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Biochemical Characterization of AtHMA6/PAA1, a Chloroplast Envelope Cu(I)-ATPase*

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Background: There is a lack of biochemical characterization of plant P_{IB-1}-type ATPases (copper transporters).

Result: Phosphorylation assays show that the plant P-type ATPase PAA1 is activated by monovalent ions Cu⁺/Ag⁺. Yeast expression validates *in vivo* the ionic selectivity of the transporter.

Conclusion: PAA1 is a high affinity Cu⁺ transporter of the chloroplast envelope.

Significance: This study provides the first biochemical characterization of a plant copper ATPase.

Copper is an essential plant micronutrient playing key roles in cellular processes, among them photosynthesis. In *Arabidopsis thaliana*, copper delivery to chloroplasts, mainly studied by genetic approaches, is thought to involve two P_{IB}-type ATPases: AtHMA1 and AtHMA6/PAA1. The lack of biochemical characterization of AtHMA1 and PAA1, and more generally of plant P_{IB}-type ATPases, is due to the difficulty of getting high amounts of these membrane proteins in an active form, either from their native environment or after expression in heterologous systems. In this study, we report the first biochemical characterization of PAA1, a plant copper-transporting ATPase. PAA1 produced in *Lactococcus lactis* is active, forming an aspartyl phosphate intermediate in the presence of ATP and the adequate metal ion. PAA1 can also be phosphorylated using inorganic phosphate in the absence of transition metal. Both phosphorylation types allowed us to demonstrate that PAA1 is activated by monovalent copper ions (and to a lower extent by silver ions) with an apparent affinity in the micromolar range. In agreement with these biochemical data, we also demonstrate that when expressed in yeast, PAA1 induces increased sensitivities to copper and silver. These data provide the first enzymatic characterization of a P_{IB-1}-type plant ATPase and clearly identify PAA1 as a high affinity Cu(I) transporter of the chloroplast envelope.

Metal ions are essential for plant growth, having both structural and catalytic roles in proteins involved in a wide variety of cellular processes. However, their concentration must be

tightly regulated as these ions become toxic when present in excess. Therefore, plants have acquired different cellular mechanisms that ensure safe handlings of metal ions, from assimilation to distribution within the plant, and storage. Among the mechanisms that contribute to metal homeostasis, metal transport plays a key role. When compared with other organisms, plant genomes encode many different types of metal transporters with their own ionic specificity, expression pattern, and subcellular localization (for review, see Refs. 1–3).

P-type ATPases are multispanning membrane proteins that translocate ions across plasma or organelle membranes at the expense of ATP consumption (4). Their catalytic cycle can be reduced to a four-step process (Fig. 1) accounting for the coupling of ion motion to ATP hydrolysis, with the formation of transient phosphorylated states resulting from ATP γ -phosphate transfer to the conserved aspartic residue of the DKTGT motif. In its free state *E*, the P-type ATPase binds cytoplasmic ions at its high affinity membrane site (Fig. 1, *step 1*). ATP bound to the large cytoplasmic domain of the transporter is then hydrolyzed, leading to the formation of a phosphorylated enzyme (Fig. 1, *step 2*). The ion binding phosphorylated form of the enzyme (Me.E~P) undergoes important conformation changes leading to metal release at the extracytoplasmic side of the membrane (Fig. 1, *step 3*). In the metal-free phosphorylated enzyme *E*-P, the aspartyl phosphate bound is hydrolyzed to bring the enzyme back in its free state *E* (Fig. 1, *step 4*). For several P-type ATPases such as the Na⁺/K⁺- or H⁺/K⁺-ATPases, a second ion binds from the extracytoplasmic side at step 3 and, on hydrolysis of the phosphorylated Asp (step 4), is released to the cytoplasmic side (4).

On the basis of sequence analysis, P-type ATPases have been classified in several subfamilies; among them, type IB corresponds to the transition metal-transporting ATPases (5). On an enzymatic standpoint, P_{IB}-type ATPases display all the major trademarks of P-type ATPases (6). According to the presence of

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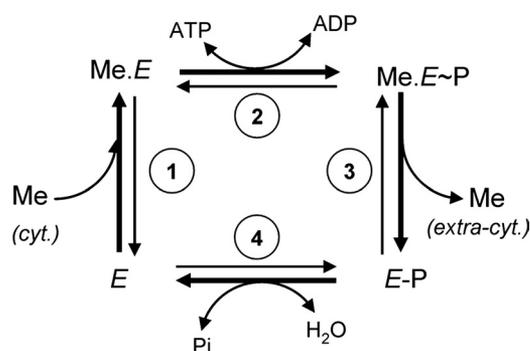


FIGURE 1. **Catalytic cycle of P-type ATPases.** The bold arrows correspond to the forward cycle of P-ATPases requiring ATP. The four steps are reversible. *cyt.*, cytoplasmic; *extra-cyt.*, extracytoplasmic.

conserved residues in transmembrane helices 6, 7, and 8, P_{IB} -type ATPases have been divided into five subgroups: (i) subgroup IB-1 of Cu^+ - and Ag^+ -ATPases; (ii) subgroup IB-2 of Cd^{2+} -, Zn^{2+} -, and Pb^{2+} -ATPases; (iii) subgroup IB-3 of Cu^+ / Cu^{2+} -ATPases; (iv) subgroup IB-4 of putative Co^{2+} -ATPases; and (v) subgroup IB-5 of P_{IB} -type ATPases with no assigned specificity (7).

The *Arabidopsis thaliana* genome encodes eight P_{IB} -type ATPases (8, 9). HMAs² 5, 6, 7, and 8 are members of the subgroup IB-1. In plants, the expression of AtHMA5 is enhanced in the presence of copper and the *hma5* knock-out mutant is hypersensitive to copper, suggesting that AtHMA5 could be involved in copper detoxification in *A. thaliana* roots (10). Originally isolated in the screening of *Arabidopsis* mutants altered in hormone receptor specificity, AtHMA7 (also called RAN1) was found to functionally replace the yeast Cu^+ -ATPase Ccc2p. It was proposed to be localized in the post-Golgi membrane, providing copper to the ethylene receptor (11–13). AtHMA6/PAA1 and AtHMA8/PAA2 have been localized in the chloroplast envelope and thylakoids, respectively. Because the outer membrane of the chloroplast envelope is not a selective barrier (due to the presence of broad specificity pores), PAA1 is expected to be a specific transporter of the inner membrane of the chloroplast envelope. PAA1 and PAA2 mutations were shown to strongly reduce chloroplast copper content (in the stroma for *paa1* and in the thylakoid for *paa2*), and consequently, copper-dependent activities (Cu/Zn-superoxide dismutase, plastocyanin-dependent photosynthetic electron transport). From *in planta* analyses, it was proposed that PAA1 provides copper first to a metallochaperone that could interact with the chloroplast Cu/Zn-superoxide dismutase and also to PAA2, which could then provide copper to the plastocyanin in the thylakoid lumen (14, 15).

HMAs 2, 3, and 4 belong to the subgroup IB-2. AtHMA2 and AtHMA4 ensure Zn^{2+} translocation from roots to the shoot (16–18). AtHMA2 is predominantly expressed at the plasma membrane of cells from vascular tissues of roots, stems, and leaves, where it works as an efflux system. When expressed in yeast, AtHMA2 displays Zn^{2+} - and Cd^{2+} -dependent (and to a lower extent, Pb^{2+} -, Ni^{2+} -, Cu^{2+} -, and Co^{2+} -dependent)

ATPase activities and forms an acid-stable phosphorylated intermediate in the presence of metals (16, 18). AtHMA4 is localized at the plasma membrane of cells from tissues surrounding the root vascular vessels. A null *hma4* mutant exhibits a lower translocation of Zn^{2+} and Cd^{2+} from roots to the shoot, whereas AtHMA4-overexpressing lines display an increase in Zn^{2+} and Cd^{2+} shoot content, suggesting that AtHMA4 is involved in providing metal to the xylem (17). When expressed in bacteria or yeast, AtHMA4 restores Zn^{2+} tolerance to *Escherichia coli zntA* mutant and Cd^{2+} tolerance to yeast $\Delta ycf1$ strain (19, 20). AtHMA3 is located at the vacuolar membrane, with a predominant expression in guard cells, hydathodes, vascular tissues, and the root apex. The *hma3* knock-out mutant was found to be more sensitive to Zn^{2+} and Cd^{2+} , whereas ectopic overexpression of AtHMA3 improved plant tolerance to Cd^{2+} , Co^{2+} , and Pb^{2+} . It was therefore suggested that AtHMA3 contributes to heavy metal detoxification by participating in their vacuolar sequestration (21). In *Saccharomyces cerevisiae*, AtHMA3 expression restores normal metal tolerance to the Cd^{2+} - and Pb^{2+} -hypersensitive $\Delta ycf1$ strain but not to the Zn^{2+} -hypersensitive $\Delta zrc1$ strain (22).

AtHMA1 was classified in subgroup IB-4 (putative Co^{2+} -ATPase) on the basis of its amino acid sequence (7). In 2006, we localized AtHMA1 in the chloroplast envelope (theoretically in the inner membrane as for PAA1) and showed that its activity was enhanced by copper (23). In addition, *hma1* mutants were found to exhibit a reduced plastidial copper content as well as a reduced chloroplast Cu/Zn-superoxide dismutase activity. These data led us to suggest that AtHMA1 functions as a copper transporter (23). Later, Moreno *et al.* (24) showed that the precursor form of AtHMA1 (*i.e.* including the chloroplast transit peptide) could complement the lack of yeast Ca^{2+} -ATPases as well as restore normal metal resistance of a yeast strain lacking the heavy metal ABC transporter Ycf1p. Additionally, ATPase activity and calcium flux measurements led them to propose that AtHMA1 could act as a Ca^{2+} , Cd^{2+} , Zn^{2+} , Cu^{2+} , and Co^{2+} transporter. More recently, *in planta* analysis and complementation assays in yeast highlighted a possible role of AtHMA1 in Zn^{2+} detoxification of *A. thaliana* chloroplasts (25).

As illustrated above, biochemical characterization of plant P_{IB} -type ATPases is either lacking or at least very partial, with the exception of the Zn^{2+} -ATPase AtHMA2 of the PIB-2 subgroup. This is mainly explained by the difficulty to get high amounts of these membrane proteins in an active form either from their native environment or after expression in heterologous systems. Recently, we have shown that *Lactococcus lactis*, a Gram-positive lactic bacterium, is an attractive system for efficient production of plant membrane proteins and especially for P_{IB} -type ATPases; AtHMA1, AtHMA3, and PAA1, produced at 300–900 $\mu g/liter$, account for 1–3% of the total membrane proteins (26). Thus, this heterologous system provides the unique opportunity to get access to enzymatic parameters of these plant ATPases, parameters that cannot be apprehended by the characterization of plant mutants alone.

As described above, the chloroplast envelope contains two P_{IB} -type ATPases, AtHMA1 and PAA1, whose specific and relative roles in metal transport are not well understood; either due to the puzzling results concerning the ionic selectivity of

² The abbreviations used are: HMA, heavy metal-associated domain-containing protein; BCA, bicinchoninic acid; BCS, bathocuproine disulfonate.

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AtHMA1 or to the lack of biochemical data on PAA1. To address a part of this issue, we have carried out a biochemical study of PAA1, heterologously produced in *L. lactis*. In this study, we provide the first enzymatic characterization of a P_{IB-1} -type plant ATPase and clearly identify PAA1 as a high affinity Cu(I) transporter of the chloroplast envelope.

EXPERIMENTAL PROCEDURES

PAA1 Expression in *L. lactis*—The expression of AtHMA6/PAA1 (At4g33520; Q9SZC9) from the pNZ8148 vector and the preparation of bacterial membranes were performed as described previously (26) with the following modifications. After induction by nisin, bacteria were collected by centrifugation, resuspended in 20 mM HEPES (pH 6.0), 6% (w/v) glycerol and kept at -80°C . After thawing, bacteria were lysed by sonication followed by two passages through a One Shot (Constant Systems Ltd., Northants, UK) at 35,000 p.s.i. (2.3 kbar). The lysate was centrifuged at $10,000 \times g$ for 13 min, at 4°C , to remove unbroken cells and large debris, and the resulting supernatant was centrifuged at $150,000 \times g$ for 70 min, at 4°C . The $150,000 \times g$ pellet, containing the membrane proteins, was resuspended in 2 ml of 20 mM HEPES (pH 6.0), 300 mM sucrose, frozen in liquid nitrogen, and stored at -80°C . The DNA sequence used in the present study to produce PAA1 in *L. lactis* codes for the mature form of the protein, *i.e.* the form lacking the 102 first amino acid residues corresponding to the chloroplast transit sequence (14). Site-directed mutagenesis (QuikChange[®], Stratagene) was performed on the pBS-RfA-PAA1 plasmid to produce the D598A mutant (afterward named PAA1-AKT). In this mutant, the aspartate residue of the DKTGT consensus sequence was replaced by an alanine, a substitution known to prevent the transient phosphorylation of any P-type ATPase. Both PAA1 and PAA1-AKT contain a *Strep*-Tag II (IBA, Goettingen, Germany) at their C-terminal end (26).

SDS-Polyacrylamide Gel Electrophoresis and Detection—Protein content was estimated using the Bio-Rad protein assay reagent (Bio-Rad). SDS-PAGE analyses were performed as described by Chua (27). Detection of the *Strep*-tag II was performed using the *Strep*-Tactin HRP conjugate (IBA) at a 1/10,000 dilution followed by a home-made ECL detection.

Phosphorylation Assays—Enzyme phosphorylation from ATP and P_i was performed as described previously (28) with the following modifications. Phosphorylation from P_i was performed at 30°C in a medium containing 1 mg ml^{-1} of *L. lactis* membrane preparations, 20 mM HEPES (pH 6.0), 10 mM MgCl_2 , 20% (v/v) Me_2SO (dimethyl sulfoxide), in the presence of various concentrations of metals, as indicated in the legends for Figs. 5 and 6, or 1 mM EGTA or a mix of 1 mM bicinchoninic acid (BCA) and 100 μM bathocuproine disulfonate (BCS). After a 5-min incubation at 30°C , the reaction was started by the addition of 100 μM $^{32}\text{P}_i$ (10–100 $\mu\text{Ci nmol}^{-1}$) and stopped, 10 min later, by the addition of 1 ml of ice-cold 1 mM KH_2PO_4 in 7% (v/v) trichloroacetic acid. Phosphorylation from ATP was performed at room temperature in a medium containing 0.5 mg ml^{-1} of *L. lactis* membrane preparations, 20 mM HEPES (pH 7.0), 100 mM KCl, 5 mM MgCl_2 , 300 mM sucrose, and metals or EGTA or BCA/BCS as indicated. The reaction was started by

the addition of 0.4 μM [γ - ^{32}P]ATP (50–500 $\mu\text{Ci nmol}^{-1}$) and stopped, 30 s later, by the addition of 1 ml of ice-cold 1 mM KH_2PO_4 in 7% (v/v) trichloroacetic acid. After a 30-min incubation on ice, precipitated proteins were collected by centrifugation (10 min at $10,000 \times g$) and washed twice with 1 ml of 1 mM KH_2PO_4 in 7% (v/v) trichloroacetic acid. Proteins were then resuspended in 35 μl of 5 mM Tris- PO_4 , pH 5, 6.7 mM urea, 400 mM DTT, 5% (w/v) SDS, 0.004% (w/v) orange G and loaded on an acidic polyacrylamide gel as described by Weber and Osborn (29). The phosphorylation signal was revealed using a phosphorimaging device (Cyclone, PerkinElmer Life Science) and analyzed using the OptiQuant software (PerkinElmer Life Sciences). The amount of loaded proteins was checked after Coomassie Blue staining.

Cu(I) Determination— Cu^+ concentration was measured with the Cu^+ chelator BCS and using $\epsilon = 12,700 \text{ M}^{-1} \text{ cm}^{-1}$ at 485 nm for the $\text{Cu}(\text{bcs})_2^3$ complex (30). This colorimetric assay specifically detects Cu^+ with a sensitivity of 0.5 μM and with a precision of 0.1 μM .

Complementation and Metal Tolerance Assays in *S. cerevisiae*—For maintenance, the *S. cerevisiae* YPH499 (31) and YPH499-ccc2 Δ strains were grown at 30°C in rich YD medium (1% (w/v) KAT yeast extract (Ohly), 2% (w/v) glucose). Cells were transformed as described previously (32). Plasmid selection was performed by growing cells in synthetic minimal Dropout Base medium (2% (w/v) glucose, 0.17% (w/v) yeast nitrogen base without amino acids, and 0.5% (w/v) ammonium sulfate (Bio 101[®] Systems)) supplemented with dropout powder without uracil and leucine (Bio 101[®] Systems) and 2% (w/v) Agar-Y (Bio 101[®] Systems). The CCC2 complementation assay was performed as described previously (33). Media containing metals (CuSO_4 and AgNO_3) at the indicated concentrations were buffered to pH 6.1 using 100 mM MES/NaOH. The CCC2 gene and the gene coding for the non-phosphorylating mutant CCC2-AKT (D627A) were expressed from a centromeric vector derived from pRS315 (31), under the control of the constitutive and strong PMA1 promoter as described previously (33). The same vector was used for the expression of the cDNA coding for PAA1 modified by the insertion of the *Strep*-tag II coding sequence at its 3' end as well as the cDNA coding for the non-phosphorylating mutant PAA1-AKT (D597A). The cDNA sequence used for expression of PAA1 codes for the mature form of the protein (*i.e.* without its chloroplast transit sequence). Vectors were purified by the MidiPrep method (Macherey-Nagel). Sequencing of PAA1 and PAA1-AKT was performed by Beckman Coulter Genomics.

RESULTS

To get the amount of proteins required for biochemical assays, PAA1 and its inactive form PAA1-AKT (in which the aspartate residue of the phosphorylation sequence DKTGT has been mutated to alanine) were produced in *L. lactis*. In the transformed *L. lactis*, PAA1 was found to stand for 2–3% of the total membrane proteins (26). As shown in Fig. 2A, PAA1 and PAA1-AKT exhibit the same expression pattern when revealed by detection of the *Strep*-tag II inserted at their C-terminal end. In addition to PAA1 forms (indicated by an arrow), three other proteins with molecular masses around 26, 36, and 118 kDa and

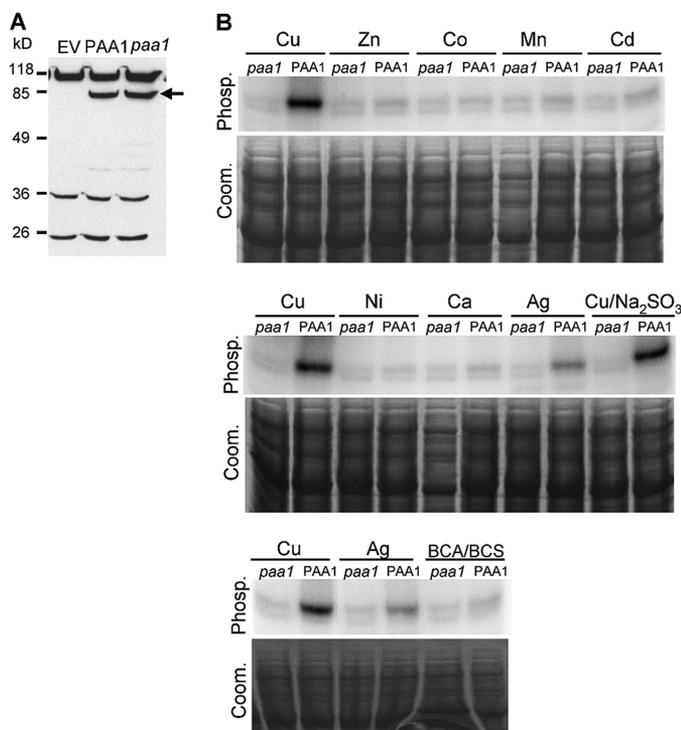


FIGURE 2. Production of PAA1 and PAA1-AKT (*paa1*) in *L. lactis* membranes and metal-dependent phosphorylation from ATP. *A*, expression of PAA1 and PAA1-AKT in *L. lactis*. Total membrane proteins (10 μ g) were separated by SDS-PAGE (10% acrylamide). *Strep*-tag II was detected using a *Strep*-Tactin HRP conjugate. The arrow indicates PAA1 and PAA1-AKT (*paa1*). *EV*, membrane proteins derived from bacteria containing the empty pNZ8148 vector. *B*, phosphorylation from ATP. Membrane preparations containing PAA1 or PAA1-AKT (*paa1*) were phosphorylated in the presence of 5 μ M of various metal ions (with or without Na_2SO_3 for copper) or Cu^+ -specific chelators BCA/BCS. Phosphorylation from ATP was performed as described under "Experimental Procedures." Samples were submitted to an acidic SDS-PAGE, and phosphorylation was detected using a phosphorimaging device. *Phosp.*, phosphorylation signal; *Coom.*, Coomassie Blue-stained gels. *paa1* corresponds to the membranes containing the mutated protein PAA1-AKT.

encoded by the bacterial genome were found to cross-react with the *Strep*-Tactin HRP conjugate. As these proteins were also detected in the membrane proteins extracted from the strain containing the empty vector (Fig. 2A), these signal were used as internal loading standards.

PAA1 Is Phosphorylated from ATP in the Presence of Monovalent Metals—In a first set of experiments, we addressed the question of PAA1 activity and selectivity by performing phosphorylation assays from [γ - 32 P]ATP in the presence of 5 μ M of various metals. As described in the legend for Fig. 1, these experimental conditions should promote forward cycling of PAA1 provided that the supplied metal is transported by the protein. As shown in Fig. 2B, CuCl_2 and AgNO_3 are the only metallic salts promoting PAA1 phosphorylation from ATP. In the presence of the other tested metals (supplied as chloride salts), the only visible signal consists of two faint bands. Also observed with membranes containing PAA1-AKT (*paa1*), a non-phosphorylatable mutant of PAA1, this signal is likely to correspond to endogenous membrane proteins.

To determine which form of copper, Cu^+ or Cu^{2+} , triggered PAA1 phosphorylation, we tested the effects of Na_2SO_3 as a reducing agent generating Cu^+ and of a BCA/BCS mixture as a Cu^+ -specific chelator decreasing the amount of available Cu^+

in the assay. Fig. 2B shows that when Cu^+ chelators are present in the assay, the phosphorylation signal of PAA1 completely disappears, suggesting that PAA1 is activated by monovalent copper. On the other hand, the addition of Na_2SO_3 does not provide any significant enhancement of PAA1 phosphorylation, suggesting that in our experimental conditions and even in the absence of a reducing agent, copper is predominantly under the Cu^+ form.

Using a BCS-based colorimetric assay that specifically detects Cu^+ , we determined that in our assays, copper was actually reduced by the buffer and the *L. lactis* membranes, even in the absence of Na_2SO_3 . Copper reduction is rapid (less than 5 min) and stable (up to 60 min) at room temperature. We measured that in the experimental conditions used in Fig. 2B, 5 μ M of total CuCl_2 corresponded to $3.5 (\pm 0.1) \mu$ M Cu^+ in the absence of Na_2SO_3 . In the presence of up to 1 mM Na_2SO_3 (more than three times the concentration used in Fig. 2B), the measured Cu^+ concentration does not exceed $4.3 (\pm 0.1) \mu$ M. In buffer alone (*i.e.* without *L. lactis* membranes), 1 mM Na_2SO_3 completely reduced 5 μ M of copper. Thus, the partial recovery of Cu^+ (4.3 instead of 5 μ M) in our experimental conditions is likely to be due to the presence of the biological membranes. This was unexpected because the sample is solubilized in detergent before analysis and suggests that membranes might tightly trap the metal (0.7 μ M Cu^+), possibly making it not available to PAA1. Together, these measurements show that Cu^+ concentrations with and without Na_2SO_3 are close, thereby explaining the similar phosphorylation intensities of PAA1 observed in these two conditions. In conclusion, phosphorylation assays from ATP suggest that PAA1 is only activated by monovalent metal ions, Ag^+ but preferentially Cu^+ .

An interesting point provided by Fig. 2B is that efficiencies of copper and silver in activating PAA1 are different. Indeed, quantifications show that if PAA1 phosphorylation intensity in the presence of 5 μ M CuCl_2 is taken as 100%, the phosphorylation intensity in the presence of 5 μ M AgNO_3 is only 40%. To determine whether this difference reflected differences in the whole reaction mechanism of PAA1 or simply in the PAA1 affinity to metals, we performed phosphorylation from ATP within a wide range of copper (with Na_2SO_3) or silver concentrations from 0.05 to 50 μ M. As shown in Fig. 3, A and B, PAA1 phosphorylation is biphasic in the two tested conditions. It displays first an increasing phase with a maximum phosphorylation reached at a metal concentration of 3 μ M followed by a steep decreasing phase. The first phase is likely to represent the normal activation process of a P-type ATPase, and using a Hill fitting, we estimated PAA1 apparent affinity around 0.5 μ M for copper and 1.1 μ M for silver. An interesting point here is that the silver-dependent phosphorylation has a sharp shape, suggesting a positive cooperative mechanism of PAA1 activation by this metal. Above 3 μ M, an increase of metal concentration (copper or silver) resulted in an inhibition of PAA1 phosphorylation almost complete at 30 μ M. As discussed above, PAA1 phosphorylation from ATP displays an unusual dependence to the transported ion (see "Discussion"). This makes the difference in the phosphoenzyme levels observed in Fig. 2B between copper and silver difficult to interpret.

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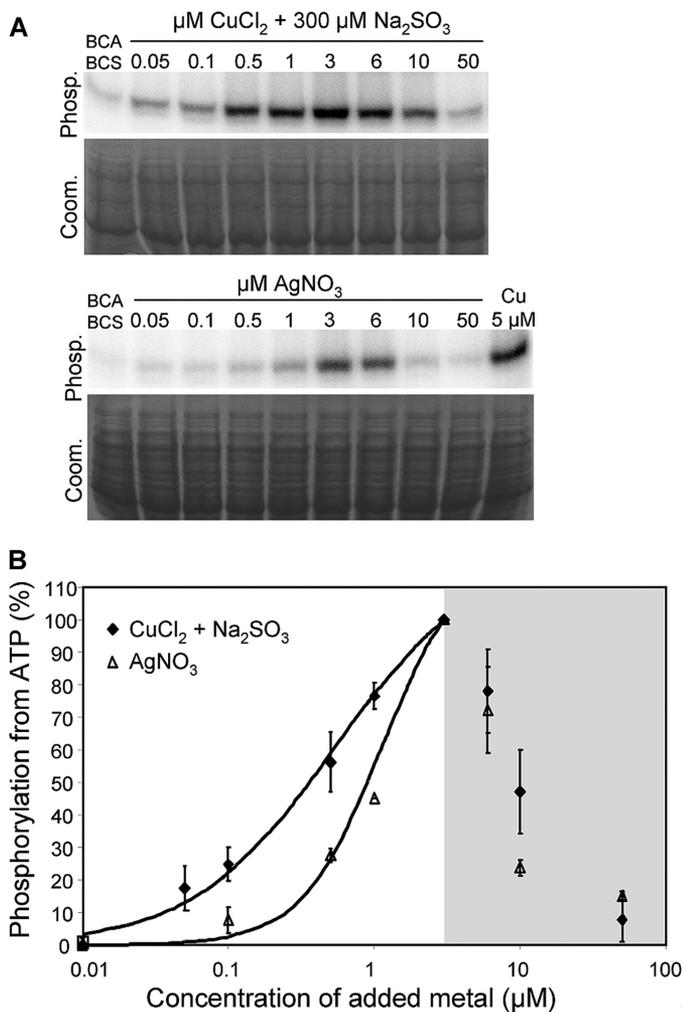


FIGURE 3. AgNO_3 and CuCl_2 dependence of PAA1 phosphorylation from ATP. *A*, *L. lactis* membranes containing PAA1 were phosphorylated as described under "Experimental Procedures." *Phosp.*, phosphorylation signal; *Coom.*, Coomassie Blue-stained gels. *B*, quantifications were made using the OptiQuant software (PerkinElmer Life Sciences). 100% corresponds to the amplitude of phosphorylation measured between the BCA/BCS and the 3 μM metal conditions. Values shown correspond to the mean of three independent experiments. *Error bars* indicate S.E.

PAA1 Phosphorylation from ATP Is Transient—As shown in Fig. 1, P-type ATPases are transiently phosphorylated when cycling in the forward direction. To demonstrate the transitory character of PAA1 phosphorylation from ATP, and thus to exclude phosphorylation from kinase, two types of experiment were performed. In the "kinetic" experiment, the reaction was carried out in the presence of 5 μM CuCl_2 as described previously in the legend for Fig. 2*B*, except that aliquots were acid-quenched at different times after $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ addition to stop the reaction. As expected, if the reaction proceeds through the enzymatic cycle shown in Fig. 1, PAA1 phosphorylation intensity progressively decreases due to ATP consumption (Fig. 4*A*, left). In the "isotopic dilution" experiment, PAA1 phosphorylation intensity decreases almost immediately upon $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ dilution by 10-fold concentrated cold ATP (Fig. 4*A*, right). Together, these two experiments demonstrate that PAA1 is active in *L. lactis* membranes and undergoes a transient phosphorylation from ATP, emblematic of P-type ATPases.

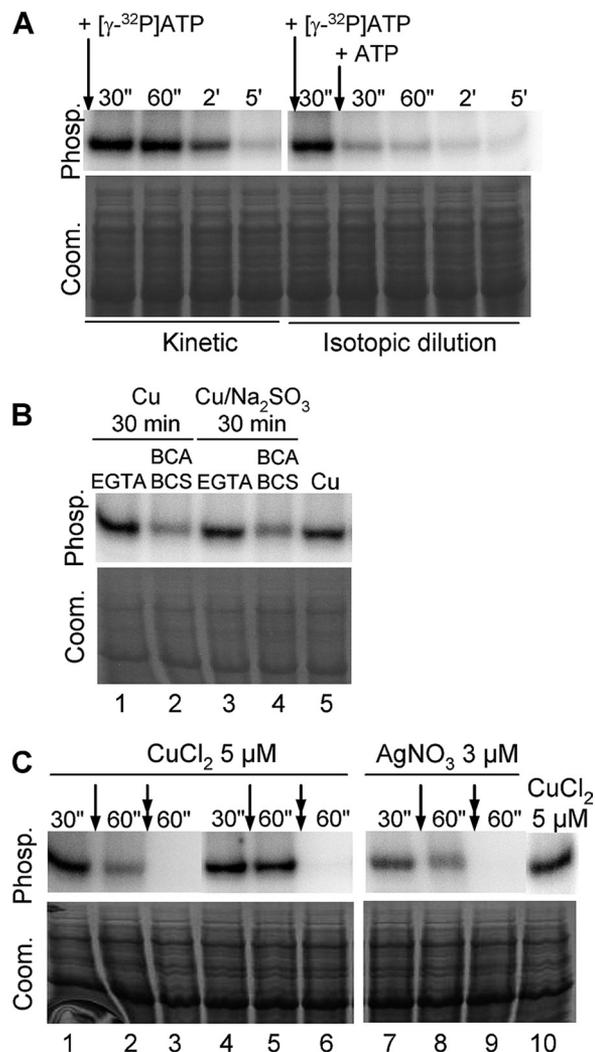


FIGURE 4. PAA1 phosphorylation from ATP is transient. *A*, kinetic and isotopic dilution experiments on *L. lactis* membrane expressing PAA1. In the kinetic experiment, phosphorylation from ATP was performed in the presence of 5 μM CuCl_2 . The reaction was stopped at different times (30 s to 5 min) after $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ addition. For isotopic dilution, membranes containing PAA1 were incubated with 5 μM CuCl_2 and 0.4 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After 30 s, an aliquot was taken and acid-quenched. On the remaining sample, ATP concentration was increased to 4 μM by the addition of cold ATP, and the reaction was stopped at the indicated times. *Phosp.*, phosphorylation signal; *Coom.*, Coomassie Blue-stained gels. *B*, impact of EGTA or BCA/BCS on PAA1 phosphorylation from ATP. *L. lactis* membranes containing PAA1 were preincubated with 5 μM CuCl_2 or with 5 μM CuCl_2 + 200 μM Na_2SO_3 . After 30 min, EGTA or a mix of BCA/BCS was added, and the phosphorylation reaction was started by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. In lane 5, copper was added just before the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, as described previously in the legend for Fig. 2. *C*, ADP induced dephosphorylation of PAA1. PAA1 was phosphorylated from ATP in the presence of 5 μM CuCl_2 (lanes 1–6 and 10) or 3 μM AgNO_3 (lanes 7–9). Following a 30-s reaction, the sample was acid-quenched (lanes 1, 4, 7, and 10) or incubated with chelators (single arrow, lane 5, EGTA; lanes 2 and 8, BCA/BCS) or with chelators plus ADP (double arrow, lane 6, EGTA + ADP; lanes 3 and 9, BCA/BCS + ADP) for 30 s prior to acid-quinching.

Confirmation of PAA1 Activation by Monovalent Metal Ions—Previous experiments depicted in Fig. 2*B* strongly suggested that PAA1 was activated by monovalent metal ions, namely Ag^+ and the most physiological one, Cu^+ . To confirm these results for copper, we treated samples before (Fig. 4*B*) and during (Fig. 4*C*) the phosphorylation reaction with the divalent chelator EGTA or the monovalent chelators BCA/BCS. In the first experiment, membranes containing PAA1 were preincu-

bated with CuCl_2 or ($\text{CuCl}_2 + \text{Na}_2\text{SO}_3$). After 30 min, EGTA or a mixture of BCA/BCS was added just before reaction triggering by ATP. As shown in Fig. 4B, only the BCA/BCS mix was able to significantly reduce PAA1 phosphorylation obtained in the presence of CuCl_2 (with or without Na_2SO_3). The presence of EGTA did not affect the phosphorylation levels no matter what copper conditions were used (Fig. 4B, compare lanes 1 and 3 with lane 5). In the second experiment, chelators were added on the ongoing reaction. As observed previously in the first experiment, phosphorylation is only impaired by the monovalent chelators BCA/BCS (Fig. 4C, compare lanes 1 and 2 with lanes 4 and 5). Together, these two experiments demonstrate that PAA1 phosphorylation from ATP strictly depends on the monovalent form of copper.

In the Presence of ATP, PAA1 Mainly Accumulates in the Me.E~P Form—As shown in Fig. 1, P-type ATPases can reach two phosphorylated intermediates; Me.E~P still binds the ion to be translocated, whereas E~P occurs after ion release. The accumulation of one of these phosphorylated intermediates versus the other depends of the relative velocities of intermediate reactions, a proper feature of the considered P-type ATPase. ADP allows discrimination between these two forms because only Me.E~P is able to provide the phosphoryl group back to ADP. Hence, an excess of ADP drives ATP synthesis from Me.E~P, and consequently, causes the disappearance of Me.E~P.

In the experiment depicted in Fig. 4C, PAA1 was first phosphorylated from ATP in the presence of copper (lanes 1, 4, and 10). We next compared the effect of ADP on the phosphorylation level remaining after the addition of chelators (see above). The comparison of lanes 2 and 3 with lanes 5 and 6 shows that in any case, ADP totally switches off the phosphorylation signal, suggesting that the PAA1 phosphorylated intermediate from ATP mainly consists of the Me.E~P form. The same results were obtained with silver (Fig. 4C, lanes 7–9).

Apparent Affinity of PAA1 to Monovalent Metal Ions Is in the Micromolar Range—P-type ATPase can be phosphorylated from ATP in the forward direction (physiological condition; Fig. 1, steps 1–4) and from P_i in the backward direction (Fig. 1, step 4). Both reactions rely on the presence of the ion to be transported. Although phosphorylation from ATP does require metal ions to occur, metal ions do competitively inhibit phosphorylation from P_i . Hence, the latter also gives access to the ionic specificity of the enzyme. In addition, involving simple equilibrium reactions (Fig. 1, steps 1 and 4), phosphorylation from P_i also provides a more realistic estimation of the metal-enzyme affinity.

Phosphorylation reactions from P_i were performed on *L. lactis* membranes containing PAA1 or the inactive form PAA1-AKT in experimental conditions previously described for other P-type ATPases, such as the SERCA1a Ca^{2+} -ATPase (34) or the CadA Cd^{2+} -ATPase (28), i.e. pH 6, no KCl, and 20% Me_2SO . As shown in Fig. 5, $5 \mu\text{M}$ CuCl_2 totally inhibits acylphosphate formation in the presence or absence of Na_2SO_3 . A lower inhibitory effect is observed with silver, whereas other metals did not prevent PAA1 phosphorylation from P_i (Fig. 5). These results are in good agreement with those obtained from phosphorylation experiments from ATP and confirm the specificity of

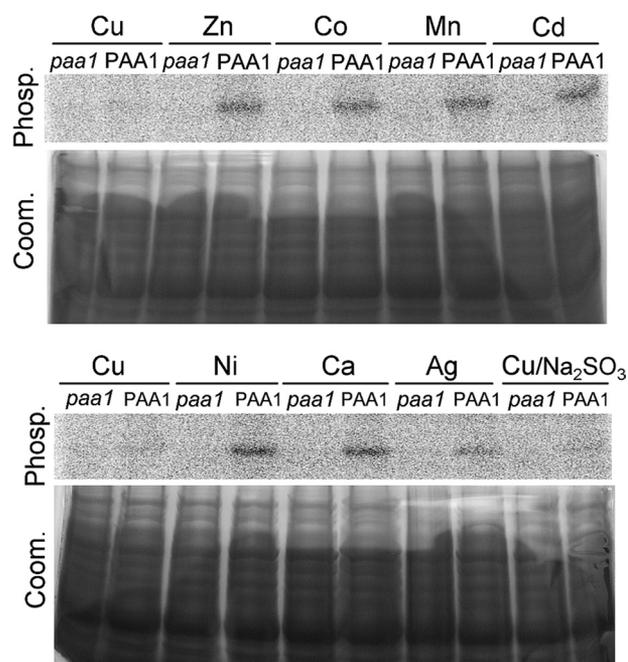


FIGURE 5. Metal-dependent phosphorylation of PAA1 from inorganic phosphate. *L. lactis* membranes containing PAA1 or PAA1-AKT (*paa1*) were preincubated for 5 min at 30 °C in 20 mM HEPES (pH 6.0), 10 mM MgCl_2 , 20% Me_2SO , and various metal ions ($5 \mu\text{M}$). Phosphorylation was initiated by the addition of $100 \mu\text{M}$ $^{32}\text{P}_i$ and stopped after 10 min (at 30 °C) by the addition of 1 mM KH_2PO_4 , 7% (v/v) TCA. Samples were resolved by SDS-PAGE in an 8% acrylamide acidic gel and visualized using a phosphorimaging device. Phosp., phosphorylation signal; Coom., Coomassie Blue-stained gels. *paa1* corresponds to the membranes containing the mutated protein PAA1-AKT.

PAA1 preferentially to copper, and to a lower extent, to silver. Fig. 6B shows a quantification of copper- and silver-dependent acylphosphate formation from P_i at concentrations of metals ranging from 0.05 to 300 μM . The apparent affinities of PAA1 determined from these data were 0.6 μM for copper and 5 μM for silver. These values are in the same range as those determined from phosphorylation experiments from ATP (0.5 μM for copper in the presence of Na_2SO_3 and 1.1 μM for silver, see above) and underline the higher affinity of PAA1 for Cu^+ .

Functional Characterization of PAA1 by Expression in Yeast—To strengthen these biochemical data, we used a yeast complementation assay to validate the ability of PAA1 to transport copper and silver ions *in vivo*. The yeast CCC2 gene encodes for a Golgi-resident Cu^+ -ATPase required to supply the multicopper oxidase Fet3p with copper. Once loaded with copper, the latter migrates to the plasma membrane, where it associates with Ftr1p to create the high affinity iron import complex Fet3p-Ftr1p. Thereby, disruption of the CCC2 gene makes yeast unable to survive under limiting iron and copper conditions (35). This complementation assay has been successfully used for the characterization of Cu^+ -ATPases from diverse origins, as illustrated with the human ATP7A (36) and ATP7B (37) or CUA-1 from *Caenorhabditis elegans* (38). As shown in Fig. 7A, PAA1 expressed from the constitutive PMA1 promoter is unable to restore the growth of the Δccc2 strain on an iron-limiting medium. PAA1, which does not possess any yeast-targeting sequence, probably stays in the endoplasmic reticulum, thereby explaining its inability to functionally replace Ccc2p,

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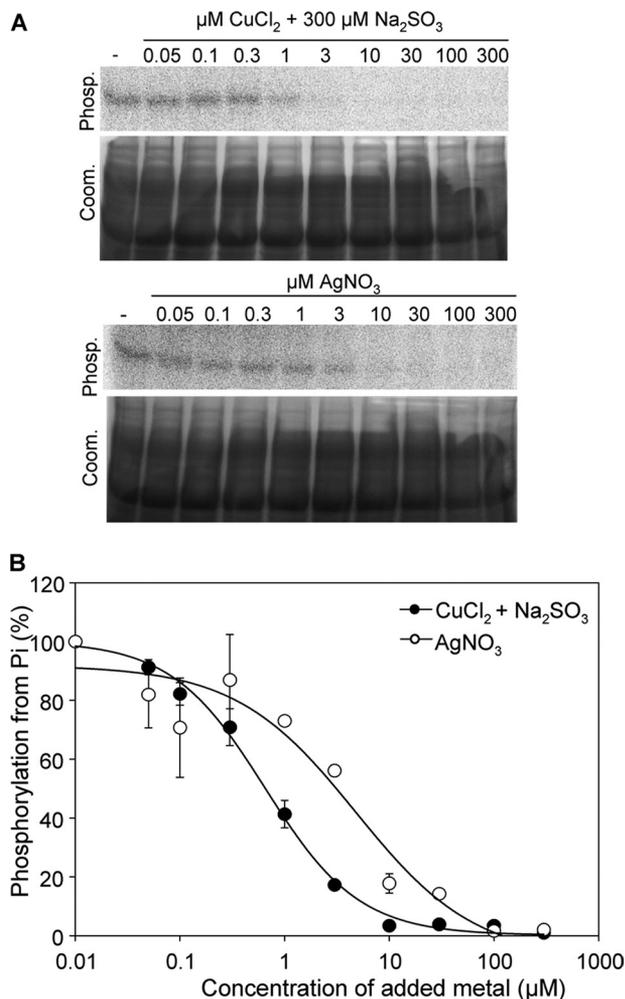


FIGURE 6. CuCl_2 and AgNO_3 concentration dependence of PAA1 phosphorylation from P_i . *A*, membrane fractions (100 μg) containing PAA1 were incubated with various concentrations of CuCl_2 or AgNO_3 . Phosphorylation was initiated by the addition of 100 μM $^{32}\text{P}_i$ and stopped after 10 min by the addition of 1 mM KH_2PO_4 , 7% (v/v) TCA. *Phosp.*, phosphorylation signal; *Coom.*, Coomassie Blue-stained gels. *B*, phosphorylation intensities were quantified using the OptiQuant software. 100% represents the phosphorylation level of PAA1 in the absence of metal. Fittings were performed using a Hill equation with a Hill number close to 1. Values correspond to the mean of three independent experiments. *Error bars* indicate S.E.

a Golgi-resident protein. As an alternative to strengthen the biochemical data, we then examined metal sensitivity of a wild-type yeast strain expressing PAA1. Our experience with CadA, the Cd^{2+} -ATPase from *Listeria monocytogenes* whose expression in yeast increases sensitivity to cadmium (39), led us to test whether PAA1 could similarly modify yeast tolerance to copper and silver. As shown in Fig. 7B, PAA1, but not the non-phosphorylating D598A mutant (PAA1-AKT), does actually increase yeast sensitivity to both copper and silver. On the other hand, expression of CCC2 or the non-functional D627A CCC2 mutant (CCC2-AKT) does not change yeast sensitivity to copper and silver at the tested concentrations. These results obtained with PAA1 can be interpreted along with those obtained with CadA (39, 40). When active in yeast, PAA1 would overload endoplasmic reticulum with copper or silver. The accumulation of these metals would impair endoplasmic reticulum functions and lead to growth defects.

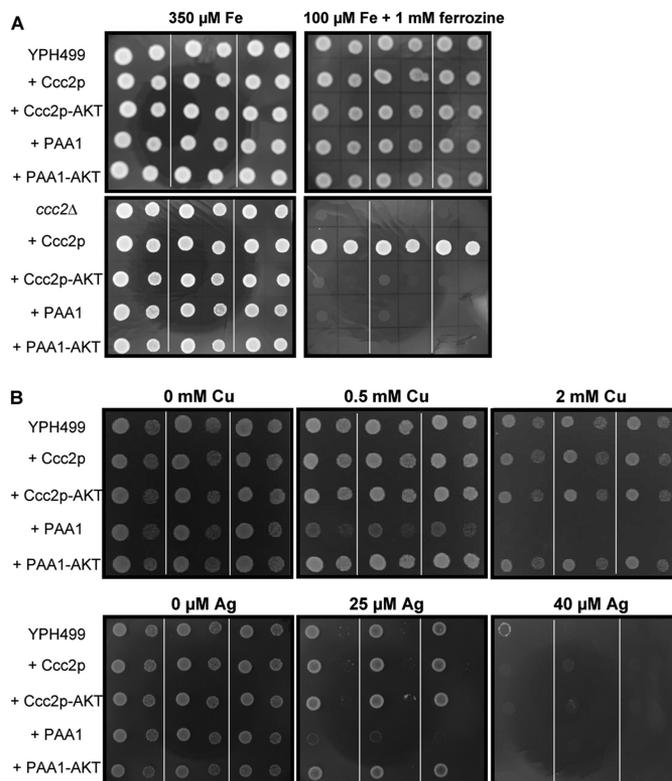


FIGURE 7. Phenotypes of yeast strains expressing PAA1. *A*, CCC2 complementation assay. The yeast strains YPH499 and YPH499-*ccc2* Δ expressing the yeast Cu^+ -ATPase Ccc2p, a non-functional Ccc2p mutant (indicated by Ccc2p-AKT), PAA1 (indicated by PAA1), or a non-functional PAA1 mutant (indicated by PAA1-AKT) were grown on selective media containing either 1 mM ferrozine + 350 μM iron or 1 mM ferrozine + 100 μM iron (iron-limiting medium). For each expression condition, three independent transformants were grown on the two media. Dilution 1 and 1/10 were spotted as 2- μl drops. Dilution 1 corresponds to an optical density of 1.5 at 600 nm. *B*, metal sensitivity of yeast strains expressing PAA1. The yeast strain YPH499 expressing the yeast Cu^+ -ATPase Ccc2p, a non-functional Ccc2p mutant (indicated by Ccc2p-AKT), PAA1 (indicated by PAA1), or a non-functional PAA1 mutant (indicated by PAA1-AKT) was grown on selective media supplemented with copper or silver at the indicated concentrations. Drop tests were performed as described above.

The sensitivity to copper and silver correlated with the presence of a functional PAA1 (no effect of the non-phosphorylating D598A mutant) is comparable with the one previously described for cadmium in yeast strains expressing CadA (39). In good agreement with the biochemical data obtained from phosphorylation assays, the results obtained using the yeast system show that PAA1 can transport copper and silver *in vivo*.

DISCUSSION

AtHMA6/PAA1 has been previously associated to the chloroplast envelope through the *in planta* expression of a PAA1::GFP fusion and the analysis of its subcellular localization using confocal microscopy (15). This envelope localization was recently confirmed, in our group, using a proteomic approach targeted to the three main compartments of the chloroplast, *i.e.* the envelope, the stroma, and the thylakoids (41; see the AT_CHLORO database). However, the copper-dependent ATPase activity, measured on the purified chloroplast envelope and attributable to PAA1, was very low (23). In purified envelope fractions, this activity did not exceed 20 nmol of hydrolyzed ATP per min and per mg of envelope proteins. Because

TABLE 1

Apparent affinities for copper and silver determined for P_{IB-1}-ATPases

ND, not determined.

P _{IB-1} ATPases	Apparent affinity for copper		Apparent affinity for silver		References
	Estimated using phosphorylation from ATP	Estimated using phosphorylation from P _i	Estimated using phosphorylation from ATP	Estimated using phosphorylation from P _i	
CopA <i>A. fulgidus</i>	3.9 μM	ND	23 μM	ND	42
CopA <i>E. coli</i>	1.5 μM	ND	ND	ND	43
ATP7A human	2.5 μM	ND	ND	ND	44
ATP7B human	1 μM	ND	ND	ND	44
PAA1 <i>A. thaliana</i>	0.5 μM	0.6 μM	1.1 μM	5 μM	This work

this low ATPase activity strongly limited further functional characterization of this transporter in its native environment, we screened alternative expression systems for their ability to produce this membrane protein in amounts compatible with further biochemical studies.

In a recent study, we reported that *L. lactis* was an efficient host for the production of plant membrane proteins and particularly of P-type ATPases (26). Indeed, AtHMA1, AtHMA3, and PAA1 were found to account for 1–3% of total bacterial membrane proteins, this enrichment being compatible with further biochemical studies. In the present study, we demonstrate that besides producing relatively large amounts of these difficult membrane proteins, *L. lactis* produces a functional form of PAA1. These data strongly support the choice of *L. lactis* as a system for the functional characterization of plant ATPases.

PAA1 had been previously associated to the PIB-1 subgroup of heavy metal-transporting P-type ATPases according to the presence of conserved residues in transmembrane segments 6, 7, and 8 (7). This subgroup contains prokaryotic and eukaryotic P-type ATPases. Some of these proteins have been already characterized, such as CopA from *Archaeoglobus fulgidus* or *E. coli* or ATP7A and ATP7B from humans (42–45). However, none of the plant ATPases classified in this PIB-1 subgroup (AtHMA5, AtHMA6/PAA1, AtHMA7/RAN, and AtHMA8/PAA2) had been biochemically studied so far. This study thus reports the first enzymatic characterization of a plant copper P-type ATPase.

Using phosphorylation assays, we demonstrated that like CopA, ATP7A, and ATP7B, PAA1 is activated in the presence of Cu⁺. Apparent affinity of PAA1 for Cu⁺, estimated using either forward phosphorylation from ATP or reverse phosphorylation from P_i, is slightly below 1 μM. This value is close to the ones reported for the previously characterized P_{IB-1}-ATPases: 1 and 2.5 μM for the human ATPases ATP7B and ATP7A and 1.5 and 3.9 μM for CopA from *E. coli* and from *A. fulgidus*, respectively (Table 1). Estimation of PAA1 apparent affinity was made using Hill equation with a Hill number equal to 1, meaning that Cu⁺ binding to the transporter does not display any cooperativity.

The apparent affinity of PAA1 for Ag⁺ is slightly lower than that for Cu⁺, as shown previously for CopA from *A. fulgidus* (42). In addition, in the presence of ATP, the PAA1 phosphorylation level reached in the presence of Ag⁺ is lower than that reached in the presence of Cu⁺. Ag⁺, which is not a physiological substrate, is generally used as a mimic of monovalent transition metal, more stable than Cu⁺ in solution. The observed

differences between Cu⁺ and Ag⁺ could be attributed to a difference of ionic radii (0.126 nm for Ag⁺ and 0.096 nm for Cu⁺) that could somehow impact on the conformational changes occurring during the enzymatic cycle and thereby affect the phosphorylation rate. Similarly, ZntA from *E. coli* was found to transport more efficiently Zn²⁺ than Cd²⁺, two metals with close chemical properties but with different ionic radii (0.074 nm for Zn²⁺ and 0.097 nm for Cd²⁺) (46).

The present results demonstrate that PAA1 behaves as a classic P-type ATPase, forming a transient phosphorylated intermediate in the presence of ATP and the transported ion. The rate of PAA1 dephosphorylation is quite similar to those observed for the yeast Ccc2p and human ATP7B ATPases (44, 47). Indeed, after 2 min, less than 50% of PAA1 is still phosphorylated, and the enzyme is totally dephosphorylated after 5 min. The transient nature of the phosphorylated intermediate was demonstrated by pulse-chase experiments with cold ATP or ADP. The addition of excess cold ATP results in an almost complete turnover of PAA1 in 60 s, which is comparable with the turnover observed for ATP7A (36). Dephosphorylation assays, in the presence of ADP, indicate a prevalence of the Me.E~P conformation of the enzyme when it is phosphorylated in the presence of both copper or silver, suggesting that PAA1 behaves similarly with copper and silver. This is not the case of the CopA from *A. fulgidus*, which is mainly in the Me.E~P form in the presence of copper and in the E-P form in the presence of silver (42).

As discussed previously, acylphosphate formation of PAA1 is copper- and silver-dependent, with a maximum level of phosphorylation reached at 3 μM. A further increase in metal concentration results in the inhibition of phosphorylation. Inhibition of P-type ATPase activity by high concentrations of the transported ion is commonly explained by a reduced dissociation of the ion from the extracytoplasmic binding site (Fig. 1, step 3), slowing down enzyme turnover rate. Such an explanation can be proposed for the inhibitory effect of copper observed on the ATPase activity of the Cu⁺-ATPase CopB from *Enterococcus hirae* (48) and the Cu²⁺-ATPase CopB from *A. fulgidus* (49). In this inhibition mechanism, the P-type ATPase, although inactive in terms of ATP hydrolysis, is nevertheless fully phosphorylated. The SERCA1a Ca²⁺-ATPase, for example, remains fully phosphorylated even at calcium concentrations far above the value corresponding to the affinity of the ion to the transporter. This was also reported for the purified Cu⁺-ATPases CopA, from *E. coli* (43) and *A. fulgidus* (42), and to a lesser extent for the human Cu⁺-ATPase ATP7B (44). What is observed with PAA1 seems therefore to contradict the

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commonly admitted inhibition scheme and has been reported for another Cu⁺-ATPase, ATP7A, a close paralog of ATP7B that shares with it a similar apparent affinity for copper. Interestingly, inhibition of ATP7A phosphorylation has been observed on total membrane preparation above 5 μM copper (36) as well as on the purified protein in detergent above 1 μM copper (45), somehow excluding an effect of protein environment on the inhibition by copper. For ATP7A, it was suggested that inhibition could be due to “substrate inhibition and/or protein denaturation” (36). The reasons why catalytic phosphorylation of PAA1 is inhibited by high metal concentrations remain obscure. However, we can exclude that PAA1 is denatured by cysteine oxidation because PAA1 inhibition is observed in the presence of reduced copper (copper/Na₂SO₃ mixture) and silver. These reducing conditions also exclude an inhibitory effect of the metal by replacement of Mg²⁺ in the Mg-ATP complex, the substrate of the reaction, because monovalent ions cannot bind ATP.

To strengthen these biochemical data, we thus performed two independent experiments aiming to validate the specificity of PAA1 *in vivo*: complementation and metal tolerance assays in the yeast *S. cerevisiae*. We first found that PAA1 was unable to functionally replace the yeast Cu⁺-ATPase Ccc2p, most probably due to a mistargeting of PAA1 in the endoplasmic reticulum, whereas Ccc2p is a Golgi-resident protein. We then analyzed the metal sensitivity of a wild-type yeast strain expressing PAA1. Similarly to what we previously observed with yeast strains expressing CadA, the Cd²⁺-ATPase from *L. monocytogenes* (39), PAA1 increases yeast sensitivity to the transported metals. On the one hand, these results show that PAA1 actually transports Cu⁺ and Ag⁺ *in vivo* and are thus in good agreement with the *in vitro* biochemical characterization of PAA1. Furthermore, when considering that Cu⁺ is the copper form present in yeast, these data also strongly support the biochemical data showing that PAA1 is a Cu⁺-, Ag⁺-ATPase.

The present data show that PAA1 enzymatic properties are similar to those of well known prokaryotic and eukaryotic Cu⁺/Ag⁺-ATPases such as CopA from *E. coli*, Ccc2p from *S. cerevisiae*, or ATP7A and ATP7B from humans. Together with the presence of the conserved motifs CPC, NY, and MXXSS in the helices 6, 7, and 8, respectively (7), these properties classify PAA1 as a P_{1B-1}-type ATPase. As extensively illustrated in the literature, P_{1B-1}-ATPases are key actors of copper homeostasis (53, 55). Most of them regulate intracellular copper levels by pumping copper out of the cell (*i.e.* CopA) or in intracellular compartments where they provide copper to copper-dependent enzymes (*i.e.* ATP7A, ATP7B, Ccc2p). In the cyanobacteria *Synechococcus* PCC7942 and *Synechocystis* PCC6803, the two P_{1B-1}-type ATPases CtaA have been proposed, from indirect measurements, to import copper into the cell (50, 51). This has also been proposed for CopA from *E. hirae* (52). How similar proteins can transport copper in two different directions (export in most of the bacteria studied so far *versus* import in cyanobacteria and *E. hirae*) is not currently understood. However, the role of PAA1 as chloroplast Cu⁺ importer, supported by physiological (14) and biochemical studies (the present work), is consistent with the evolutionary origin of chloroplast (chloroplast originated from cyanobacteria through endosym-

biosis). Abdel-Ghany *et al.* (15) have suggested that not only PAA1 and CtaA but also PAA2 and PacS, both located in the thylakoids of *A. thaliana* and cyanobacteria, respectively, might be considered as functional homologs.

The high apparent affinity of PAA1 for copper identified during this work is in good agreement with previous experiments demonstrating that *paal* mutants are defective in electron transport (plastocyanin) when grow at low copper concentrations (14). Recent studies have shown the existence of a transcriptional regulatory mechanism that allows cells to down-regulate expression of non-essential copper proteins, and thus, to preserve copper for essential functions under copper deficiency (for review, see Ref. 53). In that context, the prioritized delivery of copper to plastocyanin implies the existence of high affinity transporters that could be PAA1 in the chloroplast envelope and PAA2 in the thylakoids. In 2005, Abdel-Ghany *et al.* (15) suggested the existence of alternative low-affinity copper transporters in the chloroplast envelope because *paal* mutants could be partially rescued by the addition of copper in the plant growth medium. AtHMA1 was proposed to be one of these transporters (23). Thus, PAA1 could be the major copper import system in chloroplasts supplying copper for photosynthesis, whereas AtHMA1 could be an alternative pathway, essential under oxidative stress conditions. Another difference between these two transporters could reside in the valence of the transported ion: Cu⁺ for PAA1 and Cu²⁺ for AtHMA1 (54). Therefore, future characterization of the enzymatic properties of AtHMA1, following the approach described in the present study, should provide key information regarding the relative roles of these two ATPases, PAA1 and AtHMA1, in copper transport across the chloroplast envelope.

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**Biochemical Characterization of AtHMA6/PAA1, a Chloroplast Envelope
Cu(I)-ATPase**

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