–46

Lombi E, Zhao F-J, McGrath SP, Young SD, Sacchi GA (2001) Physiological evidence for a high-affinity cadmium transporter highly expressed in a *Thlaspi caerulescens* ecotype. New Phytol 149:53–60

Lund BO, Miller DM, Wood JS (1993) Studies on Hg(II)-induced H2O2 formation and oxidative stress in vivo and in vitro in rat kidney mitrochondia. Biochem Pharmacol 45:2017-2024

Lunin VV, Dobrovetsky E, Khutoreskaya G, Zhang R, Joachimiak A, Doyle DA, Bochkarev A, Maguire ME, Edwards AM, Koth CM (2006) "Crystal Structure of the CorA Mg^{2+} Transporter." Nature 440:833-837

Lux A, Martinka M, Vaculik PJ (2010) White root responses to cadmium in the rhizosphere:a review. J Exp Bot 62:21-37

Lux A, Sottnikova A, Opatrna J, Greger M (2004) Differences in structure of adventitious roots in *Salix* clones with contrasting characteristics of Cd accumulation and sensitivity. Physiol Plant 120:537–545

Lytle CM, Lytle FW, YangN, Qian JH, Hansen D, Zayed A (1996) Reduction of Cr(VI) to Cr(III) by wetland plants: Potential for *in situ* heavy metal detoxification. Environ Sci Technol 32:3087–3093

Ma S, Bohnert HJ (2007) Integration of *Arabidopsis thaliana* stress-related transcript profiles, promoter structures, and cell-specific expression. Genome Biol 8:R49

Macek T, Mackova M, Pavlíkova D, Száková J, Truksa M, Singh Cundy A et al (2002) Accumulation of cadmium by transgenic tobacco. Acta Biotechnol 22:101–106

Maine MA, Duarte MV, Sune NL (2001) Cadmium uptake by floating macrophytes, Water Res.35: 2629-2634.

Maksymiec W (2007) Signaling responses in plants to heavy metal stress. Acta Physiol Plant 29:177-187

Maksymiec W, Wianowska D, Dawidowicz AL, Radkiewicz S, Mardarowicz M, Krupa Z (2005) The level of jasmonic acid in *Arabidopsis thaliana* and *Phaseolus coccineus* plants under heavy metal stress. J Pl Physiol 162:1338–1346

Mandal S, Mandal N, Hazra K, Mukherjee,SK, Das PK (2004) Submergence tolerant androgenic rice dihaploids derived from intra-and interspecific crosses: I. Seed storage protein and isozyme pattern through gel electrophoresis. Pl Tissue Cult 14: 25-35

Margoshes M, Vallee BL (1957) A cadmium protein from equine kidney cortex. J Amer Chem Soc 79:4813-4818

Marrs KA (1996) The functions and regulation of glutathione S-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol 47:127–158

Martinoia E, Grill E, Tommasini R, Kreuz K, Amrhein N (1993) An ATP-dependent GSH S-conjugate `export' pump in the vacuolar membrane of plants. Nature 364:247-249

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJ, Sanders D et al (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. Pl Physiol 126: 1646–1667

Matsui K, Ishida J, Morosawa T, Mochizuki Y, Kaminuma E, Endo T et al.(2008). *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using tilling array. Pl Cell Physiol 49:1135-1149

Matzke M, Kanno T, Huettel B, Daxinger L, Matzke AJ (2007) Targets of RNA-directed DNA methylation. Curr Opin Pl Biol 10:512–519

May MJ, Leaver CJ (1994) *Arabidopsis thaliana* y-glutamylcysteine synthetase is structurally unrelated to mammalian, yeast, and *E. coli* homologs. PNAS USA 91:10059-10063

May MJ, Vernoux T, Leaver C, Van Montagu M, Inze´ D (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. J Expt Bot 49: 649–667

McCully ME (1999) Roots in soil: unearthing the complexities of roots and their rhizospheres. Ann Rev Pl Physiol Pl Mol Biol 50:695–718

McLaggan D, Logan TM, Lynn DG, Epstein W (1990) Involvement of gamma-glutamyl peptides in osmoadaptation of *Escherichia coli*.J Bacteriol 172:3631–3636

Memon AR, Schröder P (2009) Implications of metal accumulation mechanisms to phytoremediation. Environ Sci Pollut Res 16:162–175

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, van Gijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162:328–334

Metwally A, Finkemeier I, Georgi M, Dietz KJ (2003) Salicylic acid alleviates the cadmium toxicity in barley seedlings. Pl Physiol 132:272–281

Metwally A, Safronova VI, Belimov AA, Dietz KJ (2005) Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. J Exp Bot 56:167-178

Meyer AJ (2008) The integration of GSH homeostasis and redox signaling. J Pl Physiol 165:1390–1403

Meyer AJ, Brach T,Marty L, Kreye S, Rouhier N, Jacquot JP, Hell R (2007) Redox-sensitive GFP in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular GSH redox buffer. Pl J 52:973–986

Millar AH, Mittova V, Kiddle G, Heazlewood JL, Bartoli CG, Theodoulou FL, Foyer CH (2003) Control of ascorbate synthesis by respiration and its implications for stress responses. Pl Physiol 133:443–447

Mills RF, Krijger GC, Baccarini PJ, Hall JL, Williams LE (2003) Functional expression of AtHMA4, a P1B-type ATPase of the Zn/Co/Cd/Pb subclass. Pl J 35:164–176

Minglin L, Yuxiu Z, Tuanyao C (2005) Identification of genes up-regulated in response to Cd exposure in *Brassica juncea* L. Gene 363:151-158

Mishra Y, Chaurasia N, Rai LC (2009) *AhpC (alkyl hydroperoxide reductase) from Anabaena sp. PCC 7120* protects *Escherichia coli* from multiple abiotic stresses, Biochem Biophys Res Commun 381:606–611

Mithoefer A, Schulze B, Boland W (2004) Biotic and heavy metal stress in plants: evidence for common signals. FEBS Letts 566:1-5

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Pl Sci 7: 405-410

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Pl Sci 11:15–19

Miura K, Jin JB, Hasegawa PM (2007) Sumoylation, a post-translational regulatory process in plants. Curr Opin Pl Biol 10:495–502

Moons A (2003) *Ospdr9*, which encodes a PDR-type ABC transporter, is induced by HMs, hypoxic stress and redox perturbations in rice roots. FEBS Lett.23:370–376

Mori S, Uraguchi S, Ishikawa S, Arao T (2009) Xylem loading process is a critical factor in determining Cd accumulation in the shoots of *Solanum melongena* and *Solanum torvum*. Environ Expt Bot 67:127–132

Mosser DD, Theodorakis NG, Morimoto RI (1988) Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. Mol Cell Biol 8:736-4744

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113: 935–944

Muller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, DeVries EGE, Jansen PLM (1994) Overexpression of the gene encoding the multidrug resistance associated protein results in increased ATP-dependent GSH S-conjugate transport. PNAS USA 91:13033-13037.

Mullineaux P, Rausch T (2005) Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. Photosyn Res 86:59–474

Muramoto S, Oki Y (1983). Removal of some heavy metals from polluted water by water hyacinth (*Eichhornia crassipes*). Bull Environ contam Toxicol 30:170-177

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–479

Murata M, Gong P, Suzuki K, Koizumi S (1999) Differential metal response and regulation of human heavy metal inducible genes. J Cell Physiol 180:105–13

Murphy A, Taiz L (1995a) A new vertical mesh transfer technique for metal-tolerance studies in *Arabidopsis*. Pl` Physiol 108:29–38

Murphy A, Taiz L (1995b) Comparison of metallothionein gene expression and non-protein thiols in ten *Arabidopsis* ecotypes. Pl Physiol 109:945–954

Nahakpam S, Shah K (2011) Expression of key antioxidant enzymes under combined effect of heat and cadmium toxicity in growing rice seedlings. Pl Growth Regul 63: 23-35

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. Pl Physiol 140:411–432

Nakashima K, Yamaguchi-Shinozaki K (2006) Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. Pl Physiol 126:62-71

Narayan O, Kumari N, Rai LC (2010) Heterologous expression of *Anabaena* PCC 7120 all3940 (a Dps family gene) protects *Escherichia coli* from nutrient limitation and abiotic stresses. Biochem Biophys Res Commun 394:163–169

Nedjimi B, Daoud Y (2009) Cadmium accumulation in *Atriplex halimus* subsp. schweinfurthii and its influence on growth, proline, root hydraulic conductivity and nutrient uptake. Flora Morphol Distribution Funct Ecol Pl 204: 316-324

Nies DH (1999) Microbial heavy metal resistance. Appl Microbiol Biotechnol 51:730–750

Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27:313–339

Nies DH (2007) Bacterial transition metal homeostasis, 118–142.*In* D. H. Nies and S. Silver (ed.), Molecular microbiology of heavy metals, vol. 6. Springer-Verlag, Germany

Nigam R, Srivastava S, Prakash S, Srivastava MM (2001) Cd mobilisation and plant availability – the impact of organic acids commonly exuded from roots. Pl Soil 230:107–113

Nocito FF, Lancilli C, Crema B, Fourcroy P, Davidian JC, Sacchi GA (2006) Heavy metal stress and sulfate uptake in maize roots. Pl Physiol 141:1138–1148

Noctor G, Arisi ACM, Jouanin L, Foyer CH (1998) Manipulation of GSH and amino acid biosynthesis in the chloroplast. Pl Physiol 118:471–482

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Pl Physiol Pl Mol Biol 49: 249–279

Noctor G, Gomez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation, and transport in the control of GSH homeostasis and signalling. J Expt Bot 53:1283–1304

Nury D, Chabanon H, Levadoux-Martin M, Hesketh J (2005) An eleven nucleotide section of 3'-untranslated region is required for perinuclear localization of rat metallothionein-1 mRNA. Biochem J 387:419-428

Obara K, Miyashita N, Xu C, Toyoshima I, Sugita Y, Inesi G, Toyoshima C (2005) Structural role of counter transport revealed in $Ca^{(2+)}$ pump crystal structure in the absence of Ca^{2+}.PNAS 102: 14489-14496

Odjegb VJ, Fasidi IO (2007) Phytoremediation of heavy metals by *Eichhornia crassipes*. Enviromentalist 27:349-355

Ogawa K, Iwabuchi M (2001) A mechanism for promoting the germination of *Zinnia elegans* seeds by hydrogen peroxide. Pl Cell Physiol 42:286–291

Ogundiran MB (2007) Assessment and Chemical remediation of soil contaminated by lead acid battery wastes in Lalupon village Oyo State. Nigeria. Ph.D Thesis, University of Ibadan, 205.

Oram PD, Fang X, Fernando Q, Letkeman P, Letkeman D (1996) The formation constants of mercury(II)-GSH complexes. Chem Res Toxicol 9:709–712

Ortiz, DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270:4721–4728

Osterberg R, Ligaarden R, Persson D (1979) Copper(I) complexes of penicillamine and GSH. J Inorg Biochem 10:341–355

Otte ML, Haarsma MS, Broekman RA, Rozema J (1993) Relation between heavy metal concentrations in salt marsh plants and soil. Environ Pollu 82: 13-22

Ouariti O, Boussama A, Zarrouk M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Phytochem 45:1343–1350

Owttrim GW (2006) RNA helicases and abiotic stress. Nucleic Acids Res 34:3220–3230

Palmiter R (1998) The elusive function of metallothioneins. PNAS USA 95:428–430

Panda SK, Upadhyay RK, Nath S (2010) Arsenic Stress in Plants. J Agron Crop Sci ISSN:0931-2250

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee YS (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. Pl J 69:278–288

Pasternak M, Lim B, Wirtz M, Hell R, Cobbett CS, Meyer AJ (2008) Restricting glutathione biosynthesis to the cytosol is sufficient for normal plant development. Pl J 53:999–1012

Payandeh J, Pai EF (2006) A structural basis for Mg^{2+} homeostasis and the CorA translocation cycle. EMBO J 25: 3762-3773

Perfus-Barbeoch L, Leonhardt N, Vavaddeut A, Forestier C (2002) Heavy metal toxicity: Cadmium permeates through calcium channels and disturbs the plant water status. Pl J 32: 539-548

Persans MW, Nieman K, Salt DE (2001) Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. PNAS USA 98:9995–10000

Petrovic S, Pascolo L, Gallo R, Cupelli F, Ostrow JD, Goffeau A, Tiribelli, Bruschi CV (2000) The products of *YCF1* and *YLL015w* (*BPT1*) cooperate for the ATP-dependent vacuolar transport of unconjugated bilirubin in *Saccharomyces cerevisiae*. Yeast 16:561–571

Plaza S, Tearall KL, Zhao FJ, Buchner P, McGrath SP, Hawkesford MJ (2007) Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. J Exp Bot 58:1717–1728

Polle A, Schützendübel A (2004) Heavy metal signaling in plants. In:*Plant Responses to Abiotic Stress* Hirt H, Shinozaki K (eds.). Vol 4. Springer-Verlag, Berlin, pp 187–215

Pomponi M, Censi V, Di Girolamo V, de Paolis A, Di Toppi LS, Aromolo R (2006) Overexpression of *Arabidopsis* phytochelatin synthase in tobacco plants enhances Cd2+ tolerance and accumulation but not translocation to the shoot. Planta 223:180–190

Prasad MNV (1999) Metallothioneins and metal binding complexes in plants. In: Prasad MNV, Hagemeyer J, eds. Heavy metal stress in plants: from molecules to ecosystems. Berlin: Springer-Verlag, 51–72

Prasad MNV, Greger M, Landberg T (2001a) *Acacia nilotica* L. bark removes toxic metals from solution: Corroboration from toxicity bioassay using *Salixviminalis* L. in hydroponic system. Int J Phytoremed 3: 289-300

Prasad MNV, Malec P, Waloszek A, Bojke M, Strzalka K (2001b) Physiological responses of *Lemna trisulca* L. (duckweed) to cadmium and copper bioaccumulation. Pl Sci 161: 881-889

Prestridge DS (1991) SIGNAL SCAN: A computer program that scans DNA sequences for eukaryotic transcriptional elements. CABIOS 7:203-206.

Prévéral S, Gayet L, Moldes C, Hoffmann J, Mounicou S, Gruet A, Reynaud F, Lobinski R, Verbavatz JM, Vavasseur A, Forestier C (2009) A common highly conserved cadmium detoxification mechanism from bacteria to humans. J Biol Chem 284:4936–4943

Qadir S, Qureshi MI, Javed S, Abdin (2004) Genotypic variation in phytoremediation potential of Brassica juncea cultivars exposed to Cd stress. Pl Sci 167:1171–1181

Qi X, Zhang Y, Chai T (2007) Characterization of a novel plant promoter specifically induced by heavy metal and identification of the promoter regions conferring heavy metal responsiveness. Pl Physiol 143:50–59

Qin F, Sakuma Y, Tran L-S, Maruyama K, Kidokoro S, Fujita Y, Fujita M, Umezawa T, Sawano Y, Miyazono K, Tanokura M, Shinozaki, K, Yamaguchi-Shinozaki K (2008) *Arabidopsis* DREB2A-interacting proteins function as RING E3 ligases and negatively regulate plant drought stress-responsive gene expression. Pl Cell 20:1693–1707

Ramos J, Clemente MR, Naya L, Loscos J, Perez-Rontome C, Sato S, Tabata S, Becana M (2007) Phytochelatin synthases of the model legume *Lotus japonicus*. A small multigene family with different responses to cadmium and alternatively spiced variants. Pl Physiol 143:110–1118

Ranieri A, Castagna A, Scebba F, Careri M, Zagnoni I, Predieri G, Pagliari M, di Toppi LS (2005) Oxidative stress and phytochelatin characterisation in bread wheat exposed to Cd excess. Pl Physiol Biochem 43:45–54

Rascio N, Navari-Izzo N (2011) Heavy metal hyperaccumulating plants: how and why do they do it? And what makes them so interesting? Pl Sci 180(2):169–181

Raskin I, Kumar PBAN, Dushenkov S, Salt DE (1994) Bioconcentration of heavy metals by plants. Curr Opin Biotechnol 5:285–290

Rausch T, Gromes R, Liedschulte V, Muller I, Bogs J, Galovic V, Wachter A (2007) Novel insight into the regulation of GSH biosynthesis in higher plants. Plant Biology (Stuttgart) 9:565–572.

Rauser WE (1990) Phytochelatins. Annu Rev Biochem 59:61–86

Rauser WE (1995) Phytochelatins and related peptides: structure, biosynthesis, and function. Pl Physiol 109:1141–1149

Rauser WE (1999) Structure and function of metal chelators produced by plants: The case for organic acids, amino acids, phytochelatin and metallothioneins. Cell Biochm Biophys 3:19–48

Rea PA, Li ZS, Lu YP, Drozdowicz YM, Martinoia E (1998) From vauolar GS-X pumps to multispecific ABC transporters. Annu Rev Pl Physiol Pl Mol Biol 49:727–760

Reddy ASN (2007) Alternative splicing of pre-messenger RNAs in plants in the genomic era. Annu Rev Pl Biol 58:267–294

Reddy MM, Gasber EO (1971) Genetic studies of variant enzyme. III. Comparative electrophoretic

Reese RN, Mehra RK, Tarbet EB, Winge DR(1988) Studies on the gamma-glutamyl Cu-binding peptide from *Schizosaccharomyces pombe*. J Biol Chem 263:4186-4192

Reeves RD, Baker AJM (2000) Metal-accumulating plants. In: Raskin I. and Ensley BD, (eds.) Phytoremediation of toxic metals: using plants to clean-up the environment. New York, John Wiley and Sons, 193-230

Ren Z, Gao J, Li L, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37:1141–1146

Rennenberg H (1997) Molecular approaches to glutathione biosynthesis. *In* WJ Cram, LJ DeKok, I Stulem, C Brunold, H Rennenberg, eds, Sulphur Metabolism in Higher Plants. Backhuys Publishers, Leiden, The Netherlands, 59–70

Rennenberg H, Herschbach C, Haberer K, Kopriva S (2007) Sulfur metabolism in plants: are trees different? Pl Biol 9:620–637

Rivetta A, Negrini N, Coccuci M (1997) Involvement of Ca^{2+}-calmodulin in Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L) seed germination. Pl Cell Environ 20:600-608

Rivoal J, Ricard B, Pradet A (1989) Glycolytic and fermentative enzyme induction during anaerobiosis in rice seedlings. Pl Physiol Biochem 27: 43-52

Robinson NJ, Tommey AM, Kuske C, Jackson PJ (1993) Plant metallothioneins. Biochem J 295:1–10

Robinson NJ, Winge DR (2010) Copper metallochaperones. Annu Rev Biochem 79:537-562

Rodríguez-Serrano M, Romero-Puertas MC, Pazmiño DM, Testillano PS, Risueño MC, del Río LA, Sandalio LM (2009) Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. Pl Physiol 150: 229-243

Rodriguez-Serrano M, Romero-Puertas MC, Zabalza A, Corpas FJ, Gomez M, del Rio MA, Sandalio LA (2006) Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. Pl Cell Environ 29:1532–1544

Romero-Puertas MC, Corpas FJ, Rodriguez-Serrano M, Gomez M, del Rio LA, Sandalio LM. (2007) Differential expression and regulation of antioxidative enzymes by cadmium in pea. J Pl Physiol 164:1346-1357

Romero-Puertas MC, Rodriguez-Serrano M, Corpas FJ, Gomez M, del Rio LA, Sandalio LM (2004) Cadmium-induced subcellular accumulation of O^{2-} and H_2O_2 in pea leaves. Pl Cell Environ 27:1122–1134

Ronne H, Rothstein R (1988) Mitotic sectored colonies: evidence of heteroduplex DNA formation during direct repeat recombination. PNAS USA 85:2696–2700

Roth U, von Roepenack-Lahaye E, Clemens S (2006) Proteome changes in *Arabidopsis thaliana* roots upon exposure to Cd^{2+}. J Exp Bot 57:4003–13

Rouhier N, Villarejo A, Srivastava M, Gelhaye E, Keech O, Droux M, Finkemeier I, Samuelsson G, Dietz K, Jacquot J, Wingsle G (2005) Identification of plant glutaredoxin targets. Antioxid rad Signal 7:919–929

Rouhier N, Lemaire SD, Jacquot JP (2008). The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Ann Rev Plant Biol 59:143–166

Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protocols 5:725-738

Roy A, Yang J, Zhang Y (2012) COFACTOR: An accurate comparative algorithm for structure-based protein function annotation. Nucleic Acids Res 40:W471-W477

Roy A, Zhang Y (2012). Recognizing protein-ligand binding sites by global structural alignment and local geometry refinement. Structure 20: 987-997

Rucińska-Sobkowiak R, Pukacki PM (2006) Antioxidative defense system in lupin roots exposed to increasing concentrations of lead. Acta Physiol Plant 28(4):357-364

Rüegsegger A, Brunold C (1992) Effect of cadmium on γ-glutamylcysteine synthesis in maize seedlings. Pl Physiol 99:428–433

Ruis H, Schuller C (1995) Stress signaling in yeast. Bioessays 17:959–965

Rus A, Lee BH, Mu˜noz-Mayor A, Sharkhuu A, Miura K, Zhu JK, Bressan RA, Hasegawa PM (2004) "AtHKT1 facilitates Na^+ homeostasis and K^+ nutrition *in planta*. Pl Physiol 136(1): 2500–2511

Rutherford JC, Bird AJ (2004) Metal-responsive transcription factors that regulate copper homeostasis in eukaryotic. Cell 3(1):1-13

Sabale S, Jadhav V, Jadhav D, Mohite BS, Patil KJ (2010) Lake contamination by accumulation of heavy metal ions in *Eichhornia crassipes*: a case study of Rankala Lake, Kolhapur (India). J Environ Sci Eng 52: 155-156

Sachs MM, Ho HD (1986) Alteration of gene expression during environmental stress in plants. Annu Rev Pl Physiol 37:363-376

Sadowski PG, Groen AJ, Dupree P, Lilley KS (2008) Sub-cellular localization of membrane proteins. Proteomics 8:3991–4011

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406-25

Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. Pl Physiol 109:1427–1433

Salt DE, Rauser WE (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Pl Physiol 107:1293–1301

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu Rev Pl Physiol Mol Biol 49:643-668

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandalio LM, Dalurzo HC, Gomez M, Romero-Puertas MC, del Rio LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot364:2115–2126

Sanit`a di Toppi L, Gabbrielli R (1999) Response to cadmium in higher plants. Environ Exp Biol 41: 105-130

Sarry J, Kuhn L, Ducruix C, Lafaye A, Junot C, Hugouvieux V, Jourdain A, Bastien O, Fievet JB, Vailhen D (2006) The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. Proteomics, 6: 2180-2198.

Savenstrad H, Strid A (2004) Six genes strongly regulated by mercury in *Pisum sativum* roots. Pl Physiol Biochem 42:135-142

Sch¨utzend¨ubel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351–1365

Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the GSH disulfide/GSH couple. Free Radic Biol Med 30:1191–1212

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J, Bleeker PM (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumylator and non-hyperaccumuylator metallophytes. J Exp Bot 53:2381–2392

Schneider IAH, Rubio J, Misra M, Smith RW (1995) *Eichhornia crassipes* as biosorbent for heavy metal ions. Miner Eng 8: 979-988

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. PNAS USA 95: 5857-5864

Selvaraj A, Balamurugan K, Yepiskoposyan H, Zhou H, Egli D, Georgiev O, Thiele DJ, Schaffner W. (2005) Metal-responsive transcription factor (MTF-1) handles extremes, copper load and copper starvation, by activating different genes. Genes Dev 19:891-896

Seok SH, Park JH, Baek MW, Lee HY, Kim DJ, Uhm HM, Hong JJ, Na YR, Jin BH, Ryu DY (2006) Specific activation of the human HSP70 promoter by copper sulfate in mosaic transgenic zebra fish. J Biotechnol 126:406-413

Serrano A, Rivas J, Losada M (1984) Purification and properties of GSH reductase from the cyanobacterium *Anabaena sp*. Strain 7119. J Bacteriol 158: 317–324

Shao HB, Chu LY, Ni FT, Guo DG, Li H , Li WX (2010) Perspective on Phytoremediation for Improving Heavy Metal-Contaminated Soils. Pl Adapt Phytoremed 2:227-244

Shao HB, Chu LY, Jaleel CA, Manivannan P, Panneerselvam R, Shao MA (2009) Understanding water deficit stress-induced changes in the basic metabolism of higher plants - biotechnologically and

sustainably improving agriculture and the ecoenvironment in arid regions of the globe. Crit Rev Biotech 29:131–151

Shao HB, Chu LY, Lu ZH, Kang CM (2008) Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Int J Biol Sci 4:8–14

Shao HB, Liang ZS, Shao MA, Sun Q, Hu ZM (2005) Investigation on dynamic changes of photosynthetic characteristics of 10 wheat (*Triticum aestivum* L.) genotypes during two vegetative-growth stages at water deficits. Biointerfaces 43:221–227

Sharma SS, Dietz KJ (2006) The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J Exp Bot 57:711-726

Shi QH, Zhu ZJ (2008) Effects of exogenous salicylic acid on manganese toxicity, element contents and antioxidative system in cucumber. Environ Exp Bot 63:317–326

Shi X, Dalal NS (1993) Vanadate mediated hydroxyl radical generation from superoxide radical in the presence of NADH:Heber Weiss versus Fenton mechanism. Biochem Biophys Acta 307: 336-341

Shimo H, Ishimaru Y, An G, Yamakawa T, Nakanishi H, Nishizawa NK (2011) Low cadmium (LCD), a novel gene related to cadmium tolerance and accumulation in rice. J Expt Bot 62(15):5727–5734

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6:410–417.

Singhal V, Rai JPN (2003) "Biogas production from water hyacinth and channel grass used for phytoremediation of industrial effluents". Biores technol 86:221225

Siomi H, Siomi C (2009) On the road to reading the RNA-interference code. Nature 457:396–404.

Smejikalova M, Milkanova O, Boruvka L (2003) Effects of heavy metal concentrations on biological activity of soil micro-organisms. Pl Environ 7: 321-326

Sobkowiak R, Rymer K, Rucińska R, Deckert J (2004) Cadmium-induced changes in antioxidant enzymes in suspension culture of soybean cells. Acta biochimica polinica 51: 219-222

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y (2003) Engineering tolerance and accumulation of lead and Cd in transgenic plants. Nat Biotechnol 21:914–919.

Spinoza-Quinones, FR, Zacarkim CE, Palacio SM, Obregon CL, Zenatti DC *et al*. (2005) Removal of heavy metal from polluted river water using aquatic macrophytes *Salvinia* sp. Braz J Physiol 35: 744-746

Srivastava S, Tripathi RD, Dwivedi UN (2004) Synthesis of phytochelatins and modulation of antioxidants in response to cadmium stress in *Cuscuta reflexa*—an angiospermic parasite. J Pl Physiol 161:665–674

Steffens JC, Hunt DF, Williams BG (1986) Accumulation of non-protein metal-binding polypeptides (gamma-glutamyl-cysteinyl)n-glycine in selected cadmium-resistant tomato cells. J Biol Chem 261:13879-13882

Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 18(2):321-326

Sugiyama A, Nishimura J, Mochizuki Y, Inagaki K, Sekiya J (2004) Homoglutathione synthesis in transgenic tobacco plants expressing soybean homoglutathione synthetase. Pl Biotechnol 21:79–83

Suñe N, Sánchez G, Caffaratti S, Maine MA (2007) Cadmium and chromium removal kinetics from solution by two aquatic macrophytes. Environ Pollut 145: 467-473

Sunkar R, Bartels D, Kirch HH (2003) Overexpression of a stress-inducible *aldehyde dehydrogenase* gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. Pl J35:452–464

Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNA from *Arabidopsis.* Pl Cell 16:2001–2019

Suzuki H, Koyanagi T, Izuka S, Onishi A, Kumagai H (2005) The *yliA*, *-B*, *-C*, and *-D* genes of *Escherichia coli* K-12 encode a novel GSH importer with an ATP-binding cassette. J Bacteriol 187:5861–5867

Szalai G, Kellos T, Galiba G, Kocsy G (2009) Glutathione as an antioxidant and regulatory molecule in plants under abiotic stress conditions. J Pl Growth Regul 28:66–80

Szczypka MS, Wemmie JA, Moye-Rowley WS, Thiele DS (1994) A yeast metal resistance protein similar to human cystis fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance-associated protein. J Biol Chem 269:22853-22857.

Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis* -related halophyte salt cress using *Arabidopsis* microarray. Pl Physiol 135:1697–1709

Takeda S, Matsuoka M (2008) Genetic approaches to crop improvement: responding to environmental and population changes. Nat Rev Genet 9:444–457

Tama´s L, Dudikova´ J, Durcekova´ K, Haluskova´ L, Huttova´ J, Mistrı´ KI, Olle´ M (2008) Alterations of the gene expression, lipid peroxidation, proline and thiol content along the barley root exposed to cadmium. J Pl Physiol 165:1193–1203

Tan K, Sather A, Robertson JL, Moy S, Roux B, Joachimiak A (2009) Structure and electrostatic property of cytoplasmic domain of ZntB transporter. Protein Sci 18:2043-2052

Tardieu F, Tuberosa R (2010) Dissection and modelling of abiotic stress tolerance in plants. Curr Opin Pl Biol 13:206-212

The UniProt (2009) The Universal Protein Resource (UniProt). Nucleic Acids Res 37: D169–D174

Thomashow MF (2001) So what's new in the field of plant cold acclimation? Lots! Pl Physiol 125:89–93

Tomas J, Cery J, Lahucky L, Musilova J (2008) Contamination of soil and alimentary wheat in Zemplin polluted area. Chemicke listy 102: 489-490

Tommasini R, Evers R, Vogt E, Mornet C, Zaman GH, Schinkel AH, Borst P, Martinoia E (1996) The human multidrug resistance-associated protein functionally complements the yeast Cd factor 1. PNAS USA 93:6743-6748

Tommasini R, Vogt E, Fromenteau M, Hortensteiner S, Matile P, Amrhein N, Martinoia E (1998) An ABC-transporter of *Arabidopsis thaliana* has both GSH-conjugate and chlorophyll catabolite transport activity. Pl J 13:773–780

Tommasini R, Vogt E, Schmid J, Fromenteau M, Amrhein N, Martinoia E (1997) Differential expression of genes coding for ABC-transporters after treatment of *Arabidopsis thaliana* with xenobiotics. FEBS Lett 411:206-210

Tong YP, Kneer R, Zhu YG (2004) Vacuolar compartmentalization: a second-generation approach to engineering plants for phytoremediation. Trends in Pl Sci 9(1): 7-9

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early response to dehydration stress 1 promoter. Pl Cell 16:2481–2498

Tran LS, Nakashima K, Sakuma Y, Osakabe Y, Qin F, Simpson SD, Maruyama K, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K (2006) Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in *Arabidopsis*. Pl J 49:46–63

Uenishi R, Gong P, Suzuki K, Koizumi S (2006) Cross talk of heat shock and heavy metal regulatory pathways. Biochem Biophys Res Commu 341(4):1072-1077

Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Curr Opin Biotechnol 17:113–122

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shi.nozaki K (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscissic acid dependent signal transduction pathway under drought and high-salinity. PNAS USA 97:11632–11637

Uraguchi S, Fujiwara T (2012) Cadmium transport and tolerance in rice:perspectives for reducing grain cadmium accumulation. Rice 5:5

Urano K, Kurihara Y, Seki M, Shinozaki M (2010) 'Omics" analyses of regulatory networks in plant abiotic stress responses. Curr Opin Plant Biol 13:1-7

USDA (2004) United States Department of Agriculture, Plants database

USEPA (1997) Innovative uses of compost Bioremediation and pollution prevention. PNAS USA 121:404-427

Valko M, Morris H, Conin MT (2005) Metals, toxicity and oxidative stress. Curr Med Chem 12:1161–1208

Van Assche F, Clijsters H (1986) Inhibition of photosynthesis in *Phaseolus vulguris* by treatment with toxic concentration of Zn effect on ribulose1,5 bisphosphate carboxylase/oxygenase. J Pl Physiol 125:335-360

Van Hoof NALM, Hassinen VH, Hakvoort HWJ, Ballintijn KF, Schat H, Verkleij JAC, Ernst WHO, Karenlampi SO, Tervahauta AI (2001) Enhanced copper tolerance in *Silene vulgaris*(Moench) Garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. Pl Physiol 126 1519–1526

Van Vliet C, Anderson CR, Cobbett CS (1995) Copper-sensitive mutant of *Arabidopsis thaliana*. Pl Physiol 109:871–878

Vatamaniuk OK, Bucher EA, Sundaram MV, Rea PA (2005) CeHMT-1, a putative phytochelatin transporter, is required for cadmium tolerance in *Caenorhabditis elegans*. J Biol Chem 280:23684–23690

Vatamaniuk OK, Mari S, Lu YP, Rea PA (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis* : isolation and *in vitro* reconstitution. PNAS USA 96:7110-7115

Vatamaniuk OK, Mari S, Lu YP, Rea PA (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase. J Biol Chem 275:31451–31459

Vergani L, Lanza C, Scarabelli L, Canesi L, Gallo G (2009) Heavy metal and growth hormone pathways in metallothionein regulation in fish RTH-149 cell line. Comp Biochem Physiol, Part C 149: 572–580

Vierstra RD (2009) The ubiquitin–26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol 10:385–397

Villiers F, Ducruix C, Hugouvieux H et al (2011) "Investigating the plant response to cadmium exposure by proteomic and metabolomic approaches," Proteomics 11(9):1650–1663

Vitoria AP, Lea PJ, Azevedo RA (2001) Antioxidant enzymes responses to Cd in radish tissues. Phytochem 57:701–710

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG and Thomashow MF (2005) Roles of the CBF2 and ZATI2 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. Pl J 41:195–211

Vollmannova A, Tomas J, Bajcan D, Kovacak P (2008) Soil hygiene in old environmental burden areas. Chemicke listy 102: 519-520

Wang BB amd Brendel V(2006) Genome-wide comparative analysis of alternative splicing in plants. PNAS USA 103: 7175-7180.

Wawrzyński A, Kopera E, Wawrzyńska A, Kamińska J, Bal W, Sirko A (2006) Effects of simultaneous expression of heterologous genes involved in phytochelatin biosynthesis on thiol content and cadmium accumulation in tobacco plants. J Expt Bot 57:2173–2182

Weber M, Trampczynska A, Clemens S (2006) Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd-hypertolerant facultative metallophyte *Arabidopsis halleri*. Pl Cell Environ 29:950–963

Wei W, Chai T, Zhang Y, Han L, Xu J, Guan Z (2009) The *Thlaspi caerulescens* NRAMP homologue TcNRAMP3 is capable of divalent cation transport. Mol Biotechnol 41: 15-21

Weston DJ, Gunter LE, Rogers A, Wullschlerger SD (2008) Connecting genes, coexpression modules, and molecular signatures to environmental stress phenotypes in plants. BMC Syst Biol 2:16

Wildner GF, Henkel J (1979) The effect of divalent metal ion the activity of Mg^{2+} depleted ribulose1,5 bisphosphate oxygenase. Planta 146:223-228

Williams GT, Morimoto RI (1990) Maximal stress-induced transcription from the human HSP70 promoter requires interactions with the basal promoter elements independent of rotational alignment. Mol Cell Biol 10:3125-3136

Williams LE, Mills RF (2005) P1B-ATPases-an ancient family of transition metal pumps with diverse functions in plants. Trends Pl Sci 10:491–502

Williams LE, Pittman JK, Hall JL (2000) Emerging mechanisms for heavy metal transport in plants. Biochem Biophys Acta 1465: 104–126

Williams JB (2002) "Phytoremediation in wetland ecosystems: progress, problems and potential". Crit Rev Pl Sci 21:607635

Winston F, Dollard C, Ricupero-Hovasse SL (1995) Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast 11: 53-55

Wojtaszek P(1997) Oxidative burst: an early plant response to pathogen infection. Biochem J 322:681–692

Wolf AM, Asoh S, Ohsawa I, Ohta S (2008) Imaging mitochondrial redox environment and oxidative stress using a redox-sensitive fluorescent protein. J Nippon Med Sch 75:66–67

Xian X, Shokohifard G (1989) Effect of pH on chemical forms and plant availability of cadmium, zinc and lead in polluted soils. Water Air Soil Pollut 45: 265-273

Xie Y, Wu R (1989) Rice alcohol dehydrogenase genes: anaerobic induction, organ specific expression and characterization of cDNA clones. Pl Mol Biol 13: 53-58

Xing S, Lauri A, Zachgo S (2006) Redox regulation and flower development: a novel function for glutaredoxins. Pl Biol 8:547–555

Xiong J , Fu g , Tao T, Zhu C (2010) Roles of nitric oxide in alleviating heavy metal toxicity in plants. Arch Biochem Biophy 497(1-2):13–20

Xu J, Zhang Y (2010) How significant is a protein structure similarity with TM-score=0.5? Bioinformatics 26:889-895

Yadav SK (2010) HMs toxicity in plants: An overview on the role of GSH and phytochelatins in heavy metal stress tolerance of plants. South Afric J Bot 76:167–179

Yamada H, Kiozumi S (2001) Lymphocyte matallothionein-mRNA as a sensitive biomarker of cadmium exposure. Ind Health 39:29–32

Yamada K, Lim J, Dale JM et al. (2003) Empirical analysis of transcriptional activity in the *Arabidopsis* genome. Science 302:842–846

Yamaguchi-Iwai Y, Serpe M, Haile D (1997) Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. J Biol Chem 272:17711–17718

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Pl Biol 57:781–803

Yang T, Poovaiah BW (2003) Calcium/calmodulin mediated signal network in plants. Trend Pl Sci 8:505-512

Yang YY, Jung JY, Song WY, Suh HS, Lee Y(2000) Identification of rice varieties with high tolerance or sensitivity to lead and characterization of the mechanism of tolerance. Pl Physiol 124:1019–1026

Yazaki K (2006) ABC transporters involved in the transport of plant secondary metabolites. FEBS Letters 580:1183–1191

Yeh CM, Chien PS, Huang HJ (2007) Distinct signaling pathways for induction of MAP kinase activities by cadmium and copper in rice roots. J Expt Bot 58(3):659-671

Yu GY, Wang GP, He CG (2000) Comparison of physiological responses to oxidative and heavy metal stress in seedlings of rice paddy, *Oryza sativa* L. J Environ Sci 12: 458-462

Zago E, Morsa S, Dat JF, Alard P, Ferrarini A, Inzé D, Delledonne M, Breusegem FV (2007) Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. Pl Physiol 141:404–411

Zagursky RJ, Haus JB (1983) Expression of the phage lambda recombination genes exo and bet under lacPO control on a multi-copy plasmid. Gene 23:277-292

Zawoznik, MS, Groppa MD, Tomaro ML, Benavides MP (2007) Endogenous salicylic acid potentiates cadmium-induced oxidative stress in *Arabidopsis thaliana*. Pl Sci 173:190–197

Zechmann B, Mauch F, Sticher L, Muller M (2008) Subcellular immunocytochemical analysis detects the highest concentrations of glutathione in mitochondria and not in plastids. J Expt Bot 59(14):4017–4027

Zeller G, Henz SR, Widmer CK, Sachsenberg T, Rätsch G, Weigel D, Laubinger S (2009) Stress-induced changes in the *Arabidopsis thaliana* transcriptome analyzed using whole-genome tiling arrays. Pl J 58:1068–1082

Zengin FK, Munzuroglu O (2006) Toxic effects of cadmium (Cd^{++}) on metabolism of sunflower (*Helianthus annuus* L.) seedlings. Acta Agricl Scand Sect. B, Pl Soil Sci 56:224-229

Zenk MH (1996) Heavy metal detoxification in higher plants: a review. Gene 179:21–30.

Zhang Y (2003) I-TASSER server for protein 3D structure prediction. BMC Bioinformatics 9:40

Zhang Y (2008) Progress and challenges in protein structure prediction. Curr Opin Struct Biol 18:342–8

Zhang Y, Skolnick J (2004) Scoring function for automated assessment of protein structure template quality. Proteins 57: 702-710

Zhang Y, Skolnick J (2005) TM-align: A protein structure alignment algorithm based on TM-score. Nucleic Acids Res 33: 2302-2309

Zhang ZH, Zheng ZL, Gao HY, Cao HX (1988) Breeding, evaluation and utilization of anther cultured varieties "Zin Xion" and "Hua Han Zoo" in rice (*Oryza sativa* L.). *In* : IRRI and Academia Sinica (eds.) Genetic manipulations in crops, Cassel Tycooly, 36-37

Zhao H, Eide D (1996) The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. PNAS USA 93:2454-2458

Zhou GK, Xu YF, Lin JY (2005) Characterization of a rice class II metallothionein gene: tissue expression patterns and induction in response to abiotic factors. J Pl Physiol 162:686-696

Zhou J, Goldsbrough PB (1994) Functional homologs of fungal metallothionein genes from *Arabidopsis*. Pl Cell 6:875–884

Zhu JK (2000) Genetic analysis of plant salt tolerance using *Arabidopsis.* Pl Physiol 124(3):941–948

Zhu LH, Pilon-Smits EAH, Tarun AS, Weber SU, Jouanin L, Terry N (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Pl Physiol 121:1169–1177

Zhu SY, Yu XC, Wang XJ, Zhao R, Li Y, Fan RC, Shang Y, Du SY, Wang XF, Wu FQ, Xu YH, Zhang XY, Zhang DP (2007) Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in *Arabidopsis*. Pl Cell 19:3019–3036

Zivy M, Thiellement H, de Vienne D, Hofmann JP (1983) Study on nuclear and cytoplasmic genome expression in wheat by two-dimensional electrophosis studies of esterases and peroxidases for species, hybrids and amphidiploid in the genus *Nicotiana*. Bot Gazt 132:158-166

Zurbriggen MD, Hajirezaei MR, Carrillo N (2010) Development of transgenic plants with enhanced tolerance to adverse environments. Biotechnol Genet Eng Rev 27:33-56

SUMMARY

In this present study, water hyacinth (*Eichhornia crassipes*), a heavy metal (HM) hyperaccumulator plant capable of growing in HMs contaminated areas has been investigated. Attempts were made to identify the molecular mechanism of adaptation to toxic metals and hyperaccumulation in water hyacinth by cloning and characterizing a gene related to metal detoxification. The overall outcomes of the present study can be summarized as follows:

- The preliminary study on the XRD and EDX analysis of the soil samples revealed the presence of Fe, Mn, Cu, Zn, Hg, Cr, Pb, Ni, Co, As and Cd. Additionally, AAS study of soil and water ascertained the contamination of HMs that were in Pb>Cr>Cu>Ni>Cd and Pb>Cr>Ni>Cu>Cd order in soil and water respectively. The AAS analysis of plant samples quantized that HMs Pb, Cr, Ni, Cu and Cd accumulated to higher extent. The discrete variation in leaf protein profile during stress may suggested the synthesis of specific stress responsive proteins playing crucial role in the stress response. High expression of alcohol dehydrogenase (ADH), peroxidase (POX) and altered regulation of esterase (EST) were observed in root tissue during stress. The enhanced accumulation of metals in *Eichhornia* and altered regulation in proteins and enzymes indicated this plant an ideal organic indicator of HM contamination.
- The HM stress responsive gene (*Echmr*) was isolated from *E. crassipes*. The bioinformatic analysis revealed the presence of various STREs viz., DRE, ABRE, HSE and Calmudilin binding element, and HM binding motifs in EcHMR, which suggested its possible role in abiotic stress tolerance. The hydrophobic nature of EcHMR along with diverse alpha sheets in C terminal shows similarity with known HM binding proteins. The analysis also revealed that EcHMR protein has predicted molecular and biological function of metal ion transmembrane transporter activity with predicted binding site of EcHMR closest to crystal structure of the CorA Mg^{2+} transporter and Ca^{2+} ATPase pump crystal structure with its predicted location at at cell periphery or intrinsic to membrane.
- The expression of *Echmr* in Cd sensitive Δ*gsh E.coli* mutants functionally complemented and conferred HMs stress tolerance. The EcHMR was found to impart tolerance to other abiotic stresses such as heat, cold, UV apart from providing tolerance to Cd and Pd. The expression analysis of *Echmr* also confirmed the upregulation of the gene expression during above stresses advocates its role in multi-stress tolerance. The *Echmr* gene from *Eichhornia* could

rescued Cd^{2+} sensitive *E. coli* mutants from HM toxicity, heat shock, cold and UV stress which indicated that *Echmr* is functional.

- The functional validation of *Echmr* gene in Cd^{2+} sensitive yeast Δ*ycf1* mutant and FY3 cells complemented the cells to survive in the excess of Cd^{2+} and *in vitro* conditions of 4% polyethylene glycol. This complemenmtation suggested that the *Echmr* enhances Cd(II) and desiccation tolerance in yeast *ΔYcf1* mutant and FY3 cells through the possible mechanisms of cation transport.
- The subcellular localization of the *Echmr* gene was studied in *Arabidopsis* protoplast which revealed its localization within the plasma membrane or intrinsic to membrane and cytosol as predicted by the bioinformatics tools. The localization results possibly supported the hypothesis of EcHMR having transmembrane transporter activity and this implied that the regulation of *Echmr* and its integrated functions may have crucial role in the stress tolerance responses of plants in hostile environments.

The present endeavour has revealed that *Echmr* gene from *Eichhornia* possess the potential to deliver tolerance against HM and other abiotic stresses and could be a vital candidate in genetic engineering-based strategies to develop HM tolerant crop plants for remediation of polluted soils. The varied HM stress induced proteins expression suggests that a coordinated network of proteins and genes work together to finally reach at HM response. This require effort from all the specialized laboratories and institutes throughout the world to work as unit to converge at a point of the master regulator, which can be modulated to achieve HM stress tolerance.