

Heavy metal induced gene expression in Brassicaceae

Lamija Subasic, Haris Gavranovic, Imer Muhovic and Abdul Razaque Memon*

Department of Genetics and Bioengineering, Faculty of Engineering and Information Technologies, International Burch University, 71000 Sarajevo, Bosnia and Herzegovina

E-mail: armemon@ibu.edu.com

Abstract

Plants require at least 14 mineral elements for their nutrition. These include the macronutrients nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) and the micronutrients boron (B), chlorine (Cl), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) and molybdenum (Mo). These are generally obtained from the soil. Crop production is often limited by low bioavailability of essential mineral elements and/or the presence of excessive concentrations of potentially toxic heavy metals, such as Fe, Mn, Cu, Cr, Cd, Pb, Zn and Al in the soil solution. In the past few years, responses of plants to heavy metals have received increasing attention. On one hand due to industrial activities, toxic heavy metals such as Cd, Zn, Cu, Cr, Pb have been released into the biosphere and represent a widespread environmental pollution. High concentrations of heavy metals in the soil can inhibit plant growth and reduce crop yields, which can affect sustainable development severely. In order to study the molecular response of plants to heavy metals, the gene expression data of model crop plants especially in Brassicaceae family were analyzed by searching several databases available online. In the first part of this work the publicly available online resources for these plants from websites such as <http://www.ncbi.nih.gov>, <http://www.tigr.org>, <http://www.brassica.info>, and related sites were searched to collect nucleotide sequences that encode heavy metal ATPases and transporter protein homologues. The second part of this work focuses on the expression of these genes in plants grown at different concentrations of Cu, Zn, and Cd. Real time PCR (RT-PCR) experiments will be carried out to analyze the expression of these genes in roots and shoots of *B. nigra* and *B. juncea* treated with different concentrations of metals.

Keywords: *Arabidopsis thaliana*, Brassicaceae, phylogenetic tree, Metal ATPases, phytoremediation

1.INTRODUCTION

Phytoremediation uses green plants to clean up toxic amount of inorganic and organic pollutants from the environment. The rapid industrialization and urbanization of many developed and recently developing countries (for example China, India, Brazil etc) have increased heavy metal and organic pollution in the environment (Memon and Schroder, 2009). Hyperaccumulation as a phenomenon has attracted growing attention in the past decade. Exploiting hyperaccumulating plant species, and identifying metal accumulation

genes is currently focal point for phytoremediation or phytomining (Ozturk et al 2012). Among limited number of plant species 400 are classified as heavy accumulator plants (Baker et al., 2000). Among them Arabidopsis hallari, Thlaspi, Brassica nigra, and Brassica juncea have been most studied (Memon and Schroder, 2009). Those plant species have the ability to accumulate extremely high amounts of heavy metals in their leaves (Brown et al., 1995). The plants absorb contaminants through the root system and store them in the root biomass and/or transport them up into the stems and/or leaves. A living plant may continue to absorb contaminants until it is harvested. After harvest, a lower level of the contaminant will remain in the soil, so the growth/harvest cycle must usually be repeated through several crops to achieve a significant cleanup. After the process, the cleaned soil can support other vegetation. Heavy metals that have been identified in the polluted environment include As, Cu, Cd, Pb, Cr, Ni, Hg and Zn. Different sources of heavy metals are listed in Table 1.

Table 1. Different sources of heavy metal contamination.

Heavy metals	Sources
As preservatives, animal feed plants, herbicides, volcanoes, mining and smelting	Semiconductors, petroleum refining, wood additives, coal power,
Cu biosolids	Electroplating industry, smelting and refining, mining
Cd fossil fuel burning, phosphate fertilizers, sewage sludge	Geogenic sources , anthropogenic activitie,s metal smelting and refining, application of
Cr tanneries	Electroplating industry, sludge, solid waste,
Pb of leaded gasoline, wastes enriched in Pb, paints	Mining and smelting of metalliferous ores, burning municipal sewage, industrial
Hg industries producing caustic	Volcano eruptions, forest fire, emissions from soda, coal, peat and wood burning

Se fuels, glass manufacturing (e.g., varnish, pigment formulation)	Coal mining, oil refining, combustion of fossil industry, chemical synthesis
Ni bursting and gas exchange soils and geological materials	Volcanic eruptions, land fill, forest fire, bubble in ocean, weathering of
Zn mining, biosolids	Electroplating industry, smelting and refining,

Different approaches have been used or developed to mitigate the heavy metal polluted soils. The metal ion accumulated in the aerial parts that can be removed to dispose or burnt to recover metals. The results indicate that many *Brassica* sp. such as *B. juncea* L., *B. juncea* L. Czern, *B. napus* L. and *B. rapa* L. exhibited moderately enhanced Zn and Cd accumulation. According to Brooks (1998) and Baker (2000) they were also found to be most effective in removing Zn from the contaminated soils. The plant species that have been identified for remediation of soil include either high biomass plants such as willow (Landberg and Greger, 1996) or those that have low biomass but high hyperaccumulating characteristics such as *Thlaspi* and *Arabidopsis* species (Memon et al 2000, Memon et al. 2008).

The main objective of this study is to identify genes responsible for hyperaccumulation of heavy metals such as Zn, Cu and Cd in Brassicaceae family species, namely *Brassica*, do comparative phyletic analysis among different species and characterize metal induced gene expression. The present study aims at finding homologues of heavy metal ATPases among species mentioned that might possess same specific functional similarities. Moreover, this study aids to resolve many problems of soil pollution and enhance soil clean-up methods by using fast growing and high biomass accumulator plant species. The main components of this study are: i) retrieval of heavy metal ATPase nucleotide sequences from Brassicaceae family species, *H.sapiens* and *S.cerevisiae* by searching through all sequence databases; ii) multiple alignment of found sequences, iii) phyletical analysis of heavy metal ATPases genes, where the main gene source was *A.thaliana*, compared with Brassicaceae, *H.sapiens* and *S.cerevisiae* and other related organisms with the objective of finding motifs with high or identical functional similarity.

2. Materials and Methods

2.1.Retrieval of sequences

In this study DNA sequences were retrieved from websites as a molecular evidence to classify organisms. Several publicly available online data resources were used including: <http://arabidopsis.org/> (TIGR, The Institute for Genomic Research); <http://www.ncbi.nlm.nih.gov/> (GenBank); <http://plantgdb.org/> (PGD, Plant Genome Database), <http://srs.ebi.ac.uk> (EMBL-EBI). *Homo sapiens* and yeast sequences were acquired from <http://www.ncbi.nlm.nih.gov/>. and <http://www.yeastgenome.org/> (SGD, *Saccharomyces* Genome Database), respectively. All the sequences were downloaded in FASTA format and all databases were screened for heavy metal ATPase gene homologues by

employing the BLAST algorithm (Blastn and discontinuous megablast for nucleotide databases). Discontinuous megablast as a version of megablast is used to compare slightly diverged sequences, especially sequences from different organisms, which have alignments with low degree of identity. For the initial screening, Arabidopsis, human and yeast heavy metal ATPases nucleotide sequences were used and every database scanned for the E-value of sequences of $<10^{-7}$. Phylogenetic tree was constructed from DNA sequences by using Java applet JalView. Firstly, genes responsible for hyperaccumulation of heavy metals, heavy metal ATPases were collected from www.arabidopsis.org. The sequence databases were also searched using keywords for heavy metal ATPases. In total seven of the gene nucleotide sequences from Arabidopsis thaliana were taken and put for further analyses. The identified genes from these databanks are: HMA1 (Heavy metal ATPase 1); copper-exporting ATPase, HMA2 (Heavy metal ATPase 2); cadmium-transporting ATPase, HMA3 (Heavy metal ATPase 3); ATPase, coupled to transmembrane movement of ions, phosphorylative mechanism, HMA4 (Heavy metal ATPase 4); cadmium-transporting ATPase, HMA5 (HEAVY METAL ATPASE 5); ATPase, coupled to transmembrane movement of ions, phosphorylative mechanism, PAA1 (metal-transporting P-type ATPase 1), PAA1 (metal-transporting P-type ATPase 1); ATPase, coupled to transmembrane movement of ions, phosphorylative mechanism. Subsequently, based on those identified genes sequences, nucleotide sequences for other organisms such as Brassica, Saccharomyces cerevisiae and human were collected from <http://srs.ebi.ac.uk> and <http://www.ncbi.nlm.nih.gov/> for the purpose of finding homologue sequences. After the set of related sequences were obtained, we proceeded further by using BLAST tool form NCBI website to find regions of local similarity between sequences.

2.2. Multiple alignment and phylogenetic tree construction

A total of 134 heavy metal ATPase sequences were multiply aligned by utilizing ClustalW program in order to construct phylogenetic tree. Construction of phylogenetic tree is the most convenient method to represent the significant relation among obtained sequences. The purpose of our research is to study the sequences of gene family where all sequences share the same common ancestor. Thus by implementing phylogenetic trees we will ensure that the heavy metal accumulator genes are orthologous to another well-characterized gene in another species. Two genes that are orthologous often have the same exact function (have similar roles) in the two different organisms they come from. In order to construct phylogenetic tree sequences of genes need to be aligned. In multiple sequence alignment the nucleotide sequences are being overlapped so similar features end up in the same column. The idea behind a multiple alignment is to put nucleotides or amino acids in the same column because they are very similar according to some criterion. There are four major criteria to build a multiple alignment of sequences that all have different properties. These four criteria are as follows: structural, evolutionary, functional and sequence similarity. While the first three criteria have a clear biological meaning, the fourth one does not. When the sequences are closely related, their structural, evolutionary, and functional similarities are equivalent to sequence similarity.

The criterion observed in this research is that the sequences of different metal induced genes have functional and evolutionary similarities among species. Our hypothesis is that the functionally related sequences of the genes from different species or organisms will be having conserved pattern or motif which will be possibly related to hyperaccumulation of heavy metals.

3.Results and Discussion

3.1.Heavy metal ATPase homologues

Three phylogenetic trees were constructed for collected heavy metal ATPase nucleotide sequences. In Fig.1. a total of 27 gene nucleotide sequences were obtained from 2 different organism: 24 plants and 3 human species after scanning of major sequence databases. The majority of these sequences belonged to *Arabidopsis thaliana*. Mainly mRNA sequences were taken for multiple alignment and construction of phylogenetic tree by using Neighbour Joining method in Jalview software.

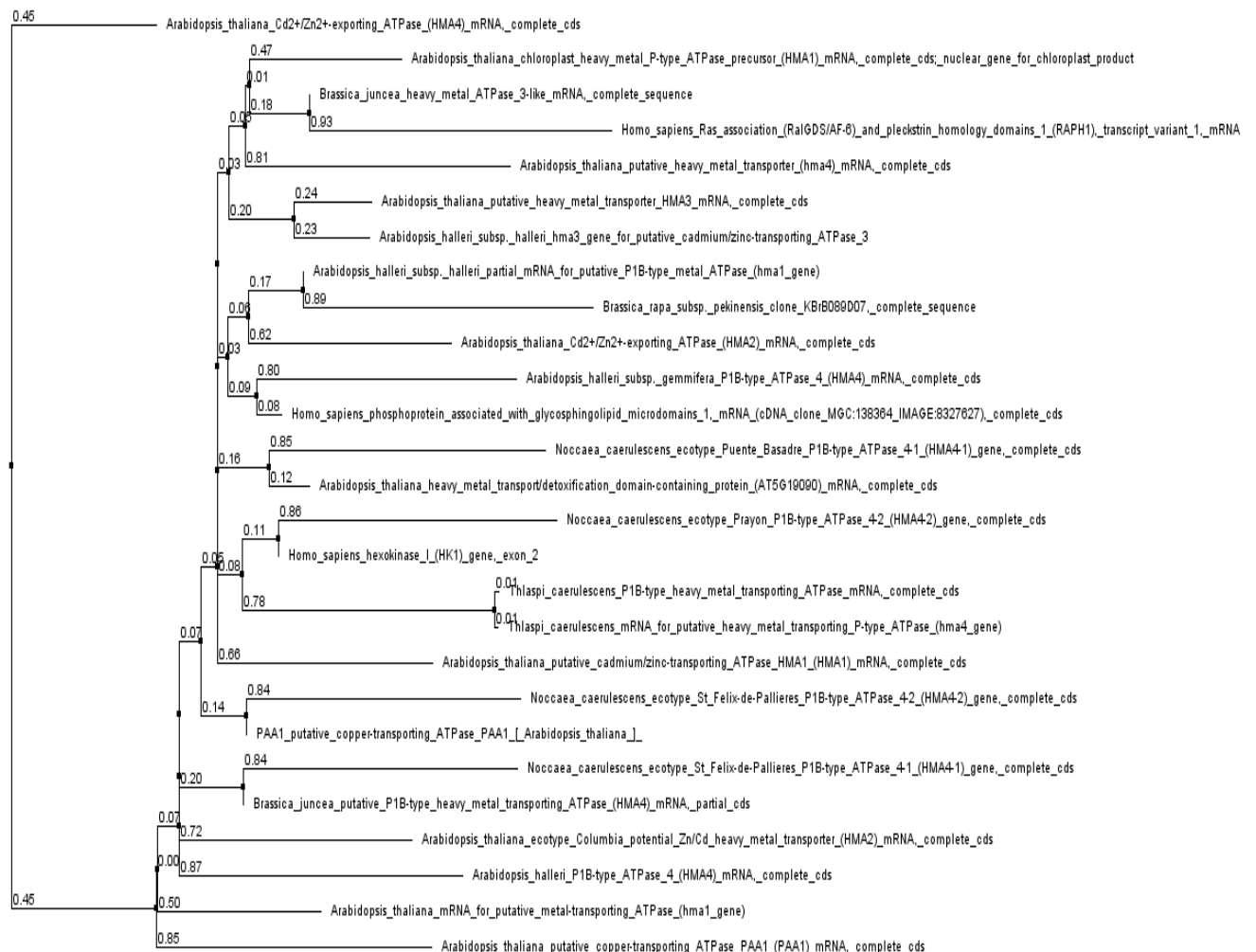


Fig.1. Linear dendrogram presenting a phylogenetic tree for metal accumulating genes.

In one study a total of 35 homologues gene nucleotide sequences were obtained from 19 different organisms: 2 plants (8 *Arabidopsis thaliana* and 3 *Oryza sativa Japonica* Group), 2 *Mus Musculus*, 2 *Rattus norvegicus*, *Caenorhabditis elegans*, 2 *Canis lupus familiaris*, *Bos taurus*, *Gallus gallus*, *Saccharomyces cerevisiae*, *Macaca mulatta*, *Anopheles gambiae* pest, *Kluyveromyces lactis*, *Pan troglodytes*, *Homo sapiens*, *Schizosaccharomyces pombe*, *Danio rerio*, *Neurospora crassa*, *Magnaporthe oryzae* and *Drosophila melanogaster* after scanning of major sequence databases. The majority of heavy metal ATPase sequences belonged to *Arabidopsis thaliana*. Mainly mRNA sequences were taken for multiple alignment and construction of phylogenetic tree by using Neighbour Joining method in Jalview software.

In another study a total of 72 homologues gene nucleotide sequences were obtained from 14 different organisms: plants (*Arabidopsis thaliana*, *Hordeum vulgare*, *Glycine max*, *Oryza sativa japonica*, *Oryza sativa indica*, *Noccaea caerulescens*, *Thlaspi caerulescens*, *Triticum aestivum*, *Sorghum bicolor*, *Populus trichocarpa*, *Medicago truncatula*, *Picea glauca*, *Solanum tuberosum*, *Hirschfeldia incana*, *Brassica juncea*, *Brassica napus*, *Vitis vinifera*, *Zea mays*, *Thellungiella halophila*, *Physcomitrella patens ssp patens*, *Selaginella moellendorffii*, *Solanum lycopersicum*, *Brachypodium distachyum*, *Sedum alfredii*, *Ricinus communis*), *Pan troglodytes*, *Pongo abelii*, *Macaca mulatta*, *Rattus norvegicus*, *Equus caballus*, *Bos taurus*, *Sparus aurata*, *Drosophila melanogaster*, *Drosophila erecta*, *Chlamydomonas reinhardtii*, *Trichoplax adhaerens*, *Candida albicans*, *Saccharomyces cerevisiae* and *Homo sapiens* after scanning of major sequence databases. The majority of heavy metal ATPase sequences belonged to *Arabidopsis thaliana* (8), and *Medicago truncatula* (6). Mainly mRNA sequences were taken for multiple alignment and construction of phylogenetic tree by using Neighbour Joining method in Jalview software. From the comparative analyses of phylogenetic trees orthologous heavy metal ATPase genes were identified from model crop plants in Brassicaceae family, such as *Arabidopsis thaliana*, *Brassica juncea*, *Brassica napus*, *Noccaea caerulescens* and *Thlaspi caerulescens*. Phylogenetic tree is comprised of: leaves or OTUs (Operational Taxonomic Units), nodes which represent an ancestral OUT, clade (a group of OTUs that includes several sequences and their common ancestor nodes), branch which defines the relation between a clade or an OTU and the rest of the tree and root which is the common ancestor of all the OTUs. Phylogenetic trees were built with distance methods by grouping OTUs according to overall similarity. These phylogenetic trees are unscaled, where branch length does not have any special meaning in terms of evolutionary time. On the other hand it indicates of orthologous heavy metal ATPase genes across different species.

Conclusion

Great efforts have been made in the last two decades to reduce pollution sources and remedy the polluted soil and water resources. Phytoremediation, being more cost-effective and fewer side effects than physical and chemical approaches, has gained increasing popularity in both academic and practical circles. Recent advances in biotechnology will play a promising role in the development of new hyperaccumulators by identifying a specific metal genes and transferring metal hyperaccumulating genes from low biomass wild species to the higher biomass producing cultivated species in the times to come. This can play a significant role in the extraction of heavy metals from the polluted soils and aid sustainable environmental development. Phytoextraction as a way of phytoremediation is environmental friendly, and causes no harm to soil quality. Moreover, it is less expensive than any other clean-up process. It takes more time than other clean-up techniques, but on the other hand its benefits certainly outweigh the time-consuming process, since it is related to plants. Although, investigations are needed to develop new methods for effective recovery of metals from the hyperaccumulator plant biomass.

REFERENCES

- Memon, A.R. and Schröder, P. 2008. Implication of metal accumulation mechanisms to phytoremediation. *Environ. Sci. Pol. Res. (ESPR)*. 16: 162-175.
- Ozturk, M., Memon, A. R. , Gucel, S. , and Dogan, Y. 2012. *Brassicas in Turkey and their Possible Role in the Phytoremediation of Degraded Habitats*. Springer-Verlag.

Memon et al 2008. Metal accumulation in crops- Human health issues, In : Trace Elements-Nutritional benefits, environmental contamination, and health implications, Ed. M. N. V. Prasad, John Wiley & Sons pp. 81-97

Memon, A. R., Aktopraklıgil, D., Özdemir, A., and Vertii, A. 2000. Gene expression of heavy metal stress protein in plants. Turkish J. Botany 25, 111-121

Memon, A. R., Yildizhan Y. and Keskin, B. C. Phytoremediation of heavy metals from contaminated areas of Turkey. 4th European Bioremediation Conference, Sept 3-6, Chania, Crete, Greece, ID04 pp1-4, ISBN 978-960-8475-12-0.

Baker, A.J.M., McGrath, S.P., Reeves, R.D., Smith, J.C.A. (2000). Metal Hyperaccumulator Plants: A Review of the Ecology and Physiology of a Biological Resource for Phytoremediation of Metal-Polluted Soils. In: Terry, N., Banuelos, G. Eds. Phytoremediation of Contaminated Soils and Water. Boca Raton, Florida, USA.

Brown, S.L., Chaney, R.L., Scott Angle, J. (1995). Zinc and cadmium uptake by hyperaccumulator thlaspi-caerulescens grown in nutrient solution. Soil Sci. Soc. Am. J., 59: 125-133.

Claverie, J.M. Cedric Notredame (2007). Bioinformatics For Dummies (2nd). Indianapolis, Indiana: Wiley Publishing, Inc.

Landberg, T., Greger, M. (1996). Differences in uptake and tolerance to heavy metals in Salix from unpolluted and polluted areas. Applied Geochem., 11(1-2):175-180.

Brooks, R.R., 1998. Plants that Hyperaccumulate Heavy Metals. CAN International, Wallington, p.379.