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antibodies revealed the localization of the GmHMA8 transporter within the chloroplast organelle. Furthermore, the precise ultrastructural distribution of GmHMA8 within the chloroplast subcompartments was demonstrated by using electron microscopy immunogold labelling. The GmHMA8 copper transporter from soybean was localized in the thylakoid membranes showing a heterogeneous distribution in small clusters.
IDENTIFICATION AND SUBCELLULAR LOCALIZATION OF THE SOYBEAN COPPER P$_{1B}$-ATPase GmHMA8 TRANSPORTER

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ABSTRACT

We have identified a copper P$_{1B}$-ATPase transporter in soybean (Glycine max), named as GmHMA8, homologue to cyanobacterial PacS and Arabidopsis thaliana AtHMA8 (PAA2) transporters. A novel specific polyclonal anti-GmHMA8 antibody raised against a synthetic peptide reacted with a protein of an apparent mass of around 180-200 kDa in chloroplast and thylakoid membrane preparations isolated from soybean cell suspensions. Immunoblot analysis with this antibody also showed a band with similar apparent molecular mass in chloroplasts from Lotus corniculatus. Immunofluorescence labelling with the anti-GmHMA8 antibody and double immunofluorescence labelling with anti-GmHMA8 and anti-RuBisCo antibodies revealed the localization of the GmHMA8 transporter within the chloroplast organelle. Furthermore, the precise ultrastructural distribution of GmHMA8 within the chloroplast subcompartments was demonstrated by using electron microscopy immunogold labelling. The GmHMA8 copper transporter from soybean was localized in the thylakoid membranes showing a heterogeneous distribution in small clusters.
Keywords: ATPase copper transporter, cell suspensions, chloroplast, immunofluorescence labelling, immunogold labelling, soybean, thylakoid membrane.
INTRODUCTION

Copper is an essential cofactor for enzymes required for a wide variety of biological processes in plants including redox reactions in photosynthetic electron transfer and detoxification of superoxide radicals. Nevertheless, copper can also be toxic at supraoptimal concentrations. Consequently, plants like other organisms possess a complex homeostasis network of metal trafficking pathways to: i) take up and distribute metals throughout the entire organism; ii) prevent high cytoplasmic concentrations of free heavy metal ions (Fox and Guerinot, 1998; Williams et al., 2000; Clemens, 2001; Hall and Williams, 2003; Krämer and Clemens, 2006). Membrane transport proteins play important roles in these processes and key roles have been identified for P1B-ATPases, also known as CPx-ATPases (Solioz and Vulpe, 1996), metal P-type ATPases (Rensing et al., 1999) and heavy metal ATPases (HMAs) (Axelsen and Palmgren, 2001). These proteins transport transition metals such as copper, zinc, cadmium, lead and cobalt across membranes. These transporters have been classified into six sub-groups (P1B1-P1B6) by analysing their amino acid sequences and topological arrangements, and combining this with their metal specificity (Axelsen and Palmgren, 1998; Argüello, 2003). Structurally, they are different from other P-ATPases and possess eight transmembrane regions, with a large cytoplasmic loop between transmembranes helices 6 and 7, a CPx/SPC motif, which is essential for metal transport, and putative heavy metal-binding domains at the N- and/or C-termini. The Arabidopsis genome encodes eight predicted P1B-ATPases (AtHMA1-AtHMA8, http://mips.gsf.de) that differ in their structure, function and regulation but all of them are specialized in specific metal ion transport to cellular compartments and target proteins (for review see...
Williams and Mills, 2005). In particular AtHMA1, AtHMA6 (PAA1), AtHMA7 (RAN1), and AtHMA8 (PAA2) P$_{1B}$-ATPases are involved in copper transport to different cellular compartments (Hirayama et al., 1999; Woeste and Kieber, 2000; Shikanai et al., 2003; Abdel-Gahny et al., 2005; Seigneurin-Berny et al., 2006). On the other hand, AtHMA2-4 and AtHMA5 P$_{1B}$-ATPases have roles in nutrition and metal detoxification, and are involved in zinc, cadmium and/or lead (Mills et al., 2003; Eren and Argüello, 2004; Gravot et al., 2004; Verret et al., 2004; 2005; Mills et al., 2005) and copper (Andrés-Colás et al., 2006) transport, respectively.

Understanding of chloroplast copper homeostasis is of particular interest because this metal is required for protein maturation and functioning, such as Cu/Zn superoxide dismutases (Cu/ZnSODs) in the stroma, and polyphenol oxidase and plastocyanin in the thylakoid lumen. Since plastocyanin is targeted to the lumen compartment as an apoprotein, a mechanism by which copper crosses the thylakoid membrane has to exist (Merchant and Dreyfuss, 1998). In cyanobacteria, the ancestor of chloroplasts, two Cu-transporting P-type ATPases, PacS and CtaA, were found in thylakoid and cytoplasmatic membranes, respectively (Kanamaru et al., 1994; Phung et al., 1994; Tottey et al., 2001). These proteins are required for copper transport to plastocyanin in the thylakoid lumen. At present, studies at a molecular level of copper homeostasis in plant chloroplasts are very limited and most of them are restricted to Arabidopsis thaliana. Recently, two Cu-ATPases, PAA1 (AtHMA6) and PAA2 (AtHMA8), homologues to CtaA and PacS, have been identified in A. thaliana (Shikanai et al., 2003; Abdel-Gahny et al., 2005). The authors proposed their participation in copper transport across the chloroplast envelope.
and the thylakoid membrane, respectively. More recently, Seigneurin-Berny et al. (2006) have shown that the copper transporter AtHMA1 is localized in the chloroplast envelope. Though experimental approach has been conducted to investigate the localization and function of those chloroplast HMA transporters in *Arabidopsis*, it is still necessary to gain an insight into detailed aspects of their precise localization as well as their structural organization. This information is needed to better understand the function and implication of HMA transporters in chloroplast copper homeostasis. Up to know there is no structural information of HMA transporters in plants. In addition, it is of interest to extend these investigations to different plant species, especially to those relevant in food and healthy, in order to know the implications of these transporters in metal tolerance. The present work reports the identification of GmHMA8, a copper P_{1B}-ATPase in soybean related to AtHMA8 (PAA2), and its subcellular localization in the chloroplasts and more specifically in the thylakoid membranes. Results are discussed in terms of the possible transporter organization within the membrane.

**MATERIALS AND METHODS**

*In silico* sequence analysis of plant P_{1B}-ATPases.- Search of the databases was done with Blastr programme (Altschul and Lipman, 1990) using previously identified PacS (sll1920) and CtaA (slr1950) sequences from *Synechocystis* PCC 6803. Alignments were done using ClustalW (Thompson et al., 1994) and theoretical subcellular location was predicted using TargetIP and ChloroP programs (Emanuelsson et al., 1999). The complete gene sequence of
HMA8 from *Lotus corniculatus* var. *japonicus* genomic DNA (AP006090) was obtained with GenScan Web Server (Burge and Karlin, 1997). The location of possible transmembrane domains was determined using the program ARAMEMNON at http://aramemnon.botanik.uni-koeln.de/ (Schwacke et al., 2003).

**Cell suspension growth conditions.**- Photosynthetic cell suspensions from soybean (*Glycine max* var. Corsoy) SB-P line were grown as described by Rogers et al. (1987) with some modifications (Alfonso et al., 1996; Bernal et al., 2006). Liquid cultures were grown in photomixotrophic medium under continuous light (30 ± 5 µE m⁻² s⁻¹) and atmosphere with 5% CO₂ at 24 ºC on a rotatory shaker (TEQ, model OSFT-LS-R) at 110 rpm. For immunolocalization experiments, soybean cells were grown on 1.5% (w/v) agar plates with KN¹ medium at 24 ºC and atmosphere with 5% CO₂. Cells cultured in these conditions were easier to handle during the fixation and sectioning procedures than liquid suspensions.

**RNA isolation and cDNA synthesis.**- Cells (0.4 – 0.8 g wet weight) were washed with fresh KN¹ medium and pestled in liquid nitrogen. Then, a mixture of phenol:extraction buffer (0.1 M LiCl, 10 mM EDTA, 1% (w/v) SDS, 0.1 M Tris-HCl, pH 8.0) (1:1 v/v) was added to the powder (1.5 mL per 0.1 g of cells) and vortexed vigorously. Samples were centrifuged at 1,500 × g for 10 min at 4 ºC. The aqueous phase was extracted first with an equal volume of phenol:chloroform (1:1 v/v) followed by an additional wash with an equal volume of chloroform:isoamyl alcohol (24:1). The RNA was then precipitated overnight.
at 8 °C with an equal volume of 4 M LiCl, centrifuged at 15,500 × g for 30 min at
4 °C and then washed with cold 80 % (v/v) ethanol. RNA was dried out with
nitrogen and resuspended in sterile H2O containing 1% (v/v) DEPC. The RNA
concentration was determined by measuring the OD260nm. cDNAs were
synthetized from total RNA (5 µg) using 200 units of reverse transcriptase (M-
MLV reverse transcriptase, Promega, Madison, Wi, USA) and 1 µM oligo(dT)12-18
from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA), according to
the manufacturer’s instructions. Total RNA for the 5'-RACE amplification was
isolated and purified from soybean cells using a RNeasy Plant Mini Kit (Qiagen
GmbH, Hiden, Germany). cDNA was synthetized at 54 °C from total RNA (3 µg)
using a GmHMA8 specific-primer (2 pmol, GSP1) and 200 units of thermoscript
reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) with
the GeneRacer Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according
to the manufacturer’s instructions.

**Isolation of GmHMA8 sequence.**- The full-length GmHMA8 cDNA, which
contained 650 bp of EST (TC228758, TIGR Soybean Gene Index), was
obtained by RT-PCR. Degenerated oligonucleotides, which could be used as
primers for RT-PCR amplification, were designed from in silico sequence data
analysis. The 24-mer oligonucleotides designed corresponded to the sequences
of the phosphorylation domain FDKTG(L/I)T (forward-ATPase) and hinge
region GIND(S/A)P(S/A)L (reverse-ATPase) (Table 1) both located in the large
cytoplasmatic loop of P1B-ATPases. The GmHMA8 3'-end (including part of the
3'-UTR) was obtained using a forward primer designed based on the identified
GmHMA8 partial sequence (GmHMA8-1) and the reverse primer designed
according to a GmHMA8 EST (UTRGmHMA8) (TC228758). The GmHMA8 5´-end (including part of the 5´-UTR) was obtained using the GeneRacer Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The primary PCR was performed using an adaptor primer (GeneRacer 5´ Primer) and a GmHMA8 specific-primer (GSP2) followed by a nested PCR with a nested adaptor primer (GeneRacer 5´ Nested Primer) and a nested GmHMA8 specific-primer (GSP3) (Table 1). The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced (CNIO service, Madrid, Spain) to check their identity.

Isolation of intact chloroplasts and thylakoid membranes.- Soybean cells from 18-day-old cultures were collected (3-4 g wet weight) and filtered through a layer of Miracloth (Calbiochem, EMD Biosciences Inc, San Diego, CA, USA). Cells were then resuspended in buffer containing 400 mM NaCl, 2 mM MgCl₂, 0.2% (w/v) sucrose, and 20 mM Tricine, pH 8.0 at a cell to buffer ratio of 1:2 (w/v) and broken with a teflon homogeneizer during 10 min with appropriate stops to avoid sample heating. Broken cells were gently stirred for 10 min and centrifuged at 300 x g for 2 min. Intact chloroplasts and thylakoid membrane isolation were done as described in van Wijk et al. (1995) and Bernal et al. (2006), respectively. All procedures were carried out at 4 ºC under dim light. Chlorophyll determination was done as described by Arnon (1949). Protein determination was done as described by Bradford (1976).

GmHMA8 antibody production.- The rabbit polyclonal anti-GmHMA8 antibody was produced from a 15 amino acid synthetic peptide designed from the
identified partial sequence of \textit{GmHMA8} (Genosphere Biotechnologies, Paris, France). The selected peptide sequence was HERFQTRANPSDLTN, located within the putative large cytoplasmatic loop of \textit{GmHMA8} (DQ418731).

\textbf{Immunoblotting analysis.-} The electrophoretic separation of chloroplast and thylakoid proteins was performed by SDS-PAGE according to Laemmli (1970) using 15\% and 12\% (w/v) acrylamide gels containing 6 M urea as described in Bernal et al. (2006) with some modifications. Gels were electroblotted to a PVDF membrane with a BioRad transfer system and the immunodetection was done using the rabbit polyclonal antibody against \textit{GmHMA8} described above. A goat anti-rabbit IgG coupled to horseradish peroxidase was used as a secondary antibody (Biorad, Palo Alto, CA, USA). Bands were revealed by the peroxidase method. Immunoreactive bands were revealed by washing with buffer containing 150 mM NaCl and 25 mM Tris-HCl, pH 7.5, and then with a solution containing 0.05\% H$_2$O$_2$ and 2.8 mM 4-chloro-1-naphtol for the peroxidase conjugate (Sambrook et al., 1989)

\textbf{Sample processing for microscopical structural analysis and immunofluorescence.-} Soybean cells were fixed overnight at 4 °C in formaldehyde 4\% (w/v) in phosphate buffered saline solution (PBS), pH 7.3, and washed with PBS. Some samples were stored at 4 °C for direct sectioning in a vibratome and further use for immunofluorescence. Other samples were dehydrated through an acetone series [30\%, 50\%, 70\% and 100\% (v/v)], infiltrated and embedded in Historesin 8100 at 4 °C. Semithin sections (1 µm thickness) were obtained and used for light microscopy observations. Toluidine-
blue stained semithin sections were observed under bright and phase contrast field for structural analysis in a Leitz microscope fitted with a digital camera Olympus DP10.

**Cytochemical stainings for starch and DNA.** - Starch was detected by $I_2K_I$ staining (O’Brien and McCully, 1981) on Historesin semithin sections and observed under bright field (Barany et al., 2005). DAPI staining for DNA was applied to semithin sections (Testillano et al., 1995) and observed under UV light in a Zeiss Axiophot epifluorescence microscope fitted with a CCD camera.

**Immunofluorescence and Confocal Laser Microscopy.** - Immunofluorescence was performed on vibratome sections as previously described (Fortes et al., 2004). Vibratome sections of 30 µm (Vibratome1000, Formely Lancer) obtained from fixed soybean cells (see above) were placed onto 3-aminopropyltriethoxysilane coated slices and treated for permeabilization purposes. First, they were dehydrated [30%, 50%, 70%, 100% (v/v)] and rehydrated [100%, 70%, 50%, 30% (v/v)] in PBS:methanol series. Second, sections were treated with 2% (w/v) cellulase (Onozuka R-10) in PBS for 40 min at room temperature. After three washes in PBS for 5 min, sections were treated with 0.5% (v/v) Triton X-100 in PBS for 20 min and washed with PBS for 5 min. Subsequently, sections were incubated with 5% (w/v) bovine serum albumine (BSA) in PBS for 5 min and then in either anti-\textit{GmHMA8} or anti-\textit{RuBisCo} (large subunit, kindly provided by Dr. R.T. Besford from Horticultural Research International, Little Hampton, West Sussex, UK) polyclonal antibodies, diluted 1:10 and 1:25, respectively, in 1% (w/v) BSA for 1 h at room temperature. After washing twice with PBS for 10 min each, the signal was
revealed with either ALEXA 488 (green fluorescence)- or ALEXA 546 (red fluorescence)-conjugated anti-rabbit antibodies (Molecular Probes, Eugene, OR) diluted 1:25 in 1% (w/v) BSA for 1 h in the dark at room temperature. Finally, the sections were washed twice with PBS for 10 min, stained with DAPI (4,6-diamidine-2-phenylindol; Serva, Heilderberg, Germany) for 10 min, washed with milli-Q H₂O and mounted with Mowiol 4-88 (Polysciences, Eppelheim, Germany).

A double immunofluorescence labelling with anti-RuBisCo and anti-GmHMA8 polyclonal antibodies was also performed to assay the putative co-localization of both antigens. Double immunofluorescence was performed following a sequential incubation protocol, essentially as previously described (Silva et al., 2004). Controls replacing the first antibody by preimmune antiserum were also assayed. Immunofluorescence assays were observed by Confocal Laser Microscopy (CLSM) (Leica TCS-SP2-AOBS) and Z-series of optical sections of 0.5-1.0 µm were collected. Images were taken from the projections of series of 15 to 20 optical sections. Differential Interference Contrast (DIC, Nomarski) images were also taken.

**Low temperature processing for immunoelectron microscopy.**- Fixed soybean cells (see above) were cryoprotected by immersion in sucrose:PBS solution at the following concentrations and times: 0.1 M for 1 h, 1 M for 1 h, and 2.3 M overnight, at 4 °C. Then, the specimens were put in cryoultramicrotomy pins and cryofixed by rapid plunging into nitrogen liquid at -190 °C. After that, the samples were dehydrated by freeze substitution in an Automatic Freeze-substitution System (AFS, Leica, Vienna, Austria) essentially
as described by Seguí-Simarro et al. (2005). Cryofixed samples were immersed in pure methanol containing 0.5% (w/v) uranyl acetate at -80 °C for 3 days, and then the temperature was slowly (during 18 h) warmed to -30 °C. After three washes in pure methanol, 30 min each, at -30 °C, samples were infiltrated and embedded in Lowicryl K4M at -30 °C, under UV. Ultrathin sections were obtained in an ultramicrotome (Ultracut Reichert, Vienna, Austria) and collected on 200-mesh nickel grids having a carbon-coated Formvar-supporting film.

**Immunogold labelling.**- Nickel grids carrying ultrathin Lowicryl sections were sequentially floated in PBS, 5% (w/v) BSA in PBS, and undiluted anti-GmHMA8 antibody, for 1 h. After several washes in 0.1% (w/v) BSA in PBS, the grids were incubated with a secondary antibody, anti-rabbit IgG conjugated to 10 nm gold particles (Biocell, Cardiff, UK) diluted 1:25 in 1% (w/v) BSA, for 1 h at room temperature, washed in PBS and water, air dried, counterstained with uranyl acetate and lead citrate and observed in a JEOL 1010 EM at 80 kV. Controls were performed excluding the primary antibody.

**Quantitative analysis of immunogold labelling density.**- Sampling was carried out over selected samples on each grid. The number of micrographs to be taken was determined using the progressive mean test, with a maximum confidence limit of $\alpha=0.05$. The labelling density was defined as the number of gold particles per area unit ($\mu m^2$). Particles were hand-counted over the cellular compartment under study (chloroplast) and over other subcellular compartments and cytoplasmic regions, where no presence of the protein was expected (as determined by other approaches), as an estimation of the
background level. The area in $\mu m^2$ was measured using a square lattice composed by squares of 15 x 15 mm each. The labelling density was expressed as the mean labelling density (MLD) ± standard deviation (s.d.).

**Quantitative evaluation of immunogold labeling distribution: Clustering test.** To assess the distribution pattern of gold particles, a clustering test was performed. Sampling was carried out over a number of micrographs taken randomly from all the cells in different grids. The number of micrographs was determined using the progressive mean test with minimum confidence limit of $\alpha=0.05$. Clustered particles were defined as 2 or more gold particles close to each other (Seguí-Simarro et al. 2003), taking into account the relative length of primary and secondary antibodies coupled to colloidal gold. For each micrograph, the isolated and clustered gold particles on chloroplasts were counted. The percentage of clustered particles versus the total number of particles was calculated.

**RESULTS**

**Isolation of GmHMA8 cDNA.**

*In silico* analysis using the sequences of PacS and CtaA from the cyanobacterium *Synechocystis* PCC 6803 identified five homologue sequences in *Arabidopsis thaliana* [At4g37270 (AtHMA1); At1g63440 (AtHMA5); At4g33520 (AtHMA6 or PAA1); At5g44790 (AtHMA7 or RAN1); At5g21930 (AtHMA8 or PAA2)] and one homologous sequence in *Brassica napus* (AY045772) as possible orthologs of the $P_{1B}$-ATPase family in plants. All $P_{1B}$-ATPases identified with this analysis correspond to putative copper transporting
ATPases. Soybean cDNA amplification was done by RT-PCR using degenerated primers designed according to in silico data analysis (for details see Materials and Methods). Two groups of cDNA bands were reproducibly amplified under these conditions (Fig. 1). One group of bands migrated at positions corresponding to 0.7-0.8 kb, and other group at positions above 1.0-1.2 kb. It is well-known that the large cytoplasmic loop of \( P_{1B} \)-ATPases is smaller than that of classical \( P \)-type ATPases. Between the phosphorylation and ATP binding domains, the Arabidopsis \( P_{1B} \) pumps have approximately 150 amino acid residues less than the Arabidopsis \( Ca^{2+} \) pump, \( AtECA1 \) (Williams et al., 2000). Thus, we assumed that the ca. 1.0 kb band encoded classical \( P \)-type ATPases, and the ca. 0.7-0.8 kb bands encoded \( P_{1B} \)-ATPases. Bands were cloned and positive clones were sequenced and analyzed. Eleven clones homologues to Cu-ATPases (the lowest molecular size bands), and seven clones homologues to Ca-ATPases plus six clones homologues to \( H^{+} \)-ATPase (the largest molecular size band) were obtained. Among those clones homologous to Cu-ATPases, only four encoded a desirable open-reading-frame (ORF) whose predicted amino acid sequence was highly similar to the corresponding regions of PacS and \( AtHMA8 \). Sequence analysis revealed the identity of a 793 bp region as encoding a new copper transporter in soybean homologue to the copper transporters PacS and \( AtHMA8 \), which we named as \( GmHMA8 \). Subsequently, the full-length \( GmHMA8 \) cDNA was obtained (for details see Materials and Methods). This provided us with a 2832 bp sequence of \( GmHMA8 \) that contained part of the 3'-UTR and part of the 5'-UTR regions of the gene. The sequence was deposited in GenBank as DQ418731.
Analysis of the predicted sequence of GmHMA8 (908 aa) indicated that this protein belongs to Cu-transporting P-type ATPases. It is an integral membrane protein with an N-terminal chloroplast transit peptide, eight transmembrane (TM) domains (Fig. 2A, B) with a small cytoplasmatic loop between TM domains 4 and 5, and a large cytoplasmatic loop between TM domains 6 and 7 (Fig. 2C). Like all P-type ATPases, it has all the characteristic conserved domains: the ATP binding (GDGxNDx) and phosphorylation (DKTGTTLT) domains in the large cytoplasmatic loop and a phosphatase domain in the small cytoplasmatic loop. GmHMA8, also possesses the specific domains present in P_{1B}-ATPases: the GMxCxxC metal binding motif in the N-terminal region, the CPx ion transduction domain in the sixth TM domain and the HP domain that is involved in the translocation of copper (Fig. 2C) (Axelsen and Palmgren, 2001; Argüello, 2003; Williams and Mills, 2005). The comparison of GmHMA8 derived amino acid sequence with the rest of the HMA8-related sequences available in databases indicated 45% similarity to PacS, 83% to AtHMA8, 75% to OsHMA8, and 88% to LcHMA8 in the overlapping amino acids (Fig. 2A).

**Production of a polyclonal antibody anti-GmHMA8.**

A specific polyclonal anti-GmHMA8 antibody was raised against a 15 amino acid synthetic peptide corresponding to a region between the phosphorylation and ATP binding domains in the large cytoplasmatic loop of the GmHMA8 protein sequence identified (Fig. 2A). This region was chosen to produce this antibody due to its high specificity for the GmHMA8 protein; it is not present in the protein sequences of other HMA family members. Since a
chloroplast target for this protein was predicted, we assayed the validity of this antibody in intact chloroplasts and thylakoid membranes isolated from soybean cell cultures. Figure 3A shows the polypeptide composition of the thylakoid fraction. Immunoblot analyses depicted a single band around 180-200 kDa in the chloroplast and thylakoid fractions assayed that may correspond to the GmHMA8 protein (Fig. 3B, D, left pannel). The analyses also revealed that the relative abundance of this band increased in those fractions enriched in thylakoid membranes (Fig. 3B). No signals were found with bovine serum albumin extract protein and the preimmune antiserum (Fig. 3B,C) demonstrating the specificity of the antibody. To better validate the produced antibody we assayed it in chloroplasts isolated from soybean and Lotus corniculatus plants. As previously, immunoblot analyses showed a high molecular weight band that migrated slightly faster in the case of Lotus compared with soybean (Fig. 3D, right pannel). The apparent molecular mass revealed by the immunoblot analysis was higher than the theoretical molecular mass predicted for GmHMA8 and LcHMA8 proteins based on their amino acid sequence, 97 kDa and 95 kDa, respectively. In general, it has been reported that P-type ATPases have a molecular mass between 70 to 150 kDa (Kühlbrandt, 2004). These findings could be interpreted as HMA8 protein might migrate differently in the electrophoretic gel that one expected (See and Jackowski, 1990) or form oligomeric structures. It is worth mentioning that according to our knowledge this is the first antibody raised against a HMA8 Cu-ATPase in plants and the third one available against a member of the P$_{15}$-ATPases family (for details see Hussain et al., 2004; Seigneurin-Berny et al., 2006).
Subcellular localization of GmHMA8 by immunofluorescence labelling.

The structural organization of soybean photosynthetic cells, as visualized in toluidine blue-stained semithin sections (Fig. 4A) was similar to that of mesophyll cells from young leaves. They showed a large and central cytoplasmic vacuole, chloroplasts distributed along the peripheral layer of the cytoplasm and an ellipsoid nucleus (Fig. 4A). Most chloroplasts contained large granules which appeared as clear inclusions in toluidine blue-stained sections visualized under bright field (Fig. 4A) and in unstained sections observed under phase contrast (Fig. 4E); the content of these inclusions was revealed by iodide-based cytochemistry as starch (Fig. 4E, F).

Immunofluorescence experiments with anti-GmHMA8 provided specific signals as intense green fluorescence in defined cytoplasmic spots (Fig. 4B). Labelling was not found in the nucleus that was revealed by DAPI with an intense blue fluorescence, or in the vacuole that appeared as a large dark central area (Fig. 4B). Confocal images of anti-GmHMA8 immunofluorescence green signals were overlapped with the corresponding differential interference contrast (DIC) image of the section (Fig 4C). The result showed that the green fluorescence was localized on small rounded cytoplasmic structures, frequently at their periphery (Fig. 4C). This pattern of distribution of the immunofluorescence labelling and its comparison with the structural organization of the soybean cells strongly suggested the localization of the GmHMA8 in the chloroplast. Controls with preimmune antiserum showed no labelling (Fig. 4D).

To further confirm the chloroplast localization of GmHMA8 in soybean cells a double immunofluorescence labelling with anti-RuBisCo, a good
chloroplastic marker, and with anti-GmHMA8 antibodies was performed (Fig. 5). Anti-GmHMA8 labelling, revealed by green fluorescence (Fig. 5A) and anti-RuBisCo labelling, visualized as red fluorescence (Fig. 5B) were specifically found in the same cytoplasmic rounded structures, the chloroplasts, showing a central dark area which probably corresponded with the starch deposits found in most chloroplasts (Fig. 5D, E). The overlay of both green and red fluorescence signals showed the co-localization of both antigens, as a yellow signal, in the chloroplast (Fig. 5C, F); some individual green and red small spots were also observed in the merged images (Fig. 5F) indicating that the co-localization was not complete.

Subcellular localization of GmHMA8 by immunogold labelling.

The resolution of the confocal microscope does not inform about the precise localization of the GmHMA8 antigen inside the different chloroplast subcompartments: outer and inner interenvelope space, stroma, thylakoid membrane and thylakoid lumen. In fact, the immunofluorescence signal provided by the RuBisCo, a typical stromatic protein, could overlap with immunofluorescence signals from antigens localized in the thylakoid membranes, as it seems to be the case of the GmHMA8 protein. To go further on the ultrastructural distribution analysis of the GmHMA8 protein, electron microscopy immunogold labelling was performed (Fig. 6). Cryofixation and freeze-substitution have been reported as very convenient processing methods for immunogold assays of membrane-associated antigens in plant cells (Risueño et al., 1998; Seguí-Simarro et al., 2003; 2005; review in Koster and Klumperman, 2003). The results showed that the protocol used was adequate.
for soybean cell cultures, which maintain a good ultrastructural preservation of the different subcellular compartments, including chloroplasts and thylakoid membranes (Fig. 6A, B). Immunogold labelling was specifically found on the thylakoid membranes of chloroplasts (Fig. 6C, D).

Quantification studies of the labelling density as the number of gold particles per area unit were performed over the chloroplasts and over other cell compartments (vacuoles, cell walls, etc) and cytoplasmic regions where no presence of the protein was expected, as demonstrated by the immunofluorescence and western results. The quantification results revealed a labelling density in the chloroplasts of $20.55 \pm 4.90$ particles/$\mu m^2$, which was much higher than in the rest of cellular regions where a few isolated gold particles could be found, being the mean labelling density, as estimated background, less than 5% of the quantified signal in chloroplasts. The possibility that a few of the scarce cytoplasmic particles could correspond to a small subset of the newly formed protein in transit to the chloroplast, only detected with the highest sensitivity of the immunogold labelling technique, could not be excluded.

To assess the distribution pattern of immunogold particles over the chloroplasts, a quantitative clustering test was performed. Taking into account the labeling distribution observed, particles were considered clustered when two or more particles appeared close to each other. When considering the clusters of particles decorating the chloroplastic thylakoids, the clusters containing 2-4 particles were the most numerous and only a few of them displayed more than 4 particles. The percentages of clustered and isolated particles versus the total number of particles were calculated. Results showed that the percentage of
clustered particles was much higher (73.92%) than the isolated ones (26.35%), indicating a grouped distribution of the antigen in discrete locations of the chloroplastic thylakoids. No clustering was observed in the few gold particles found over the cytoplasm.

DISCUSSION

In this paper we present the identification of a soybean copper P-type ATPase transporter, \( GmHMA8 \), homologous to \( PacS \) and \( AtHMA8 \) (PAA2) transporters that are members of Cu/Ag transporting group of \( P_{1B} \)-ATPases (Tottey et al., 2001; Abdel-Ghany et al., 2005). Comparison of \( GmHMA8 \) with sequences from other organisms showed that HMA8 protein sequences are evolutionary conserved, which may reflect a critical function for this protein. Immunofluorescence assays demonstrate that \( GmHMA8 \) is localized in chloroplasts. Furthermore, a well-defined immunogold signal in the thylakoid membrane with no evidences of labelling in the chloroplast envelopes was observed, demonstrating that \( GmHMA8 \) is located in the thylakoid membrane. The results are consistent with data reported for PAA2 (\( AtHMA8 \)) in protoplasts of \textit{Arabidopsis thaliana} by using green fluorescence fusion proteins (PAA2-GFP) and \textit{in vitro} import protein experiments with a non-full length PAA2 (\( AtHMA8 \)) protein (Abdel-Ghany et al., 2005). It is worth mentioning that the approach presented here constitutes a direct method providing advantages to localize the full-length protein. The use of the specific antibody against \( GmHMA8 \) is a valuable tool for this purpose and future studies.
Interestingly, the labelling pattern frequently appeared in clusters of 2-3 gold particles. This type of distribution in cluster would indicate the presence of GmHMA8 transporter enriched sites within the thylakoid membrane. This finding is consistent with the current idea that thylakoid membranes are heterogeneous and contain domains or regions with specific functions. On the other hand, the observed distribution might suggest an oligomeric structure for this copper transporter. In this respect, it is worth mentioning that the apparent molecular mass of the HMA8 proteins detected in denaturing gel electrophoresis was around 2-fold higher than the predicted theoretical molecular mass. At present, studies concerning to structural aspects of P-type ATPases are very limited (Kühlbrandt, 2004) and in particular those concerning to copper-transporting P$_{1B}$-ATPase subfamily. It is to note the great difficulties that in general membrane proteins present for structural studies. The X-ray structure of the sarcoplasmic reticulum Ca$^{2+}$-ATPase, a P$_{2}$-type ATPase, and the homology models of P-ATPases proposed provide a basis for understanding the molecular structure of these transporters (Toyoshima et al., 2000, 2002; Kühlbrandt, 2004). Based on those models, P-type ATPases of types II and III seem to form oligomeric structures (Kühlbrandt, 2004). Although this information cannot be straightforward extrapolated to members of P$_{1B}$-ATPases subfamily, since they differ in overall architecture, it might not be ruled out a similar organization for P-ATPases of type I. More recently, progress in structural characterization of P$_{1B}$-ATPases have been done, but these investigations were restricted to specific protein domains (Dmitriev et al., 2006; Achila et al., 2006; Sazinsky et al., 2006a,b). Hence, information of the possible homodimerization or oligomerization for P$_{1B}$-ATPases is not still known. In this
sense structural data of other copper transporters could be of interest. The structure at 6Å-resolution of the human copper transporter hCTR1, responsible for the initial uptake of copper into cells and homologue to plant COPT transporters (Sancenón et al., 2003), revealed a symmetrical trimeric structure for hCTR1 that was <40 Å wide (Aller and Unger, 2006). The formation of a putative pore for metal ions at the interface of three identical subunits has been proposed, which could be related with its metal transport function. Further investigations are required to understand the functional implications if any of the observed GmHMA8 protein organization within the thylakoid membrane.

ACKNOWLEDGEMENTS

The authors thank M.V. Ramiro for her helpful technical assistance. The SB-P line was kindly provided by Prof. Jack M. Widholm (Department of Agronomy, University of Illinois at Urbana, Urbana IL). This work was supported by the Aragón Government (Grant P015/2001 and GC E33 DGA programme) and the Ministry of Education and Culture of Spain (BFU2005-07422-C02-01; AGL2005-05104; BFU2005-01094). M. Bernal is recipient of a predoctoral fellowship from Consejo Superior de Investigaciones Científicas (I3P Programme financed by the European Social Fund).
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ATPasa HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J. 45, 225-236.


FIGURE LEGENDS

Figure 1. Expression of P-ATPase family in soybean photosynthetic cell suspensions. Lane 1, 1.0 kb plus DNA ladder; lane 2, RT-PCR of P-ATPase family with degenerated primers: 1.0-1.2 kb bands correspond with classical P-ATPases (Ca-ATPase, H⁺-ATPase) and 0.7-0.8 kb bands correspond with P₁B-ATPases (Cu-ATPases).

Figure 2. A) Sequence alignment of soybean GmHMA8 (DQ418731) with HMA8 proteins from Arabidopsis thaliana [AtHMA8 (PAA2), At5g21930], Oryza sativa (OsHMA8, XP_470523), Lotus corniculatus (LcHMA8) and Synechocystis PCC 6803 (PacS, sll1920). Identical residues are in black and conservative residues are shaded. The eight putative transmembrane domains (TM1-TM8) for GmHMA8 are underlined. Functional regions and 15 amino acid peptide sequence chosen to produce the specific antibody anti-GmHMA8 are boxed. B) Hydrophobicity profiles of GmHMA8. C) Topological model of GmHMA8.

Figure 3. Protein composition of thylakoid membranes from soybean cells obtained by SDS-PAGE and visualized by Coomassie Blue (A). Immunoblot with antiserum anti-GmHMA8 protein (B) and with preimmune antiserum (C) of intact chloroplasts (Chl) and thylakoid membranes (Thy) from soybean cells, and bovine serum albumine protein (BSA). D) Immunoblot with antiserum anti-GmHMA8 of chloroplasts from soybean cells (Chl, left pannel), and chloroplasts from soybean plants (Gm Chl, right pannel) and Lotus corniculatus plants (Lc Chl, right pannel). Loading protein amount was 12 µg (A), 26 µg (B,C), 21 µg
(D, Thy), 58 µg (D, Gm Chl, Lc Chl). Note the different MW markers for each electrophoresis.

**Figure 4.** A) Structural organization of soybean photosynthetic cell cultures. Historesin semithin sections after toluidine blue staining. Vacuole (v), cytoplasm (c), and chloroplasts (arrows). B) Immunofluorescence with anti-GmHMA8 in soybean photosynthetic cells. Confocal laser microscopy observations of 30 µm vibratome sections. The images represent projections of 15-20 optical sections. Positive immunofluorescence (green) is observed in chloroplasts. Nuclear DNA was stained with DAPI (blue). C) Overlay of Nomarski picture (grey) with anti-GmHMA8 immunofluorescence (green). Arrows showed the chloroplast localization of GmHMA8. D) Preimmune antiserum immunofluorescence. E) Cell structure with chloroplasts, many of them containing clear starch deposits (arrows). F) Starch grains revealed as dark inclusions by iodide-base cytochemistry, observed under bright field. Bars in A, B, C and D represent 2.0 µm; bars in E and F represent 1.0 µm.

**Figure 5.** Double immunofluorescence with anti-GmHMA8 and anti-RuBisCo in soybean photosynthetic cell cultures. Confocal laser microscopy observations of 30 µm vibratome sections, the images represent projections of 15-20 optical sections. A) Immunofluorescence with anti-GmHMA8 and detected with anti-rabbit immunoglobulins labelled with Alexa 488. B) Immunofluorescence with anti-RuBisCo and detected with anti-rabbit immunoglobulins labelled with Alexa 546. C, F) Co-localization of staining for GmHMA8 and RuBisCo (yellow). D) Cell structure observed at higher magnification: cells show chloroplasts, many
of them containing clear starch deposits. E) Starch grains revealed as dark inclusions by iodide-base cytochemistry, observed under bright field. Images in A and B were taken with CLSM for the red channel reduced to zero and for the green channel reduced to zero, respectively. Images in C and F were obtained by simultaneous acquisition of data in both channels. Bars in A, B and C represent 1.0 µm; bars in D, E and F represent 2.0 µm.

**Figure 6.** Anti-GmHMA8 immunogold labelling on soybean photosynthetic cell. Ultrathin Lowicryl sections. A) General view of soybean cells. B) General view of chloroplasts. C) and D) Gold particles are localized in thylakoid membranes (arrows). V (vacuole); N (nucleus); S (stroma); T (thylakoid membranes); C (cytoplasm). Bars in A, B represent 0.5 µm, and in C, D represent 200 nm.
Table 1. Primers used for the isolation of *GmHMA8*.

<table>
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<th>Primer name</th>
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<tr>
<td></td>
<td>reverse</td>
<td>(GTCA)A(AG)(AGT)GC(AGT)GG(AGT)G(AG)TC(AG)TT(AT)AT(TCAG)CC’</td>
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<td>UTRGmHMA8</td>
<td>reverse</td>
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<td>5' GeneRacer nested primer</td>
<td>forward</td>
<td>GGACACTGACATGAGAGGAGA’</td>
</tr>
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</table>
Fig. 1
Figure 2

A

GmHMA8 1 67
LcHMA8 1 68
AthHMA8 1 61
OsHMA8 1 70
PacS 1 1

HMB
phosphatase
ion transduction TM 6
TM 1
TM 2
TM 3
TM 4
TM 5
ion transduction TM 6
Fig. 4
Fig. 5