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Comparative Structural Analysis of Heavy Metal ATPases in *Arabidopsis thaliana, Arabidopsis halleri, Brassica rapa,* and *Brassica juncea*[#]

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ABSTRACT

Arabidopsis thaliana has eight genes encoding members of the type P_{1B} heavy metal–transporting ATPase, subfamily of the P-type ATPases. We focused our study on four ATPases, mainly HMA1, HMA2, HMA3, and HMA4, which are closely related and most similar in their sequences. We carried out the bioinformatics analysis of these metal ATPases and obtained their structure in *A. thaliana*, A. *halleri*, and the other heavy metal accumulators in *Brassica* spp. *A. thaliana* is a model plant for research because of the duplications and other evolutionary events. These evolutionary events provided a chance to elucidate their regulation and function in the cell. All previous bioinformatics analyses have given some information about their structure, but not much work has been done on their structural components and interactome analysis. Experimental determination of 3D structures is essential to understand better these proteins' function, which is crucial for the proper functioning of all plant cellular processes. Especially, docking sites and domains need to be worked out to understand the role of these transporter proteins and their interaction in plant cells. These bioinformatic analyses will help the researcher understand these ATPases' role in detoxifying the toxic metals from the cells of accumulator plants. Further research on gene cloning, gene expression, and generating new accumulator plants for phytoremediation is needed to reclamation polluted soils from toxic heavy metals.

Türk Tarım – Gıda Bilim ve Teknoloji Dergisi, 10(sp2): 2988-2995, 2022

Arabidopsis thaliana, Arabidopsis halleri, Brassica rapa ve *Brassica juncea*'daki Ağır Metal ATPaz'ların Karşılaştırmalı Yapısal Analizi

MAKALE BİLGİSİ

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Hiperakümülatör bitkiler
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ÖZ

Model bir bitki olan *A. thaliana*, P-tipi ATPaz'ın alt ailesi olan P_{1B}-tipi ATPazın sekiz üyesine sahiptir. *Arabidopsis thaliana*, duplikasyonlar ve diğer evrimsel olaylar nedeniyle HMA'ların ve diğer birçok proteinin araştırılmasında tercih edilmektedir. Bu evrimsel olaylar, HMA'ların düzenlenmesinde ve hücredeki işlevlerinin açıklanmasında önemli rol oynamaktadır. Önceki biyoinformatik araştırmalarda, HMA'ların bazı yapıları hakkında bilgi mevcut olmasına rağmen, bunların yapısal bileşenleri ve interaktomları üzerinde yeterli çalışma bulunmamaktadır. Bu çalışmada, bu sekiz üye arasından yakın ilişkili ve yüksek benzerlik oranına sahip olan HMA1, HMA2, HMA3 ve HMA4 üzerinde detaylı biyoinformatik analizi gerçekleştirilmiştir. *A. thaliana* ile aynı familyaya ait *A. halleri* ve bazı *Brassica*'nın akümülatör türlerinden HMA1-HMA4 dizileri elde edilerek biyoinformatik veritabanları aracılığıyla karşılaştırılmıştır. Bu biyoinformatik çalışmalar, akümülatör bitki hücrelerinden toksik metalleri detoksifiye etmedeki HMA'ların rolü hakkında bilgi vermektedir. Ayrıca, bitkide hücresel süreçlerin aktif bir şekilde sürdürülebilmesi için bu taşıyıcıların 3D yapılarının deneysel olarak belirlemek oldukça önemlidir. Bu bağlamda, bu taşıyıcıların yapısal analizleri bazı protein veritabanları kullanılarak yapılmıştır. Bu sonuçlar, toksik ağır metallerce kirlenmiş toprakların ıslahı için mevcut akümülatör bitkilerin üretimine ve ayrıca gen klonlama, gen ekspresyon bilgileri ile fitoremediasyon için uygun yeni akümülatör bitkilerin geliştirilmesine katkı sağlayacaktır.

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Introduction

Heavy metals (HMs) are classified into two categories: i) essential metals such as copper (Cu), iron (Fe), and zinc (Zn), and ii) non-essential metals such as cadmium (Cd), lead (Pb), and mercury (Hg) (Ozturk et al., 2012; Pinto and Ferreira, 2015; Memon et al., 2021). The essential elements are indispensable, and a small amount is required for plant growth and development. But a high amount of these metals above their normal ranges are highly toxic to plant and animal life (Van der Zaal et al., 1999; Dehkordi et al., 2010). The toxic amount of heavy metals in the soil adversely affects soil ecology, reduces agricultural production or product quality, deteriorates groundwater quality, and ultimately harms living organisms' health.

Specific plant species can uptake large amounts of metals from the soil, translocate efficiently from the roots to shoots, and deposit them in different parts of the leaves (Memon et al., 2001; Memon and Schroder, 2009; Memon and Zahirovic, 2014). These plant species can detoxify metals by either compartmentalizing at the subcellular level or depositing them in leaf trichomes in an innocuous form. The natural phenomenon of heavy metal tolerance became of most significant concern and interest in plant research to study the gene expression and regulation in some well-known accumulator plants such as *Arabidopsis halleri*, *Noccaea caerulescens, Pteris vittata*, and *Brassica juncea* (Hall, 2002; Mills et al., 2005; Kraemer, 2009, Meraklı et al., 2022).

Arabidopsis thaliana as a Model Plant for Identifying Genes for Metal Transport and Accumulation

Arabidopsis thaliana, (mouse-ear cress) is a small flowering plant native to Eurasia and is widely used as a model organism in plant biology. *Arabidopsis* is a member of the mustard (Brassicaceae) family, and its genome of 125 Mbp has been sequenced and annotated (The Arabidopsis Genome, Initiative 2000). It has a short life cycle of about six weeks from germination to seed maturation. Another reason why it is advantageous in research is that this plant can grow in broad climatic conditions and is easily cultivated under different laboratory conditions. The Arabidopsis Information Resource (TAIR) collects and makes available information from scientific research related to Arabidopsis (https://www.arabidopsis.org/). Like other plants, *A. thaliana* possesses specific mechanisms that regulate the cell's metal homeostasis. Through this work, we show that the structures of heavy metal ATPases and regulatory mechanisms are primarily similar to other plants. Since it is mainly used in plant research, the most helpful way is to study its transporter proteins with co-relation to proteins from other plant species, especially in the Brassicaceae family. We describe here the structure of heavy metal ATPases in *Arabidopsis thaliana* and compare the similarities and differences among different plant species, especially *Arabidopsis lyrata, Arabidopsis halleri, Noccaea caerulescens,* and *Brassica rapa.*

Metal Hyperaccumulator Plants

A specific group of plants has shown a great potential to absorb and translocate metals from roots to shoots and are termed hyperaccumulators (Baker and Whiting, 2002; Chaney et al., 2007, Sytar et al., 2021). The distinctive characteristic of hyperaccumulator plants is to efficiently translocate toxic metals from roots to shoots,, sub-compartmentalize them in the cell, and keep the metal away from the metabolic processes (Van der Ent et al., 2013; Memon et al., 2021, Sytar et al., 2021). A set of transporter proteins such as P-type ATPase, ZIP, MTPs, and NRAMP are essential in plant cell metal homeostasis (Hall and Williams, 2003; Williams and Mills, 2005; Memon, 2016). For instance, P_{1B}-ATPases, also known as heavy metal ATPases (HMAs), play a critical role in phytoremediation via the long-distance transport of various metals between plant organs (Colangelo and Guerinot, 2006). In recent years, significant scientific progress has been made in understanding the role of metal transporters in model plants like *A. halari*, *A. lyrata*, *N, caerulescens,* and some other accumulator plant species in *Brassicaceae* (Memon et al. 2021). Here we intend to elucidate the heavy metal tolerance and accumulation mechanisms in the plant species in the family Brassicaceae with the role of the HMAs (HMA1-HMA4) in response to heavy metal stress.

Material and Methods

Retrieving HMA Sequences and Multiple Sequence Alignment

The sequences for all chosen HMA proteins (HMA1, HMA2, HMA3, and HMA4 from *Arabidopsis thaliana* species) were obtained from the National Center for Biotechnology Information (NCBI) database and TAIR (The Arabidopsis Information Resource) database (Lamesch et al., 2012).

Multiple sequence alignment (MSA) was carried out to check for variability and conserved patterns among all 11 sequences in all three species (Sievers et al. 2011). Clustal Omega program was used in our analysis work because it produces high-quality MSAs and can handle data sets of hundreds of thousands of sequences in a reasonable time (Larkin et al. 2007). In addition, the quality of alignments is more efficient and valuable than in previous versions of the programs (Sievers et al.2011). The Clustal Omega tool is available at the European Bioinformatics Institute (EMBL-EBI). MView, a multiple-alignment viewer, was used to visualize the alignment (Brown et al. 1998, Larkin et al. 2007).

3D Structure Prediction and Validation

The 3D structure prediction and validation is one of the most critical parts of Bioinformatics. It is known that the structure of one protein affects the function of the other related proteins; an alteration of the structure of one protein may result in the improper functioning not only of the candidate protein but also the different proteins which interact with that protein. The 3D structure analysis of all four heavy metal ATPases (HMA1-HMA4) for three plant species (*A. thaliana, A. halleri and B. rapa)* was carried out to understand the similarities and differences among these transporter ATPases (Berman et al. 2000, Westbrook et al. 2003). Phyre2 server was used for 3D structure prediction. Phyre2 servers predict a protein sequence's three-dimensional structure using homology modeling principles and techniques (Kelley et al. 2015). Because the structure of a protein is more conserved in evolution than its amino acid sequence, this software can model a protein sequence of interest with reasonable accuracy. One of the most significant advantages of using Phyre2 modeling is that it is fast and reliable. Structure visualization was done by

PyMOL (The PyMOL Molecular Graphics System), a molecular visualization tool that provides viewing, customizing, and rapid generation of the desired molecule (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC, Yuan, et al. 2016). Prediction of protein-protein interactions was performed by using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) online tool (Franceschini et al. 2012). The interactions include physical and functional associations, and many organisms apply to this tool.

Results and Discussion

Heavy Metal ATPases

Heavy metal transporting ATPases (HMAs) are membrane-bound proteins having 6–8 predicted transmembrane helices (Axelsen and Palmgren 2001; Smith et al., 2014). They contain 31 amino acids (aa) of heavy metal-associated domain featuring GMTCxxC, a short C-terminal domain, and a very long N-terminal domain (Bull and Cox, 1994).

Genomic analysis of the *Arabidopsis* genome helped identify many reported transporters, including heavy metal ATPases. Eight genes (AtHMA1-AtHMA8) are identified in the *Arabidopsis* genome, but only four analyzed in this research. Two (AtHMA2 and AtHMA4) are of most significant interest because of their role in metal transport from roots to shoots. They function as putative transporters of divalent cations ($Zn^{2+,}$ Cd^{2+}, Pb^{2+}, $Co^{2+,}$ and Cu^{2+}). HMA1 functions as a transporter of metals in the chloroplast, and HMA3 is putative metal transporter located in the vacuolar membrane (Memon et al., 2001: Meraklı et al. 2022).

Given the main functions of HMA proteins in *A. thaliana*, we tried analyzing the phylogeny, multiple sequence alignments, and 3D structure prediction to investigate the presence of these and other interacting proteins present in different species of Brassicaceae. Study on these protein-protein interactions is vital to understand the complexity of the function of HMA proteins.

3D Structure Prediction and Validation of HMA Transporters

Arabidopsis thalian Heavy metal ATPase 1 (AtHMA 1)

The protein sequence of HMA1 obtained from NLM-NCBI is cadmium/zinc-transporting ATPase. It is also reported as a Cu-transporting ATPase and is involved in Cu homeostasis in the chloroplast. It contains 819 amino acids, and the gene encoding this protein is located on chromosome 4, which has 13 exons (NLM-NCBI database). Using PyMol, a visualization tool, a protein structure of HMA1 was obtained (Figure 1) and the active site was shown to be on the 453rd residue and metal binding sites on 682 and 686 residues (See Figures 2A, B). Using BLAST (Basic Local Alignment Search Tool), homologs of HMA1 in other plant species were found and given in Table 1.

Arabidopsis thaliana heavy metal ATPase 2 (AtHMA2)

The 3D structure of AtHMA2 is shown in Figure 3. The AtHMA2 is cadmium/zin transporting ATPase and is 951 amino acids long. The AtHMA2 gene is located on chromosom 4 and contains nine exons (NCBI database). It is located in the plasma membrane and maintaining heavy metal homeostasis inside the cell. It also utilizes energy in the form of ATP, producing ADP. The AtHMA *g*ene is generally expressed during the final stage of leaf development, flowering stage, petal differentiation, and expansion stage. The active site is in the 391st position and has two metal-binding sites, 592 and 596 residues (see Figures 3 and 4). The homologs of AtHMA2 in other plant species are given in Table 2.

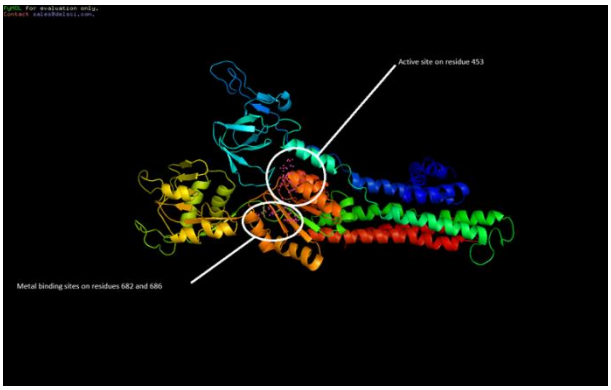
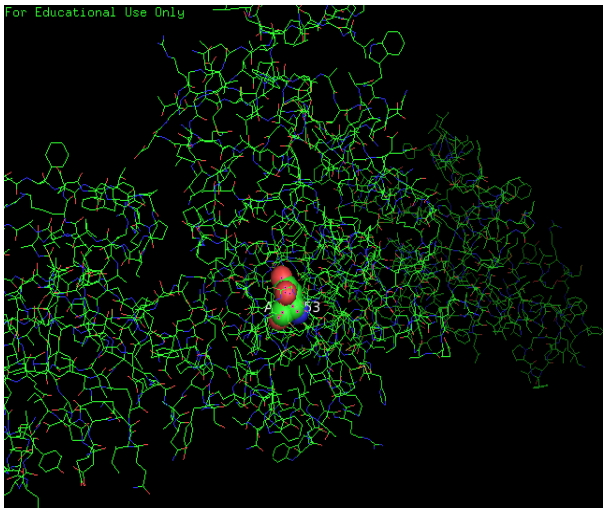
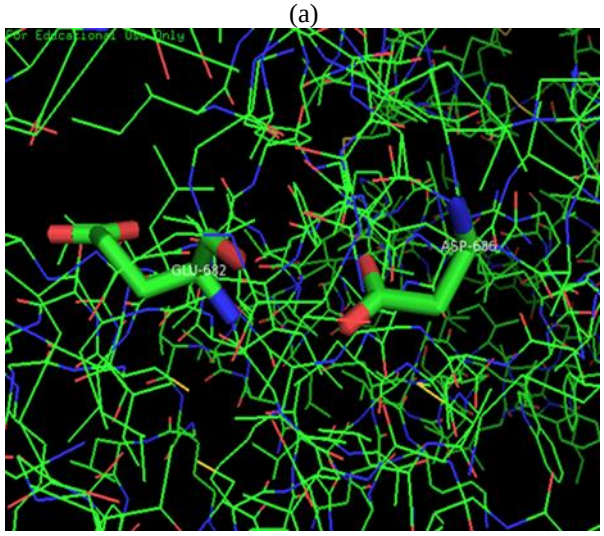


Figure1. The predicted 3 D structure of AtHMA1 using PyMol software



(a)



(b)

Figure 2. A. Active site of AtHMA1 protein located on 453rd residue, B. Metal-binding site located on 682nd and 686th residues

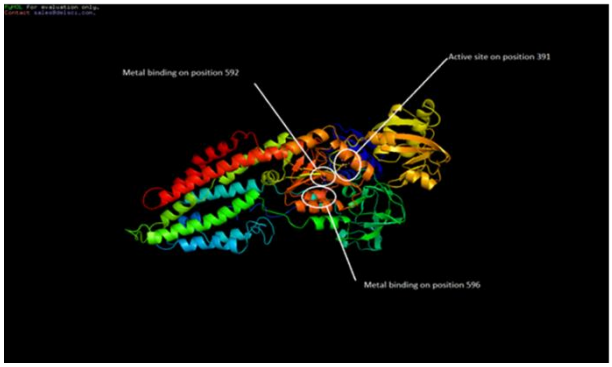


Figure 3. The 3D structure of AtHMA2 shows the positions of the active site and metal binding

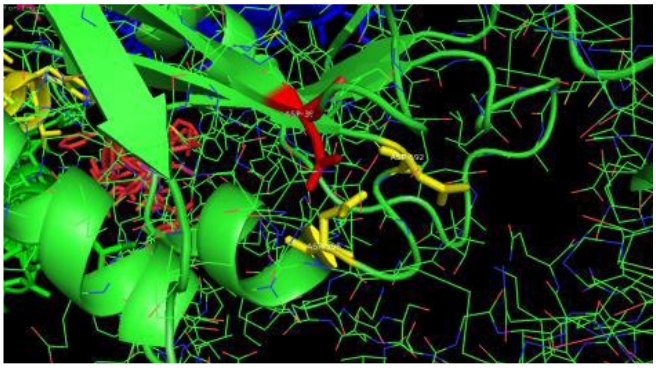


Figure 4. The 3D structure of At HMA2 shows an active site (red) and metal binding sites (yellow)

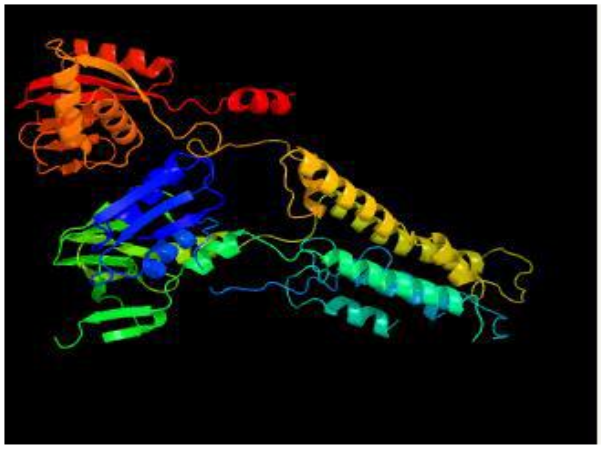


Figure 5. The 3D structure of AtHMA3 from *Arabidopsis thaliana*

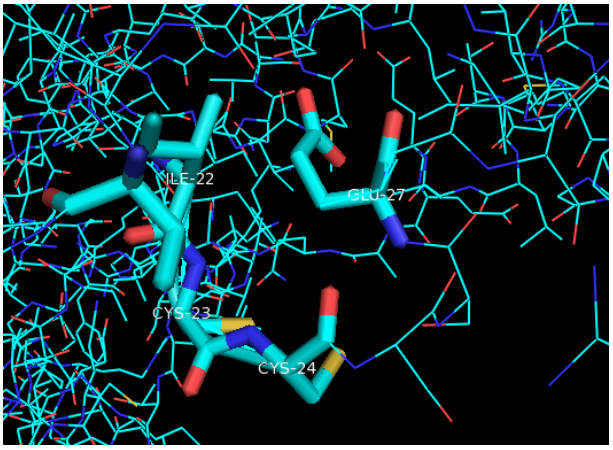


Figure 6. The metal binding site of At HMA3. Metal-binding site is positioned on ILE-22, CYS-23, CYS-24, and GLU-27

Table 1. Homologs of At HMA1 in other plant species.

Proteins	Organism	Query cover	Identity	Accession number
A putative P1B-type metal ATPase	*Arabidopsis halleri subsp. halleri*	42%	97%	CAE45019.1
A hypothetical protein	*Arabidopsis lyrata subsp. lyrata*	99%	96%	XP_002866960.1
A probable cadmium/zinc-transporting ATPase HMA1, chloroplastic	*Brassica rapa*	99%	93%	XP_009137459.1

Table 2. The homologous proteins of AtHMA2 are predicted in other plant species.

Protein	Organism	Query cover	Identity	Acc. Number
P1B-type ATPase 4-1	*Noccaea caerulescens*	98%	66%	ADZ73050.1
Predicted protein	*Arabidopsis lyrata subsp. lyrata*	100%	87%	XP_002867367.1
PREDICTED: cadmium/zinc-transporting ATPase HMA2	*Brassica rapa*	99%	77%	XP_009128090.1
Zn/Cd P(IB)-type ATPase	*Arabidopsis halleri subsp. halleri*	98%	69%	ACC68167.1

Table 3. The homologs of AtHMA3 in other plant species.

Name of the protein	Organism	Query cover	Sequence identity ; matches	Accession number
Predicted protein	*Arabidopsis lyrata subsp. lyrata*	100 %	93 % 505/542	XP_002867366.1
P1B-type ATPase 4	*Arabidopsis halleri*	97 %	71 % 379/531	ABB29495.1
P1B-type ATPase 4-2	*Noccaea caerulescens*	97 %	70 % 367/527	ADZ73052.1
PREDICTED: cadmium/zinc-transporting ATPase HMA3	*Brassica rapa*	99 %	89 % 479/541	XP_009128077.1

Table 4. Homologs of AtHMA4 in other plant species.

Name of the protein	Organism	Query cover	Sequence identity; matches	Accession number
hypothetical protein	*Arabidopsis lyrata subsp. lyrata*	100 %	94 % 89/95	XP_002886241.1
P1B-type ATPase 4	*Arabidopsis halleri*	100 %	97 % 92/95	ABB29495.1
P1B-type ATPase 4-2	*Noccaea caerulescens*	82 %	94 % 73/78	ADZ73052.1
PREDICTED: cadmium/zinc-transporting ATPase HMA3-like	*Brassica rapa*	82 %	88 % 69/78	XP_009102202.1

Table 5. A detailed description of HMAs interacting proteins is given in Figure 11.

Gene ID	Interacting protein name	Amino acids (aa)
CCH	Cu chaperone	121 aa
NAKR1	Sodium potassium root Defective 1	319 aa
HIPP27	Heavy associated isoprenylated plant protein 27	147 aa
FP3	Farnesylated protein 3	355 aa
AT5G60800	Heavy metal transport/detoxification domain-containing protein	302 aa
AT5G50740	Heavy metal-associated domain-containing protein	290 aa
AT5G37860	Heavy metal transport/detoxification domain-containing protein	262 aa
AT5G27690	Heavy metal transport/detoxification domain-containing protein	352 aa
AT5G26690	Heavy metal-associated domain-containing protein	114 aa
AT5G24580	Heavy metal transport/detoxification domain-containing protein	319 aa

Arabidopsis thaliana heavy metal ATPase 3 (AtHMA3)

The 3-D structure of AtHMA3 is shown in Figure 5. This transporter protein is located in the vacuolar membrane and is a putative cadmium/zinc-transporting ATPase. The gene's coding sequence (CDS) is 1629 bp long and contains 542 amino acids (NCBI database). The metal binding site is located on 22-24th and 27th residues (Figure 6). The phosphorylation site is on the residues from 397th to 403th (see Figure 7). It contains five domains. Four are transmembrane domains, and one is a low-complexity domain (analyzed by the SMART sequence program). The homologs of AtHMA3 in other plant species are given in Table 3.

Arabidopsis thaliana heavy metal ATPase 4 (AtHMA4)

Heavy metal ATPase 4 protein is a transporter protein located in the plasma membrane and is involved in cadmium/zinc transport. HMA 4 can rescue Zn deficiency in yeast and Cd resistance, suggesting a role in Zn and Cd transport. The HMA4 gene is located on chromosome 2, and the polypeptide chain contains 1172 amino acids (Zapta et al., 2016). The 3D structure is shown in Figures 8 and 9 show active sites and metal binding sites, respectively.

It contains soluble N-terminal metal binding domains essential for Zinc transport. There are five residues where Zn is binding, and they are GLY-25, ILE-26, CYS-27, CYS-28, and GLU-31, and they are located in chain A of HMA4, determined by NMR spectroscopy (see Figure 10). Chain A consists of 96 amino acids. The AtHMA4 homologs in other plant species are given in Table 4.

Intracome Analysis of Meal-Binding Proteins in Plants

Figure 11 shows the interactome analysis of metal transporters, including AtHMA1 to ATHMA4. A detailed description of protein transporters interacting with different heavy metal ATPase (HMA1- HMA4) is given in Table 5.

Discussion

Genomic sequencing analysis of the *Arabidopsis* genome helped identify many reported transporters. Many transporters belong to the family of heavy metal ATPases that regulate HM homeostasis (Eren and Arguello, 2004; Williams and Mills, 2005, Memon 2020). HMA transporters belong to the P-type ATPase protein family that plays a vital role in metal transport and detoxification processes. All HMA proteins that were analyzed in this study showed a considerable amount of similarities with each other. Our previous work with phylogenetic analysis showed that AtHMA1, AhHMA1, and BrHMA1 belong to the same group (Memon, 2016). In addition, AtHMA2 and BrHMA2 are most similar to each other. AtHMA3, AhHMA3, and BrHMA3 also showed similarities to each other. AtHMA4 and BrHMA4 were also phylogenetically close to each other (Memon, 2016; Meraklı et al., 2022).

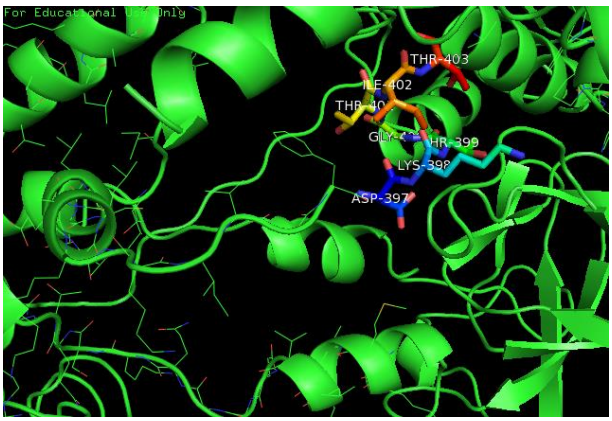


Figure 7. Phosphorylation sites of AtHMA3 are located on residues from Asp-397th to Thr-403rd

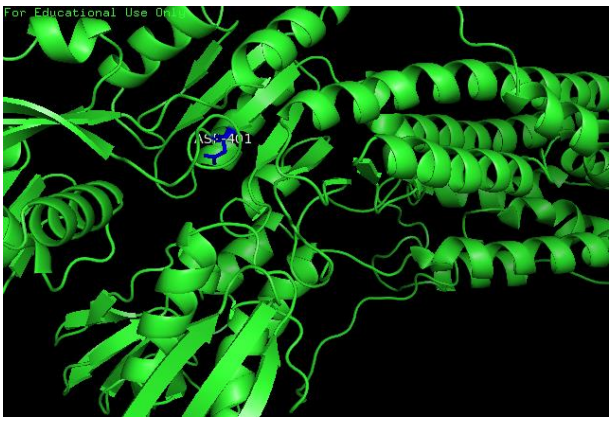


Figure 8. The 3D structure of HMA4 showing an active site of HMA4 protein located on 401st Asp residue (Aspartic acid).

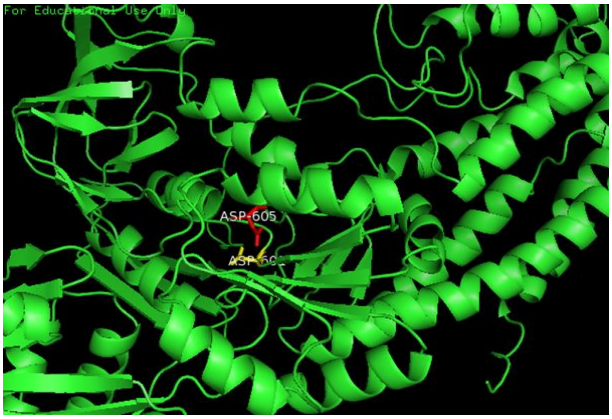


Figure 9. The 3D structure of AtHMA4 showing metal binding sites located Metal-binding site located on 601st and 605th Asp residues

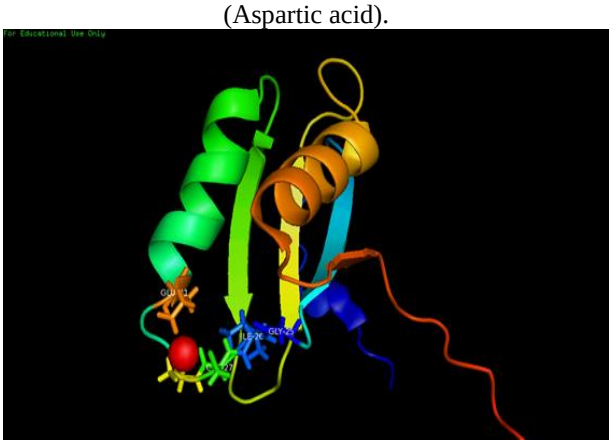


Figure10. 3D structure showing the Chain A of AtHMA4 consists of 96 residues. Among them, five residues bind Zn, which are GLY-25, ILE-26, CYS-27, CYS-28, and GLU-31.

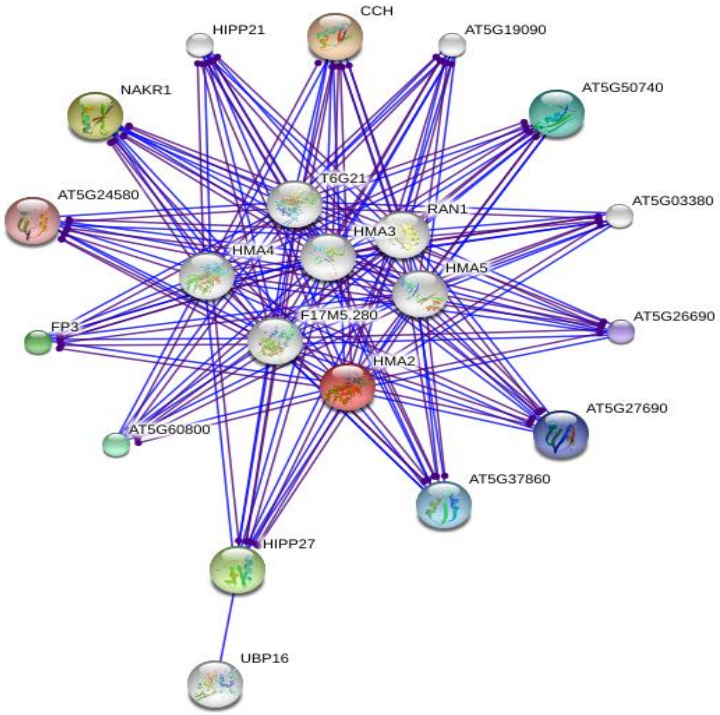


Figure 11. Protein-protein interaction prediction shows the interaction of other proteins with HMA1, HMA2, HMA3, and HMA4 in Arabidopsis thaliana. The interactome is generated by STRING software. A detailed description of interacting proteins is given in Table 5.

In the 3D structural analysis of proteins, we concluded that all HMAs had similarities in their secondary structural elements, including the number of alpha helices, beta sheets, and loops (See Figures 1, 3, 5 and 8). Our data in Tables 1-4 show the homologs of these transporters in other plant species in Brassicaceae, which generally do a similar function as in *Arabidopsis thaliana* (Hussain et al., 2004, Memon et al., 2021). They are suggested to do the same function as their counterpart in *Arabidopsis thaliana*. Interactome analysis showed that all four HMAs interact with different metal transporters and metal chaperons (Memon 2020). The primary approach to generating interactome protein analysis is to check for the specificity in structure and function between plant species in *Brassicaceae* and compare this data to that of *A. thaliana.* Studies on these protein-protein interactions are essential to understand the complexity of the function of HMA proteins.

Our data show that all these transporters except HMA4 are encoded from the genes located on chromosome 4. The most challenging part was predicting the structures and finding the protein's active and metal-binding sites. Structures were predicted using Swiss-Expasy, one of the most accurate computational techniques. All the pictures are edited in PyMOL to visualize these transporters' better resolution. These data were generated through in silico analysis, which can benefit further detailed experimental research in the laboratory.

All previous bioinformatics analyses have given some information about their regulation, but not much work has been done on their structural and interactome studies. This research shows their interaction with other proteins (see Figure 11 and Table 5). Some structural differences have been detected in the domain analysis, subnuclear and subcellular localizations of these proteins (data not shown). In this connection, detailed research is being carried out in our laboratory.

To enhance the root-to-shoot translocation (a hallmark of efficient hyperaccumulators), HMA2 and HMA4 are the most important candidate gene for hyperaccumulation (Hussein et al., 2004). Multiple lines of research have provided valid evidence for their role in the xylem loading of heavy metals and shown its crucial role in metal hyperaccumulation in *A. halleri* (Nouet et al., 2015).

These performed bioinformatic analyses are critical for future work on heavy metal ATPases. This work represents an excellent comparative structural analysis that leads to the prediction and estimation of the main functions of proteins.

In conclusion, these bioinformatic analyses show that HMA transporters are divided into three groups:1. Cu/Ca/Zn/Cd/Co-ATPases group, 2. the Zn/Cd/Pb/Co-ATPases group, and 3. the Cu-ATPases group. The 3D structural analysis of HMAs in *Brassica* species showed many similarities among plant species. All these have similarities in their secondary structural elements and play a similar role in metal transport in the cell. The present paper will help the researcher understand these ATPases' role in detoxifying the toxic metals from the cells of accumulator plants.

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