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Short sequence-paper

Cloning of a cDNA encoding a putative metal-transporting P-type ATPase from *Arabidopsis thaliana* [1]

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Abstract

Metal-transporting P-type ATPases were recently proposed to constitute a newly emerged sub-family of cation-transporting P-type ATPases, and are known to occur widely in prokaryotes and eukaryotes. However, no instance has been reported for higher plants. A cDNA clone encoding a metal-transporting P-type ATPase was thus searched for, if present, and was identified in *Arabidopsis thaliana*. The amino acid sequence, predicted from the determined nucleotide sequence for the cloned cDNA, shows all the critical features common to known metal-transporting P-type ATPases. This plant P-type ATPase has a typical metal-binding motif at its N-terminal portion. The newly isolated *Arabidopsis* gene, named PAA1, provides us with the first instance of putative metal-transporting P-type ATPases in higher plants. Some results of genomic analyses for this gene are also presented.

Keywords: ATPase, P-type; Cation transport; Metal transport; cDNA sequencing; (*A. thaliana*)

Members of a large family of cation-transporting P-type ATPases are known to participate in some system that controls a wide variety of intracellular cation homeostasis [1–3]. Most P-type ATPases function as cation pumps, either for uptake, efflux, or exchange of each specific cation (H^+, Na^+, K^+, Ca^{2+}, or Mg^{2+}). The classical and familiar P-type ATPases are the Ca^{2+}-ATPase of the muscle sarcoplasmic reticulum and the Na^+/K^+-exchange ATPase of animal cell membranes [1–3]. The number of identified P-type ATPases is growing rapidly. In this context, an intriguing recent finding is that certain P-type ATPases have a short consensus motif in the N-terminal portion, which is implicated in metal-binding [4–6]. This dithiol motif (GMxCxxC) was first found in a set of bacterial P-type ATPases, e.g., CadA (a Cd^{2+}-ATPase of *Staphylococcus aureus*) [7] and CopA (a Cu^{2+}-ATPase of *Enterococcus hirae*) [8]. More recently, certain eukaryotic P-type ATPases (Menkes- and Wilson-ATPases) were also found to contain this putative metal-binding motif [4–6]. These human P-type ATPases are implicated in syndromes caused by defects in copper metabolism [9–12]. Putative Cu^{2+}-ATPases were also found in lower eukaryotes (e.g., yeast) [13]. Therefore, such metal-transporting ATPases can be classified as a new family of P-type ATPases, and appear to occur widely both in prokaryotes and eukaryotes [14,15].

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank databases under the accession number D89981.

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We and others recently identified Cu^{2+}-ATPases in a cyanobacterium, *Synechococcus* species [16–18], which harbors a photosynthetic apparatus (thylakoid) similar in structure and function to that located in the chloroplasts of higher plants. The *Synechococcus* Cu^{2+}-ATPase, named PacS, was shown to be involved in Cu^{2+}-resistance of cells [17]. This finding supports further the idea that metal-transporting P-type ATPases occur widely in many cell types. Nevertheless, so far, no instance has been reported for higher plants, in which a number of classical P-type ATPases have been found [14]. Needless to say, the metal-homeostasis is particularly important for plant physiology. We report, for the first time, cloning a cDNA that encodes a putative metal-transporting P-type ATPase in *Arabidopsis thaliana*.

A comparison of the amino acid sequences of a set of metal-transporting ATPases enables us to design a pair of degenerated oligonucleotide mixtures, which could be used as primers for PCR-amplification. These two 17-mer oligonucleotides correspond to the sequences, DKTGTL (5′-primer; dGA(TC)AA(AG) AC(AGCT)GG(AGCT)AC(AGCT)CT,) and DG (T/I/V)ND(A/S) (3′-primer; dG(CA)(AG)TC(AG) TT(AGCT)(AG)(TC)(AGCT)CC(AG)TC), respectively. Using these primers, PCR amplification was carried out for a cDNA plasmid-bank of *Arabidopsis* (94°C, 1 min/55°C, 2 min/72°C, 3 min; 25 cycles). This *Arabidopsis* cDNA library in the expression vector pBluescript™ was constructed by a modified vector-primer method of Mori et al. (poly(A)$^+$ RNA was prepared from 7-day-old dark growth seedling of *Arabidopsis thaliana* ecotype Lansberg *erecta*) [19]. Two groups of DNA segments were reproducibly amplified under the conditions used. One group migrated at positions corresponding to 600–700 bp, and the other group migrated at positions corresponding to above 1000 bp. It was assumed that the larger segments originated from cDNAs encoding classical P-type ATPases. Thus, the former ones were cloned onto pUC19, and then several insert DNAs were subjected to nucleotide sequencing. Among these PCR segments thus sequenced, only one was revealed to encode a desirable open-reading-frame (ORF), whose predicted amino acid sequence is highly similar to the corresponding regions of the P-type ATPases, including PacS Cu^{2+}-ATPase and Wilson Cu^{2+}-ATPase (Fig. 1). Analyses by the FASTA program (provided by an e-mail server of DDBJ, Japan) revealed that the *Arabidopsis* sequence is 42.6% identical to PacS ATPase in the overlapping 204 amino acids, and is 37.9% identical to Wilson ATPase in the overlapping 132 amino acids. However, the same analysis showed that the *Arabidopsis* sequence is only 28.4% identical to the human classical Ca^{2+}-transporting ATPase in the overlapping 102 amino acids. Therefore, the

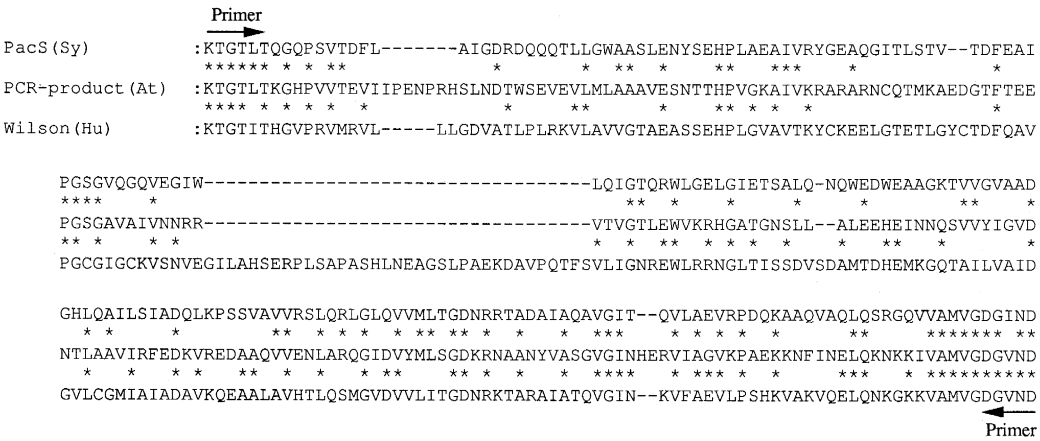

Fig. 1. Alignment of the amino acid sequence of the PCR-product of *Arabidopsis* (At: middle line), with the each corresponding region of PacS Cu^{2+}-ATPase of *Synechococcus* (Sy: upper line) and Wilson Cu^{2+}-ATPase of human (Hu: lower line). Identical amino acids between each pair (Sy/At and At/Hu) are indicated by asterisks. The amino acid sequences corresponding to the primers used for PCR are indicated by arrows. The polymerase chain reaction (PCR) was carried out with a Thermal Cycler (Perkin-Elmer Cetus) with Taq polymerase (Pharmacia). The conditions were those recommended by the supplier.

predicted sequence is significantly more similar to those of known metal-transporting ATPases than others. Note also that no sequence identical to the one isolated in this study was registered in the *Arabidopsis* databases.

To isolate a cDNA encompassing the region corresponding to the PCR-segment, a cDNA λgt11-bank of *Arabidopsis* was screened by means of plaque hybridization with the cloned PCR-segment as a probe. This *Arabidopsis* cDNA library in λgt11 expression vector was a gift from Shinozaki (RIKEN, Japan) (poly(A)$^+$ RNA was prepared from greening rosettes of *Arabidopsis thaliana* ecotype Columbia). Several positive cDNAs were thus identified under the conditions of relatively high stringency (hybridization, 68°C; washing, 68°C). Restriction analyses showed that these cDNA with different lengths encompass each overlapping sequence. Thus, the largest representative (about 3.3 kb) was subjected to nucleotide sequencing. The result revealed that the insert DNA consists of 3229 nucleotides with a poly(A)$^+$ tail (DDBJ/GenBank/EMBL, accession No. D89981). The longest ORF, then predicted, is shown as the amino acid sequence starting with a methionine residue (Fig. 2).

An extensive inspection of this predicted ORF consisting of 949 amino acids revealed several noticeable characteristics. The amino acid sequence of the PCR-segment was found in the relatively C-terminal portion of ORF (positions 599–812) (note that a few amino acid substitutions were found, this may be due to an ecotype variation). In any case, a crucial issue is whether or not this ORF has features characteristic of P-type ATPases. This is indeed the case, as judged by the fact that the predicted amino acid sequence shows several conserved regions common to all P-type ATPases, as follows (Fig. 2). At least four regions with ascribed biochemical functions are known to be common to all P-type ATPases, namely, 'phosphatase', 'ion-transduction', 'phos-

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MESTLSAFSTVKATAMARSSGGPSLPLLTISKALNRHFTGARHLHPLLLARCSPSVRRLG  60

GFHGSRFTSSNSALRSLGAAVLPVIRHRLECLSSSSPSFRSISSGGGSGFGGYNGGSGGG 120

GGGGSESGDSKSKLGANASDGVSVPSSDIIILDVGGMTCGGCSASVKKILESQPQVASAS 180
                                  Metal-binding
VNLTTETAIVWPVPEAKSVPDWQKSLGETLANHLSNCGFQSTPRDLVTENFFKVFETQPK 240

DKQARLKESGRELAVSWAPCAVCLVGHLTHFLGVNAPWIHAIHSTGFHVSLCLITLLGPG 300

RKLVLDGIKSLLKGSPNMNTLVGLGALSSFSVSSLAAMIPKLGWKTFFEEPVMLIAFVLL 360

GRNLEQRAKIKATSDMTGLLSVLASKARLLLDGDLQNSTVEVPCNSLSVGDLVVILPGDR 420

VPADGVVKSGRSTIDESSFTGEPLPVTKESGSQVAAGSINLNGTLTVEVHRSGGETAVGD 480
Phosphatase
IIRLVEEAQSREAPVQQLVDKVAGRFTYGVMALSAATFTFWNLFGAHVLPSALHNGSPMS 540

LALQLSCSVLVVACPCALGLATPTAMLVGTSLGARRGLLLRGGDILEKFSLVDTVVFDKT 600
                          Ion-transduction                Phosphorylation
GTLTKGHPVVTEVIIPENPRHNLNDTWSEVEVLMLAAAVESNTTHPVGKAIVKAARARNC 660

QTMKAEDGTFTEEPGSGAVAIVNNKRVTVGTLEWVKRHGATGNSLLALEEHEINNQSVVY 720

IGVDNTLAAVIRFEDKVREDAAQVVENLTRQGIDVYMLSGDKRNAANYVASVVGINHERV 780

IAGVKPAEKKNFINELQKNKKIVAMVGDGINDAAALASSNVGVAMGGGAGAASEVSPVVL 840
                          ATP-binding
MGNRLTQLLDAMELSRQTMKTVKQNLWWAFGYNIVGIPIAAGVLLPLTGTMLTPSMAGAL 900

MGVTSLGVMTNSLLLRYRFFSNRNDKNVKPEPKEGTKQPHENTRWKQSS             949
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Fig. 2. Deduced amino acid sequence of PAA1. The longest amino acid sequence was predicted from the nucleotide sequence of the cDNA clone, named PAA1, of *Arabidopsis*. Four regions with ascribed biochemical function, which are known to be common to all P-type ATPases, are underlined. In these regions, each highly conserved motif is boxed. In addition, two motifs (i.e., metal-binding and CPC), both of which are particularly characteristic for metal-binding P-type ATPases, are also boxed. Analyses of nucleotide and amino acid sequences were carried out with several analytical programs, including the FASTA and BLAST searches, which were provided by appropriate WWW servers.

phorylation', 'ATP-binding'. All of these characteristics were found in the predicted amino acid sequence with an appropriate fashion, as shown in Fig. 2. Besides these, it would be also worth mentioning that the HP-dipeptide that is highly conserved in P-type ATPases was found at an appropriate position carboxy-terminal to the presumed phosphorylation site in the *Arabidopsis* sequence. From these results, we conclude that the isolated cDNA clone corresponds to an *Arabidopsis* gene encoding a novel P-type ATPase, and hereafter this gene is named PAA1 (P-type ATPase of *Arabidopsis*).

The entire structural design of PAA1 is highly similar to the metal-transporting P-type ATPases including the *Synechococcus* Cu^{2+}-ATPase, PacS, and the human Menkes Cu^{2+}-ATPase (Fig. 3). More importantly, PAA1 was found to have a sequence (GMTCGGC) at its relatively N-terminal end, which is highly similar to the metal-binding motif mentioned above (Fig. 3). In this sense also, PAA1 is much more similar to the members of the metal-transporting P-type ATPase family than those of other classical P-type ATPase families. Furthermore, in the case of known metal-transporting P-type ATPases, another characteristic sequence, CPC, is found in the region involved in ion-transduction [15]. This sequence was proposed to be significant for metal-transduction. In fact, PAA1 has this sequence at an appropriate position (see Figs. 2 and 3).

For PAA1, standard genomic analyses including Southern and Northern hybridization were carried out. A sample of genomic DNA from *Arabidopsis* was digested either with *Eco*RI or *Hin*dIII, and then analyzed by Southern hybridization with an appropriate probe (i.e., the original PCR-segment). In each case, a single discreet band was detected (Fig. 4A). Then, samples containing poly(A)$^+$ mRNA were also isolated from whole plants, which were grown for 24 h with a medium supplemented with exogenous copper ($CuSO_4$: 0 mM, 0.01 mM, or 1 mM). These samples were subjected to Northern hybridization with the same probe described above under the standard conditions. However, no significant positive signal was detected. Then, a method of reverse transcriptase (RT)-PCR was applied for these mRNA samples with an oligo-T primer, and subsequently with the specific 5′/3′-primers. The PCR-products were analyzed by Southern hybridization with the same specific probe described above. In this case, a hybridized band with the predicted size (about 630 bp) was detected for each preparation, as shown in Fig. 4B. The intensity of each hybridized band did not vary depending upon the concentration of $CuSO_4$ added in the growth medium. Note here that we intended to carry out this RT-PCR solely to detect mRNA which would be expressed in a very low amount. In any event, these results of genomic analyses suggest that at least a single copy of the PAA1 gene is present in the *Arabidopsis* genome, and its expression appears to be very low under the growth conditions tested.

The conservation of all essential motifs of P-type ATPases in PAA1 leaves little doubt that the gene specifies a P-type ATPase. Importantly, alignments of the entire P-Type ATPase sequences in the current protein databases clearly place PAA1 closest to

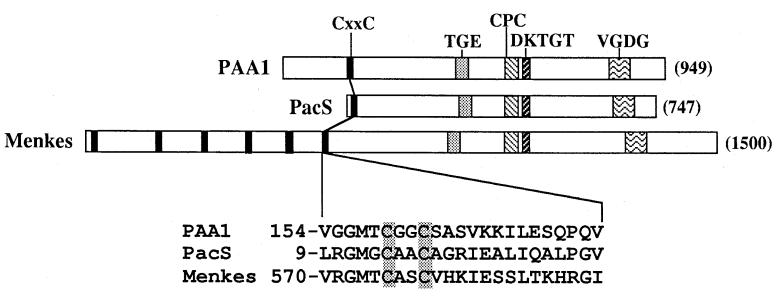

Fig. 3. Schematic representation of the structural design of PAA1, in comparison with the previously characterized typical metal-transporting P-type ATPases (the PacS and Menkes ATPases). Four regions with ascribed biochemical function, which are known to be common to all P-type ATPases, are indicated (see Fig. 2). In these regions, each highly conserved amino acid motif is specified. Each presumed metal-binding motif is indicated by a black box. In addition, the corresponding sequences are aligned to highlight the characteristic dithiol motif (CxxC).

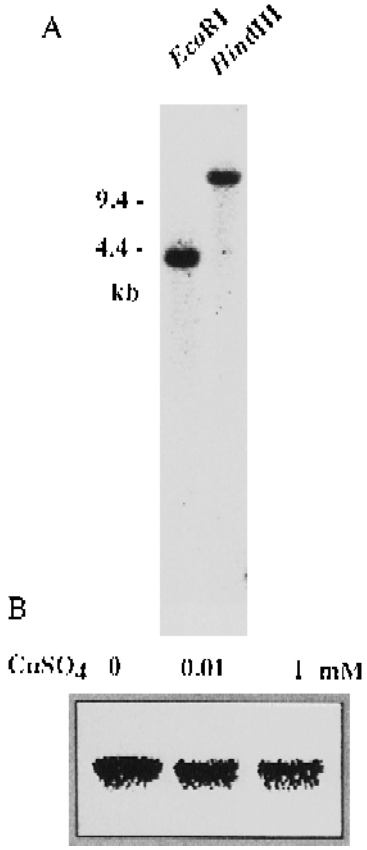

Fig. 4. Hybridization analyses for the *Arabidopsis* APP1 gene and its transcript. (A) Total genomic DNA from *Arabidopsis* was digested either with *Eco*RI or *Hin*dIII, and then the digests were analyzed by Southern hybridization with an appropriate probe (i.e., the original PCR-segment, note that neither *Eco*RI site or *Hin*dIII site is in this probe). (B) Total poly(A)$^+$ mRNA was also isolated from whole plants, which were treated for 24 h with exogenous copper in a growth medium ($CuSO_4$: 0 mM, 1 mM, or 10 mM). These samples were subjected to RT-PCR with an oligo-T primer, and subsequently with specific D/G-primers. The PCR-products were analyzed by Southern hybridization with the same specific probe described above. Recombinant DNA techniques including Southern hybridization and Northern hybridization were carried out according to conventional laboratory methods [20]. The reverse transcriptase (RT)-PCR was also according to the method described in [20].

cyanobacterial PacS-ATPase [16,17], and CtaA-ATPase [18], both of which were previously proposed to function as a Cu^{2+}-pump in the photosynthetic bacteria. Furthermore, a second group of P-type ATPases, to which PAA1 exhibits an extensive similarity, turned out to be the human putative Cu^{2+}-ATPases from Menkes syndrome and Wilson disease, and their related mammalian homologues. Based on these, together with the fact that PAA1 contains a characteristically conserved metal-binding motif, one can assume that this is a member of metal-transporting P-type ATPases. Even so, it should be emphasized that the presumed metal-substrate of PAA1 could be Cu^{2+}, Cd^{2+}, or perhaps others. In this respect, an extensive analysis should be carried out not only to demonstrate that PAA1 is indeed a metal-transporting ATPase, but also to determine its metal-selectivity.

Whatever the metal-substrate of PAA1 is, the discovery of metal-transporting ATPase in higher plants is intriguing as in the case of human Menkes and Wilson ATPases. Some heavy metals, like copper, appear to be essential trace elements that are maintained at relatively constant levels in the tissues of all organisms. As well as being an essential trace element, these are often very toxic. Therefore, the heavy metal-homeostasis appears to be an essential physiological event for all cell types. Consequently, regulated transport mechanisms must exist for their absorption, delivery and excretion. These conceptual views are true even for higher plants. Nevertheless, studies on such a heavy metal-homeostasis mediated by metal-pumps in higher plant cells are still at a very early stage. In this regard, our finding of occurrence of a putative metal-transporting ATPase in the model plant should provide us with an avenue to address the relevant issues in higher plants.

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