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Isolation of two novel isoforms encoding zinc- and copper-transporting P_{1B}-ATPase from Gouan (*Aeluropus littoralis*)

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Abstract

Two new isogenes encoding heavy metal ATPase have been partially cloned from *Aeluropus littoralis* genome. One of them was 849 bp in length and related to an isoform which encodes a copper-transporting P_{1B}-ATPase (*AlHMA1*), and the other one was 403 bp in length and related to an isoform encoding a zinc-transporting P_{1B}-ATPase (*AlHMA2*). When southern blot hybridization was performed under low-stringency, 6 to 7 hybridization bands appeared, indicating that in this plant heavy metal ATPase is encoded by a multigene family. RT-PCR assay displayed that the *AlHMA2* highly expresses in leaf and root and its transcription level is the same in both organs. Phylogenetic tree showed that copper transporters from different plants were located in one group and zinc transporters were located in another group, although all heavy metal P_{1B}-ATPasees have the same function. It means the different isoforms in single plant that involve in transporting of different heavy metals, may come from different sources.

Keywords: gene cloning, tissue expression pattern, Gouan, heavy metals ATPase, metal-transporting.
Abbreviations: cDNA - Complementary DNA, NCBI- National Center for Biotechnology Information, IPTG- Isopropyl β-D-1-thiogalactopyranoside, DDBJ- DNA Data Bank of Japan, CTAB- Cetyl trimethylammonium bromide.

Introduction

Heavy metals are a group of elements with specific weight higher than 5 g/cm^3 that exhibit metallic properties. Some of them (Co, Cu, Fe, Mn, Mo, Ni and Zn) known as essential micronutrients because they are vital for plant normal growth as well as they have many biological and structural functions as metalloproteins or metabolites. In contrast, no physiological function has reported for Cd, Cr, Hg and Pb that thought to be toxic and non-essential for plants (Michalak, 2006). Copper as a redox-active transition metal has many functions such as cofactor for many enzymes, in photosynthesis and respiration precesses, signaling of transcription and hormens, protein trafficking machinery, lignin formation in cell walls, and oxidative stress responses (Hirayama et al., 1999; Yruela, 2005). But in high concentration, this element can be toxic for plants in several ways by oxidative damage to biomolecules, decrease in availability and absorption of other essential mineral nutrients and reduce the efficiency of enzymes by binding to enzyme SH groups (Yruela, 2005). Zinc is another essential minor element which has many functional, regulatory and structural roles in many enzymes (Brennan and Bolland, 2006). This element is also important for auxin production, carbohydrate and protein metabolism, protecting cells against oxidative stress, photosynthesis reaction and maintaining the membrane structure and functions (Brennan and Bolland, 2006). Zn toxicity induces chlorosis in plant by competition with the uptake of other elements or reduction in chlorophyll synthesis due to Fe-deficiency (Broadley et al., 2007). So plants developed a number of different hemostasis mechanisms to maintain heavy metals concentrations at cellular level. These mechanisms comprised of regulation of transport, chelation and compartmentalization (Clemens, 2001). Previous studies reported several types of heavy metal transporters that have different sub-cellular location, metal specificity and expression pattern (Colangelo and Guerinot, 2006). The most important role in metal efflux from cytoplasm identified for P_{1B}-ATPases family. P_{1B}-ATPases are a subfamily of P-type ATPases that transport heavy metals (Cu^+, Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+}) against their electrochemical gradient by the energy of ATP hydrolysis (Williams and Mills, 2005). P_{1B}-ATPases observed in both prokaryotic and eukaryotic species. Plants have more number of P_{1B}-ATPases in comparison with other organisms (Williams and Mills, 2005). Also plants are the only eukaryotic species which possess Zn^{2+} -ATPase. Plant P_{1B}-ATPase has important roles in nutrition and detoxification (Colangelo and Guerinot, 2006). Previous studies have revealed that most P_{1B}-ATPases have eight transmembrane domains (TMs), signature CPX motif (CPC, CPH, CPS, SPC and TPC) in sixth domain, with a key role in metals translocation, and carboxyl- or amino-terminal regions (N-MBD and C-MBD) with putative metal-binding domains (Argüello, 2003). Also there are two cytoplasmic loops between helices 4-5 (A domains) and 6-7 (P and N domains). According to substrate specificity and phylogenetic analyses, P_{1B}-ATPases are grouped into two subgroups: monovalent cation transporter Cu^+/Ag^+ and divalent cation transporter ($Zn^{2+}/Cd^{2+}/Pb^{2+}/Co^{2+}$) (Axelsen and Palmgren, 2001). Previous studies showed that P_{1B}-ATPases play a key role in heavy metals detoxification by sending out from cytoplasm.

It is demonstrated that these pumps are specific for substrate for example in Arabidopsis, AtHMA3 has active role in the sequestration of Pb, Zn, Cd and Co (Morel et al., 2009) while AtHMA5 has a role in the compartmentation of Cu (Williams and Mills, 2005). It has also been revealed that P_{1B}-ATPase are necessary for a many physiological activities in plants such as providing Cu for chloroplast proteins, photosynthetic electron transport, ethylene signaling pathway, cell expansion and Cu mobilization in leaf during senescence process (Hirayama et al., 1999; Woeste and Kieber, 2000; Himelblau and Amasino, 2001; Abdel-Ghany et al., 2005; Waraich et al., 2011; Jaradat, 2011). Although P_{1B}-ATPases have important role in plants but unfortunately only a few number of genes that encoding these proteins have been identified in plants species by molecular approaches. By analysis of genome sequences, eight isoforms in Arabidopsis (AtHMA1-AtHMA8), nine ones in rice (OsHMA1- OsHMA9), and 10 isofomrs in barley (HvHMA1- HvHMA9) were detected (Lee et al., 2007). In this work we tried to describe and characterize a new isoform of P_{1B}-ATPase genes from a C4 halophyte monocotyledonous grass (*A. littoralis*) that its small genome and tolerant to a number of abiotic stresses (drought, heat and salinity) turn it to a suitable research source for biotechnological researches (Zouari et al., 2007).

Results

PCR results and sequences analysis

The band corresponding to extracted DNA was distinctly visible on agarose gel, indicating high quality and the 260/280 nm ratio was 1.95 that verified the purity of DNA.
On the basis of conserved region in the genes encoding heavy metals ATPase a pair of degenerate primer was designed as described later. As expected, a 849 bp fragment (*AlHMA1*) was amplified by PCR from *A. littoralis* genomic DNA was extracted from leaves (Fig. 2A). After sequencing, it was revealed that the predicted amino acid sequence has similarity to copper-transporting P_{1B}-ATPase in other plants (Fig. 3). AlHMA1 had the highest similarity (91%) to copper-exporting ATPase in *Sorghum bicolor* (NCBI accession No. AAT42167.1) and copper-transporting ATPase sequence in *Zea mays* (NCBI accession No. AAT42153.1), respectively. Based on the sequences of the genes encoding heavy metal ATPases in plants a pair of specific primer (HEMF2 and HEMR2) was designed as described in Materials and Methods section. An expected fragment with 403 bp in length (*AlHMA2*) was amplified by PCR from *A. littoralis* genomic DNA was extracted from leaves (Fig 2B). Its sequence revealed that the fragment was related to a gene encoding a zinc-transporting P_{1B}-ATPase (Fig 4).
The second fragment exhibited more than 70% homology to some plants heavy metal ATPase in *Arabidopsis thaliana, Arabidopsis halleri* and *Noccaea caerulescens*.

Analyses of tissue-specific expression

The expression of *AlHMA2* was investigated in different tissues. Total RNA was isolated from different tissues has high quality. In all samples, bands corresponding to 5.8S, 18S and 28S rRNA were distinctly visible, indicating high quality and non-degraded RNA (Fig. 1). The A260/A280 ratios of extracted RNA from different tissues were 1.9 to 2 with a peak at 260 nm, indicating low amounts of contaminating protein, polysaccharides and polyphenol

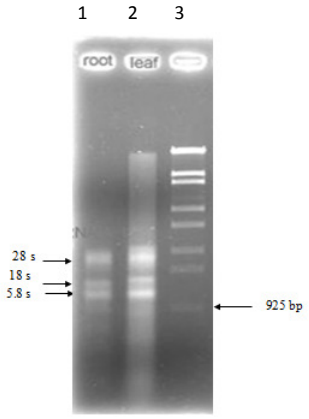

Fig 1. RNA extraction from different tissues using Qiagen kit. Lane 1: Total RNA isolated from root; Lane 2: Total RNA isolated from leaf; Lane 3: marker λ/*Sty*I.

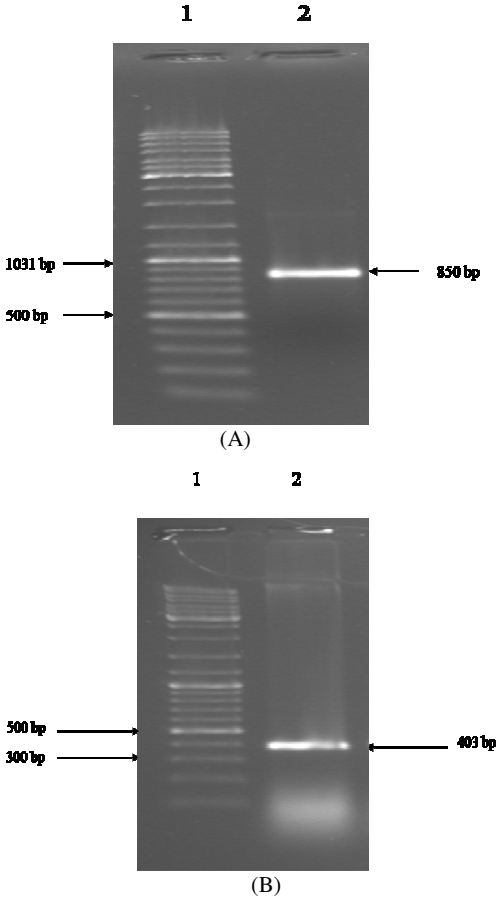

Fig 2. A. PCR amplification of 849 bp fragment from genomic DNA. Lane 1: Gene Ruler ™ DNA Ladder Mix; Lane 2: The amplification of 849 bp fragment by PCR. **B.** PCR amplification of 403 bp fragment from genomic DNA. Lane 1: Gene Ruler ™ DNA Ladder Mix; Lane 2: The amplification of 403 bp fragment by PCR.

Fig 3. Sequence homology between AlHMA1 and other isogenes encoding copper-transporting P_{1B}-ATPase in other plants. SbHMA1 (*Sorghum bicolor*, accession No. AAT42167), ZmHMA1 (*Zea mays*, accession No. AAT42153), RcHMA1 (*Ricinus communis*, accession No. XP_002509783), RcHMA2 (*Ricinus communis*, accession No. XP_002509784), RcHMA3 (*Ricinus communis*, accession No. XP_002513473), BnHMA1 (*Brassica napus*, accession No. AAL02122), AtHMA7 (*Arabidopsis thaliana*, accession No. NP_199292.1) and OsHMA1 (*Oryza sativa*, accession No. BAD25508.1).

compounds. As described in Materials and Methods section, using specific primers, HEMF3 and HEMR3, a 314 bp fragment of the coding sequence of gene was amplified from leaf and root (Fig. 5). The result revealed that *AlHMA2* expresses in leaf and root and the expression level of that was the same in both tissues.

Southern blot results

Figure 6 shows the southern blot analysis of genomic *Sma*I-digested DNA with second segments cDNA as a probe. Under high stringency condition (65º C), genomic DNA displayed only one hybrid band in the thoroughly digested-sample with a specific length of 7 kbp. But 6 or 7 band fragments detected under low stringency condition (55º C) (Fig. 6).

Phylogenetic tree analysis

Phylogenetic tree analysis of copper- zinc-transporting P_{1B}-ATPase sequences from different plants showed that metal transporting ATPase involved in the transport of same metal are located in the same subgroup (Fig. 7). In the other words, the sequence of heavy metal transporting ATPase is dependent on which kind of heavy metal is transported by that. In addition, it found that the amount of homology between partial sequences of these proteins is same to complete sequences (Fig. 7).

Discussion

Plant heavy metal ATPases are different in metal specificity and number from other organisms (Williams and Mills, 2005). They have main role in maintain metal homeostasis and inhibition of their accumulation to toxic levels by absorption, distribution and efflux (Argüello and Eren, 2007). Despite their key roles, only few researches were conducted on heavy metal ATPases structure and function in monocot species. In this work we chose a halophyte monocotyledonous grass (*A. littoralis*) with a special response to heavy metals (Rastgoo and Alemzadeh, 2011) to study the genes encoding these pumps. This is the first report to indicate the isolation and characterization of genes encoding P_{1B}-ATPases from *A. littoralis*. Predicted amino acid sequence of AlHMA1 has shown high similarity to copper-transporting ATPase (Fig. 3) while AlHMA2 has the most similarity to zinc-transporting ATPase (Fig. 4). It shows that this plant processes several classes of metal transporters that involve in metals uptake and homeostasis. It has been reported for other heavy metal hyperaccumulator plants (Papoyan and Kochian, 2004). That means this plant must have high tolerance to heavy metals. Although, there are different classes of P_{1B}-ATPase heavy metal transporters in

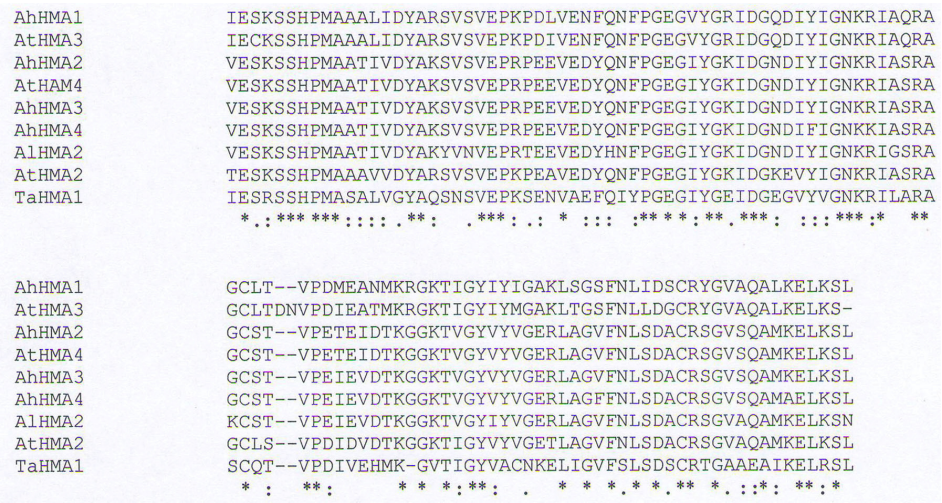

Fig 4. Sequence homology between AlHMA2 and other isogenes encoding zinc-transporting P_{1B}-ATPase in other plants. AhHMA3 (*Arabidopsis halleri*, accession No. CAD89012), AtHMA3 (*Arabidopsis thaliana*, accession No. AAL16382), AhHMA2 (*Arabidopsis halleri*, accession No. ACC68167), AtHMA4 (*Arabidopsis thaliana*, accession No. NP_179501), AhHMA1 (*Arabidopsis halleri*, accession No. ACC68153), AhHMA4 (*Arabidopsis halleri*, accession No. ACC68152), AlHMA2 (*Aeluropus littoralis*, non register), AtHMA2 (*Arabidopsis thaliana*, accession No. NP_194740) and TaHMA1 (*Triticum aestivum*, accession No. ABF55693).

plants, a recent research shows that these pumps have some common motives (Ashrafi et al., 2011). Phylogenetic tree showed that all copper-transporting ATPases were placed in group A, and all zinc-transporting ATPases were located in group B (Fig. 7). It means that in single organism, ATPase pumps that involve in transporting of different heavy metals probably have not duplicated from each others. In the other words, the ATPase pumps that involve in transporting of same metal in different plants, probably have a common ancestor. This result being consistent with that of Argüello and Eren (2007). It has been suggested that amino acids in transmembrane regions participate in determining metal specificity (Argüello and Eren, 2007). It should also be considered that although any group has specific motives, but there are some domains that conserved in all P-type ATPase (Williams et al., 2000). On the basis of dendrogram, AlHMA1 shows the most similarity to other isoforms of copper-transporting ATPase from other monocots and was located in the same subgroup with *Zea mays* and *Sorghum bicolor* (Fig. 7) that may mean these isoforms have a common ancestor. AlHMA2 has the most similarity with zinc-transporting ATPasees and is located in the same subgroup with some isoforms from *Arabidopsis*. Probably, AlHMA1 and AlHMA2 belong to different subgroups of P_{1B}-ATPases and they have come from different sources. The most knowledge about plant P_{1B}-type ATPase has been acquired from dicot species (Papoyan and Kochian, 2004; Deniau et al., 2006; Lee et al., 2007; Milner and Kochian, 2008), but our results present knowledge concerning P_{1B}-type ATPase in an important monocot species. Phylogenetic dendrogram deduced from complete sequences is completely similar to that of deduced from partial sequences (Fig. 7). On the basis of this interesting result it suggests that the homology between different parts of these sequences is the same. In addition, it was found the metal specificity is more important than taxonomy in classifying of these transporters. It is consistent with the finding of Argüello and Eren (2007). The tissue-specific expression pattern of *AlHMA2* in *A. littoralis* which carried out for the first time in this study show this isoform expresses in leaves and roots that shows this gene is under the control of a promoter that expresses in different tissues. This may indicate an important role for the product of this gene in this plant. There has not been any report investigating tissue specific expression of heavy metal P_{1B}-type ATPase in monocot species. The southern blot result shows that probably there are from 6 to 7 isogenes in the genome of *A. littoralis* for P_{1B}-ATPases heavy metal transporters. This result is exactly coincided with the result of Williams and Mills (2005) about the copy number of isoforms in dicot plants. It may indicate that the evolution of these proteins was the same in both species.

Materials and methods

Plant growth

A. littoralis's seeds were supplied by Pakan Seed Research Centre, Isfahan, Iran. The plant seeds were surface sterilized by soaking in 1% (v/v) sodium hypochlorite for 20 min and then rinsed several times with distilled water. Seeds were grown in plastic pots filled with sand and grit (1:1 v/v) in a controlled-environment growth room under a 16-h day/8-h night cycle, 60%–80% relative humidity and 25° C/16° C day/night temperature. Plants were regularly irrigated with standard MS medium (Murashige and Skoog, 1962).

Primer design

cDNA sequences of plant P_{1B} -ATPases were obtained from NCBI site. Two degenerate primers (F1: AYY GYT GCT TTY GAY AAA ACH GGD AC; R1: ATC RTT HAN HCC DTC YCC T AC C) and two specific primers (F2: GTT GAG AGC AAG TCA AGT CAT CC; R2: AGA TTT CAG TTC CTT CAT AGC TTG AG) were designated by sequence alignments using Vector NTI (Version 9) program.

Genomic DNA extraction procedure

Fresh and young leaves are used for DNA extraction. DNA was extracted from plant material by modified CTAB method (Alemzadeh et al., 2006). Leaves ground with a mortar and pestle in liquid nitrogen as finely as possible. Around 1 g ground leaves was transferred to a tube containing 10 ml extraction buffer [Tris-HCl (100 mM), EDTA (50 mM), NaCl (700 mM), hexadecyl trimethyl ammonium bromide, CTAB (2%), and bacterial mercaptoethanol (1%)]. The suspension was incubated at 65° C for 1 h with occasional inverting of the tube. After incubation, the tube kept at room temperature for 10 min to cool. After that, 4.5 ml Octanol: Chloroform (1:24) was added to each tube and inverted at room temperature for 10 min. The tube centrifuged at 1420 × g at room temperature for 15 min. Pipetted off supernatant to a new tube and added 4.5 ml Octanol: Chloroform (1:24) to the tube. The tube inverted for 10 min and then, centrifuged at 1420 × g at room temperature for 15 min. The supernatant was transferred to a new tube, added DNase-free RNase (1 U/ml) and kept it in room temperature for 1 h. To precipitate DNA, 12 ml cold absolute ethanol was added to each tube, gently mixed by inverting and centrifuged at 1420 × g at 4° C for 10 min. The plate was dissolved in 50 µl TE (Tris, 10 mM and EDTA, 1mM) and stored at - 20° C.

PCR amplification and cloning

PCR reactions in a final volume of 25 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM each primer (forward and reverse), 1 µg genomic DNA as template and 1 unit of Taq DNA polymerase were carried out under the following conditions: 5 min at 95° C, 35 cycles at 95° C for 30 s, 60° C for 1 min, and 72° C for 2.5 min, with a final extension for 7 min at 72° C. PCR product was separated on 1% agarose gel.

Cloning of PCR products

The PCR products were cloned into pTZ57R/T vector and transferred into *E. coli* DH5α cells by heat shock method and plated on LB medium containing 250 µg/ml ampicillin, 100 mM X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) and 100 mM IPTG (Sambrook and Russell, 2001).

Plasmid extraction and sequencing

After white/blue selection, white colonies were picked and grown in 3 ml LB broth at 37° C for overnight. The recombinant plasmid was extracted by alkaline lysis method (Sambrook and Russell, 2001). Extracted plasmid was digested by *Eco*R1 and *Hin*dIII enzymes and digestion products were checked on 1% agarose gel. The positive clones were selected and sequenced.

RNA extraction, preparing cDNAs and analysis of expression pattern

Total RNA was extracted from leaves and roots using RNeasy Plant Mini Kit (Qiagen, USA). The quality of RNA was assessed by electrophoresis on 1% agarose gel (Fig. 1). First-strand cDNAs were prepared from total RNA by SuperScript II kit (Invitrogen, USA), with oligo (dT) primer.

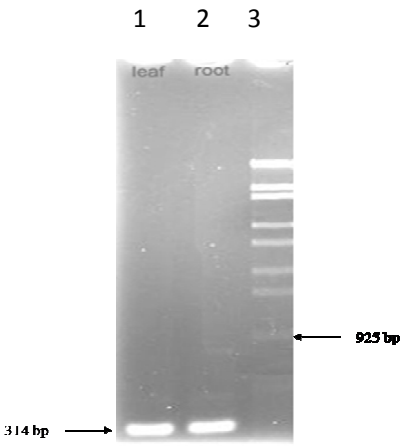

Fig 5. Expression pattern of *AlHMA2* in different tissues. Lane 1: leaf; Lane 2: root; Lane 3: marker λ/*Sty*I.

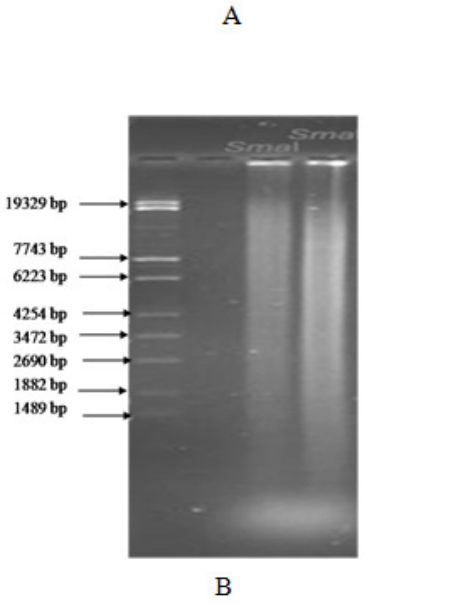

Fig 6. A. λ/*Sty*I (lane 1) genomic DNA was digested with *Sma* I (lanes 3 and 4). **B.** Southern blotting**.**

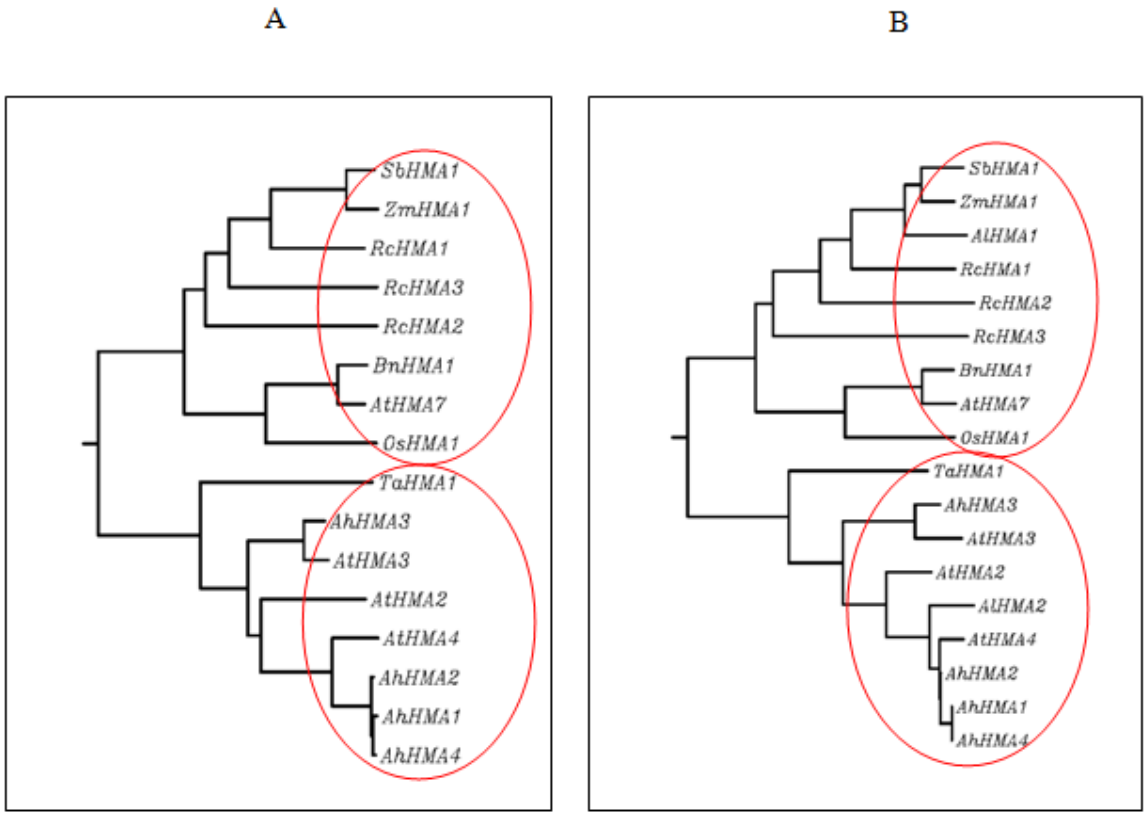

Fig 7. A) Phylogenetic dendrogram deduced from predicted amino acid sequences of 8 copper-transporting P_{1B}-ATPase and 8 zinc-transporting P_{1B}-ATPase from different plants (complete sequences). **B)** Phylogenetic dendrogram deduced from predicted amino acid sequences of AlHMA1, AlHMA2, 8 copper-transporting P_{1B}-ATPase and 8 zinc-transporting P_{1B}-ATPase from other plants (partial sequences). Copper-transporting P_{1B}-ATPase sequences containing: AlHMA1 (*Aeluropus littoralis*, no register), SbHMA1 (*Sorghum bicolor*, accession number AAT42167), ZmHMA1 (*Zea mays*, accession No. AAT42153), RcHMA1 (*Ricinus communis*, accession No. XP_002509783), RcHMA2 (*Ricinus communis*, accession No. XP_002509784), RcHMA3 (*Ricinus communis*, accession No. XP_002513473), BnHMA1 (*Brassica napus*, accession No. AAL02122), AtHMA7 (*Arabidopsis thaliana*, accession No. NP_199292.1) and OsHMA1 (*Oryza sativa*, accession No. BAD25508.1). Zinc-transporting P_{1B}-ATPase sequences containing: AlHMA2 (*Aeluropus littoralis*, no register), AhHMA3 (*Arabidopsis halleri*, accession No. CAD89012), AtHMA3 (*Arabidopsis thaliana*, accession No. AAL16382), AhHMA2 (*Arabidopsis halleri*, accession No. ACC68167), AtHMA4 (*Arabidopsis thaliana*, accession No. NP_179501), AhHMA1 (*Arabidopsis halleri*, accession No. ACC68153), AhHMA4 (*Arabidopsis halleri*, accession No. ACC68152), AtHMA2 (*Arabidopsis thaliana*, accession No. NP_194740) and TaHMA1 (*Triticum aestivum*, accession No. ABF55693).

The expression pattern of that *AlHMA2* was investigated in leaves and roots of *A.littoralis* using specific primers HEMF3 (GAG CCT AGG ACT GAA GAA GTT GAG G) and HEMR3 (GCT TGA GCT ACA CCT GAT CTA CAA GC) by semiquantitative RT-PCR.

Southern blotting

Genomic DNA (5 µg) was digested with the restriction endonuclease *Sma* I, separated by 0.8% agarose gel electrophoresis and capillary transferred to a Biodyne A nylon membrane (Sambrook and Russell, 2001) and then blotted by capillary blotting onto positively charged nylon membranes. To make a probe for P_{1B} ATPase, a 403 bp fragment was amplified by PCR with specific oligonucleotide primers (forward: GTT GAG AGC AAG TCA AGT CAT CC; reverse: AGA TTT CAG TTC CTT CAT AGC TTG AG) from the genomic sequence of *AlHMA2* gene and inserted into pGEM-T Easy Vector and sequenced. Hybridization was carried out with a fluorescein labeled probe was prepared using a Gene Images Labeling Kit (Amersham Biosciences, USA) in a mixture containing 5 × SSC, 0.1% SDS, 5% blocking reagent, and 5% dextran sulfate at 65° C (high stringency conditions) or 55° C (low stringency conditions) for 18 h. The hybridized membrane was finally washed with 0.2 × SSC containing 0.1% SDS at 65° C (high stringency) or 55° C (low stringency) for 2 × 15 min. The signals were detected with a Gene Images Detection Kit (Amersham Biosciences) using X-ray film.

Bioinformatic analysis

The homology comparison of DNA sequences and predicted amino acid sequences was carried out using ClastalW program on the DNA Data Bank of Japan, DDBJ (http://clustalw.ddbj.nig.ac.jp/top-e.html). The sequences

similarity analysis was carried out using Blast program on the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov/). Phylogenetic tree was drawn using Phylogeny.fr (Dereeper et al., 2008).

Acknowledgements

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