

Higher plants possess two different types of ATX1-like copper chaperones

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Abstract

Copper (Cu) chaperones constitute a family of small Cu⁺-binding proteins required for Cu homeostasis in eukaryotes. The ATX1 family of Cu chaperones specifically delivers Cu to heavy metal P-type ATPases. The plant *Arabidopsis thaliana* expresses the ATX1-like Cu chaperone CCH, which exhibits a plant-specific carboxy-terminal domain (CTD) with unique structural properties. We show that CCH homologues from other higher plants contain CTDs with structural properties similar to Arabidopsis CCH. Furthermore, we identify a new ATX1-like Cu chaperone in *Arabidopsis*, AtATX1, which functionally complements yeast *atx1Δ* and *sod1Δ* associated phenotypes, and localizes to the cytosol of *Arabidopsis* cells. Interestingly, AtATX1, but not full-length CCH, interacts *in vivo* with the *Arabidopsis* RAN1 Cu-transporting P-type ATPase by yeast two-hybrid. We propose that higher plants express two types of ATX1-like Cu chaperones: the ATX1-type with a predominant function in Cu delivery to P-type ATPases, and the CCH-type with additional CTD-mediated plant-specific functions.

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Eukaryotic organisms deal with copper (Cu) through complex homeostatic networks that tightly regulate intracellular Cu levels to ensure its availability, while avoiding its potential toxicity (reviewed in [1]). High-affinity Cu

acquisition at the plasma membrane depends on the conserved family of CTR Cu⁺ transporters (denoted COPT in *Arabidopsis thaliana*) [2,3]. Once inside the cell, the limited solubility and high reactivity of Cu⁺ requires the participation of cytosolic factors named Cu chaperones or metallochaperones. Cu chaperones are a set of soluble Cu⁺-binding proteins that mediate intracellular Cu trafficking to specific Cu-proteins and compartments within the cell (reviewed in [4]). Studies with the yeast *Saccharomyces cerevisiae* have allowed the identification of Atx1 metallochaperone, which delivers Cu to a Cu-transporting P-type ATPase located at the *trans*-Golgi network, for its translocation and incorporation into Cu-proteins within the secretory pathway [5,6]. Atx1 protein exhibits a

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conserved overall $\beta\alpha\beta\beta\alpha\beta$ -fold structure that coordinates one atom of Cu^+ to the cysteines of a MxCxxC motif [7]. In addition to the P-type ATPase-conserved features, including the kinase, ATP-binding and phosphatase domains, Cu-transporting P-type ATPases possess from two to six amino-terminal metal-binding domains (MBDs) with MxCxxC motifs and an overall tertiary structure similar to Atx1 [8]. Cu^+ transfer from Atx1 metallochaperones to the MBDs of Cu-transporting P-type ATPases is a vectorial Cu^+ interchange involving mixed intermediates of double and triple coordination to the cysteine ligands in the MxCxxC motifs [9]. Arabidopsis Responsive-to-AN-antagonist 1 or RAN1 (HMA7) was the first Cu-transporting P-type ATPase identified in plants [10,11]. Given that Cu coordination to the Arabidopsis ethylene receptor ETR1 is required for ethylene binding [12], RAN1 has been proposed to supply this metal cofactor to ETR1, thus allowing biogenesis of functional ethylene receptors [10,11].

The Arabidopsis CCH protein was the first Cu chaperone described in plants [13]. At its amino-terminal domain, CCH possesses the conserved features of the ATX1-like family of metallochaperones including the predicted overall $\beta\alpha\beta\beta\alpha\beta$ -fold structure and an MxCxxC Cu^+ -binding motif [7,13]. Interestingly, CCH exhibits a plant-specific carboxy-terminal domain (CTD) (amino acids 72–121), which is absent in all the identified non-plant ATX1-like metallochaperones [14–16]. This domain adopts an extended conformation in solution, which in the presence of anionic detergents displays altered electrophoretic mobility and forms well-ordered amyloid-like fibrils [16]. The CCH metallochaperone is mostly found in vascular cells that lack nuclei, suggesting that the protein is transported from neighbour cells through plasmodesmata [14], and its expression is increased by oxidative stress, senescence, and Cu deficiency [13].

In this report, we show multiple experiments including heterologous functional complementation of specific yeast mutants, subcellular localization in Arabidopsis protoplasts, gene expression profile at different growth stages, and protein–protein interaction assays, which strongly suggest that At1g66240, named hereafter AtATX1, is a functional homologue in *A. thaliana* of yeast Atx1 metallochaperone. Furthermore, structural studies on the CTD of the CCH Cu chaperone from rice and soybean plants, and protein–protein interaction studies with Arabidopsis CCH, strongly suggest a conserved functional and regulatory role for CCH CTD in plants. Taken together, we conclude from these results that higher plants express two functionally different ATX1-like metallochaperones.

Experimental procedures

Plant material and sequence analysis. Arabidopsis ecotype Columbia-0 plants were grown under a 16 h light/8 h dark cycle on soil at 23 °C. For growth on agar media, seeds were surface-sterilized and sown on solid Murashige and Skoog (MS) medium including 3% sucrose and 0.4% agarose. TAIR and MIPS databases suggest AtATX1 to be a 106 amino acid protein with a probable amino terminal signal sequence of 30 amino

acids length, or alternatively a shorter 76 amino acid protein. In this report a full-length 106 amino acid AtATX1 has been used for expression in Arabidopsis, while a 76 amino acid AtATX1 has been used for expression in yeast and for sequence alignments.

Yeast complementation. The entire coding regions of *S. cerevisiae* ATX1, and Arabidopsis CCH, AtATX1 and AtATX1-GFP were cloned into p426GPD yeast vector. Yeast *atx1Δ* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *atx1::KanMX4*) and *sod1Δ* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *sod1::KanMX4*) mutant strains (Research Genomics) were used for complementation.

Expression of green fluorescent reporter protein in isolated Arabidopsis protoplasts. Protoplasts were isolated and transformed essentially as previously described [17]. A confocal laser-scanning microscope (FVX-IHRT Fluoview Confocal LSM, Olympus, Melville, NY, USA) with Kr/Ar laser excitation (488 nm) was used to monitor green fluorescence (530 nm) and red chlorophyll auto fluorescence (660 nm). Image captures and processing were by Fluoview software (Olympus). Images were captured at 90× magnification at a scan speed of 0.45 s for 256 × 256 pixel area. Scan slices were 1.0 μm thick.

Yeast two-hybrid assays. Arabidopsis AtATX1 coding sequence was cloned into the pGADT7 plasmid generating pGAD-ATX1 plasmid. The *RAN1* coding region encoding the 289 amino-terminal amino acids containing the two MBDs was cloned into the pGBKT7 plasmid generating pGBK-RAN1 plasmid. AH109 cells cotransformed with GAD and GBK based plasmids (Matchmaker, BD Biosciences) were assayed for growth. Otherwise, yeast two-hybrid assays were performed as previously described [18].

Expression and purification of recombinant proteins. Glutathione-S-transferase (GST) fusion constructs were created by subcloning the appropriate fragments of the CCH cDNA into the pGEX-5X vector (Pharmacia Inc.). The insert DNA for construction of GST-Arabidopsis CTD was prepared as described previously [15]. To construct the GST-rice and soybean CTD fusions, the corresponding sequences were amplified by PCR using the primer sets: 5'-GCTCCGATCCCGTGGGAGGCTG CAGAAGCCGC-3' and 3'-GGCCGGTTCCGACTACGAACACTGCT TAAGGCCTG-5' for *Oryza sativa*, and 5'-GCTCCGGATCCCGTGG GTGGATGAAGCACCAC-3' and 3'-CAACTTTGACAACGAATT CCGGCTTAAGGCCTG-5' for *Glycine max*, and cloned into pGEX-5X. Expression in *Escherichia coli*, purification and removal of the GST tag were performed as indicated [15]. All purified recombinant proteins were analyzed for purity and integrity by SDS-PAGE. Identity was confirmed by matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry.

Results

Two types of ATX1-like metallochaperones are present in higher-plant genomes

We have analyzed the distribution of ATX1-like sequences among photosynthetic organisms present in the databases, as compared to *S. cerevisiae* ATX1, including two cDNAs from *O. sativa* (OsATX1) and *G. max* (GmATX1), whose corresponding sequences have been deposited in the databases (Accession Nos. AF198626 and AF198627, respectively) (Supplementary Fig. S1). Sequence analysis suggests that higher plant genomes, as opposed to the rest of eukaryotic organisms, possess two types of ATX1-like metallochaperones based on the absence or presence of an extra CTD when compared to yeast Atx1 (Supplementary Fig. S1). Sequence comparison at the amino acid level among the plant proteins reveals an 87% identity at the amino-terminal domain (the ATX1-like

portion of the proteins), while the identity drops down to 24% at the CTD.

Higher-plant CCH carboxy-terminal domains exhibit conserved structural properties

The lack of sequence similarity between the CTDs from different plant CCH proteins raises the question of its functional significance. Given that Arabidopsis CCH CTD possesses unusual characteristics including an extended conformation in solution and in the presence of anionic detergents, as well as altered SDS/PAGE mobility [14,15], we decided to analyze the CCH CTD properties from other higher plants including rice and soybean. Both CTDs fused to GST were separately expressed and purified in *E. coli*, and their electrophoretic mobility analyzed by SDS-PAGE gel. The CTDs from rice (6.8 kDa) and soybean (6.1 kDa) fused to GST (29 kDa) migrated at the molecular weights expected for the respective monomeric fusion proteins (35.8 and 35.1 kDa) (Fig. 1A). Strikingly, after releasing the CTDs by cleavage with factor-Xa, the *G. max* CCH CTD fractionated as two weak protein bands of an estimated molecular weight around 28 and 35 kDa (Fig. 1B). Furthermore, the *O. sativa* CTD polypeptide was barely observed in SDS-PAGE gels as a band of

35 kDa (Fig. 1B), although its presence was confirmed by HPLC and MALDI-TOF analysis (results not shown). The apparent molecular weight obtained from the electrophoretic mobilities (Fig. 1A and B) differs from the molecular weights obtained by MALDI-TOF (results not shown), which are in close agreement with their expected molecular weight derived from the amino acid sequence. These results indicate that both rice and soybean CCH CTDs exhibit abnormal electrophoretic properties as previously shown for Arabidopsis CCH CTD [15].

The rice and soybean CCH CTD conformation was further analyzed by CD spectroscopy in the absence or presence of 40% trifluoroethanol (TFE) (Fig. 1C). The far-UV CD spectra of the recombinant rice and, especially, soybean CTDs in the presence of TFE exhibited a strong negative ellipticity at 198 nm ($\sim -10,000^\circ \text{ cm dmole}^{-1}$) as compared to their spectra in the absence of TFE, which is consistent with a disordered polypeptide structure. In addition, the rice and soybean CTDs show a stabilization of secondary structure at alkaline pH (data not shown), as indicated by an increase in the intensity of the negative peak at 198 nm. Taken together, these results strongly suggest that the structural properties of Arabidopsis CCH CTD are conserved in other higher-plant CCH-like chaperones.

Identification of *AtATX1* as a functional homologue of yeast *Atx1* in Arabidopsis

The Arabidopsis genome contains a gene encoding a putative short *Atx1* homologue, which we have named *AtATX1* (MIPS code: At1g66240). To ascertain whether *AtATX1* is a functional homologue of yeast *Atx1*, the Arabidopsis protein was expressed in *atx1Δ* and *sod1Δ* yeast mutants. Yeast *ATX1* (*ScATX1*) and the Arabidopsis long member CCH were used as positive controls. Yeast *atx1Δ* mutants exhibit growth defects on iron-deficient conditions as a consequence of impaired Cu delivery to the multicopper-oxidase Fet3, which is essential for high-affinity iron uptake [9]. We tested *atx1Δ* functional complementation by growth on media containing both the extracellular Cu chelator bathocuproine disulfonic acid or BCS, and the extracellular iron chelator bathophenanthroline disulfonic acid or BPS (BPS/BCS), or on media containing the membrane permeable iron chelator ferrozine. *AtATX1* fully complements the growth defect of yeast *atx1Δ* mutants on iron-deficient media (Fig. 2A). Yeast cells defective in the Cu/Zn superoxide dismutase *Sod1* exhibit a growth defect in the presence of menadione, an agent that generates the formation of radical oxygen species, and are auxotrophic for lysine due to the oxidative damage of enzymes required for lysine biosynthesis [19]. Yeast *Atx1* was identified as a suppressor of *sod1Δ*-associated oxidative defects [5]. Arabidopsis *ATX1* fully complements the antioxidant properties of yeast *Atx1*, as shown by growth on lysine-defective and on menadione-containing media (Fig. 2B). Taken together these results strongly suggest that

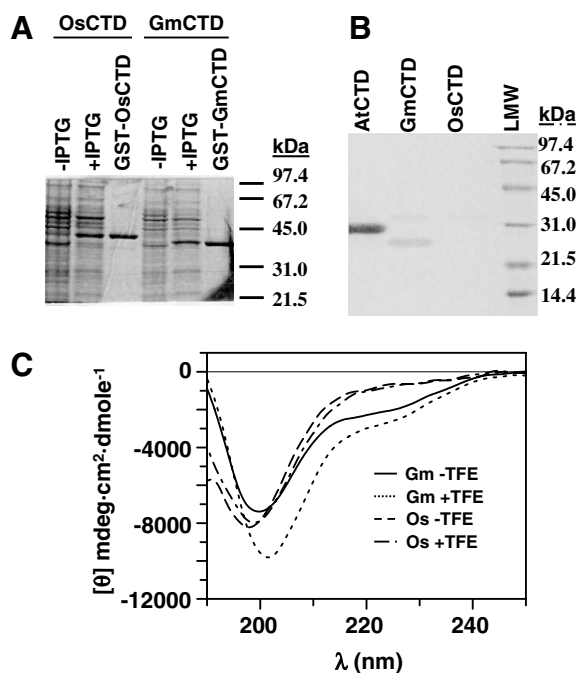


Fig. 1. Rice and soybean CCH CTDs exhibit similar structural properties to Arabidopsis CCH CTD. (A) Purification of GST-CTDs from rice and soybean. Electrophoretic profile of *E. coli* cell lysates before (minus) and after (plus) IPTG treatment, and purified GST-CTD fusion proteins. (B) Electrophoretic mobility of CTDs after digesting with Xa Factor. (C) CD spectra of CCH CTDs in the absence and presence of 40% trifluoroethanol (TFE). Rice CTD in the presence of TFE is represented as a broken line, and in the presence of TFE as a broken-dotted line. Soybean CTD in the absence of TFE is represented as a continuous line, and in the presence of TFE as a dotted line.

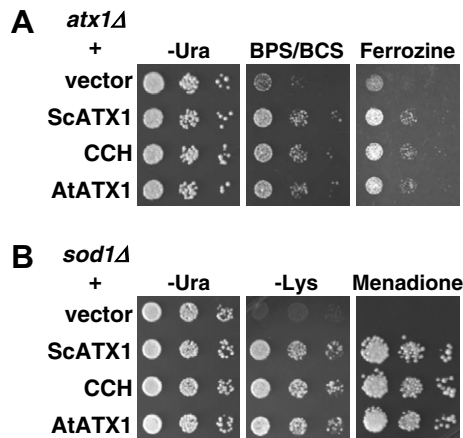


Fig. 2. Arabidopsis ATX1 rescues yeast *atx1Δ* and *sod1Δ* mutant phenotypes. (A) Complementation of the iron-deficient growth of *atx1Δ* yeast mutant strain. Yeast *atx1Δ* cells transformed with p426GPD vector alone (vector), *S. cerevisiae* ATX1 (ScATX1), Arabidopsis CCH (CCH), and Arabidopsis ATX1 (AtATX1) were assayed for growth on SC lacking uracil, SC medium containing 30–100 μ M BPS and 100 μ M BCS (BPS/BCS), or 850 μ M ferrozine (Sigma). (B) Complementation of the aerobic lysine auxotrophy and menadione toxicity of yeast *sod1Δ* mutant. *sod1Δ* cells transformed with the vector and plasmid constructs indicated in panel A were assayed for growth on SC-Ura, SC lacking lysine (SC-Lys) and YPD medium containing 25 μ M menadione (Sigma). Cells were spotted on one-tenth serial dilutions starting at $A_{600\text{ nm}}$ 0.1.

AtATX1 is a functional homologue of yeast Atx1 Cu chaperone.

Arabidopsis ATX1 is induced during senescence and oxidative stress

We have previously shown that Arabidopsis CCH mRNA and protein levels increase during plant senescence and oxidative stress, and decrease by Cu treatment [13,14]. To further analyze AtATX1 function in Arabidopsis as compared to CCH, we studied its response to these stresses. Both Northern blot and RT-PCR analyses indicate that *AtATX1* mRNA levels increase by oxidative stress treatments and at senescence (Supplementary Fig. S2, panels A and B). Whereas CCH is down-regulated by Cu treatment *AtATX1* is not (Supplementary Fig. S2, panel C). These results, together with previous data on CCH [13,14], show that both genes are induced by senescence and oxidative stress, but only CCH metallochaperone levels decrease by Cu treatment.

AtATX1 protein localizes to the cytosol of Arabidopsis cells

In order to test AtATX1 subcellular localization, the coding sequence for full-length AtATX1 protein was fused to the green fluorescent protein (GFP). AtATX1-GFP fusion protein was fully functional since it complemented the yeast *atx1Δ* and *sod1Δ* growth defects (data not shown). AtATX1-GFP was cloned in a transient expression vector under the control of the Cauliflower Mosaic

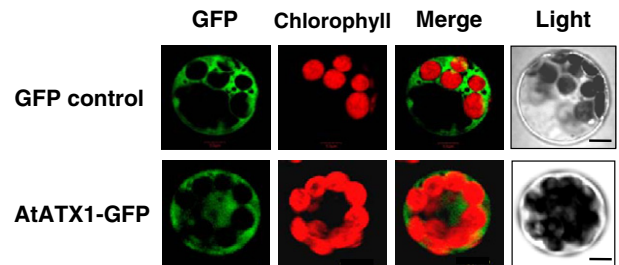


Fig. 3. AtATX1 localizes to the cytosol of Arabidopsis cells. AtATX1-GFP alone were transiently expressed in Arabidopsis protoplasts. Sixteen hours after transformation, protoplasts were analyzed by confocal microscopy for green fluorescence (GFP), red fluorescence (chlorophyll), green and red overlay fluorescence (Merge), and bright transmitted light. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Virus 35S constitutive promoter. Isolated *Arabidopsis* protoplasts transformed with AtATX1-GFP were analyzed for green and red fluorescence indicative of GFP and chlorophyll localization, respectively. A construct expressing GFP in the cytosol was used as a control. The pattern for the AtATX1-GFP fusion signal is very similar to the cytosolic control, as indicated by a GFP-like distribution pattern that is diffuse throughout the cell but absent from vacuoles, plastids and other discrete subcellular locations (Fig. 3). These results strongly suggest that AtATX1 is a soluble protein that localizes to the cytosol of Arabidopsis cells.

RAN1 copper-transporting P-type ATPase interacts with ATX1-like copper chaperones

Biochemical and genetic studies in bacteria, yeast and mammals have established that Cu delivery to metal P-type ATPases occurs through specific interactions between the MBDs of the ATPase and a cytosolic ATX1-like Cu chaperone. We have recently shown that AtATX1 interacts by yeast two-hybrid with the Arabidopsis P-type ATPase HMA5, which is involved in Cu detoxification [18]. To further characterize the pathway involved in Cu delivery to the secretory pathway, we ascertained whether RAN1 interacts *in vivo* with the Arabidopsis CCH and AtATX1 metallochaperones by using the yeast two-hybrid system. AtATX1, CCH and CCH lacking the CTD plant-specific domain (CCH Δ) were fused in frame to the GAL4 activation domain (GAD), and the amino-terminal metal binding domains (MBDs) of RAN1 were fused to the GAL4 DNA-binding domain (GBD). *HIS3* and *ADE2* genes, under the control of the *GAL4*-regulated promoters *GAL1* and *GAL2* respectively, were used as reporter genes for the yeast two-hybrid assay. As shown in Fig. 4, cells co-expressing the RAN1-MBDs and CCH did not grow in media without histidine (–His) or adenine (–Ade), which is indicative of a lack of interaction. Interestingly, cells co-expressing RAN1-MBDs with CCH Δ or ATX1 grow in the absence of histidine or adenine indicating a RAN1-chaperone inter-

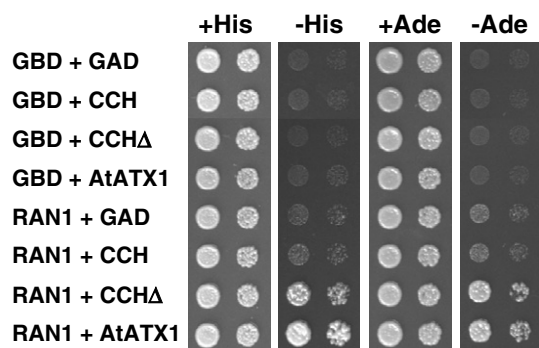


Fig. 4. Interaction between RAN1 Cu-transporting P-type ATPase and ATX1-like Cu chaperones. AH109 cells were cotransformed with pGAD and pGBK-based plasmids, which express GAL4 activation domain (GAD) and GAL4 DNA binding domain (GBD), respectively. RAN1 represents the fusion of 289 amino-terminal amino acids (MBDs) from RAN1 protein to the GBD, CCH represents full length CCH coding sequence fused to the GAD, CCH Δ represents the fusion of the 68 amino-terminal amino acids of CCH protein to GAD, and AtATX1 represents full length AtATX1 coding sequence fused to the GAD. Cotransformed cells were spotted on SC-Leu-Trp (+His) and SC-Leu-Trp-His + 10 mM of the His3 inhibitor aminotriazol (–His), SC plus adenine (+Ade) and SC minus adenine (–Ade).

action. These results demonstrate that RAN1 is able to interact with ATX1-like Cu chaperones *in vivo* and suggest a potential regulatory role for the CCH CTD present in higher plants.

Discussion

A protein alignment with ATX1-like Cu chaperones, including the higher-plant metallochaperones cloned in this study (AtATX1, OsCCH, and GmCCH), shows two types of ATX1-like Cu chaperones in higher plants, which are distinguishable by the presence or absence of an extended CTD. We show here that both Arabidopsis Cu chaperones are capable of complementing the growth defects under iron deficient conditions of yeast *atx1Δ* mutant cells, as well as the aerobic lysine growth deficiency and menadione toxicity of yeast *sod1Δ* mutants. These results indicate that both AtATX1 and CCH are able to carry and transfer Cu to the yeast Ccc2 P-type ATPase in the secretory pathway, and they also confer antioxidant protection against oxidative stress damage. Furthermore, AtATX1 and CCH share similar expression characteristics regarding their up-regulation during senescence and oxidative stress processes. This gene regulation pattern suggests a putative function of AtATX1 during plant senescence processes as previously suggested for CCH [15]. Moreover, taken together with the yeast *sod1Δ* complementation, these results also point to a putative antioxidant function for the Arabidopsis ATX1-like metallochaperones, as initially proposed for yeast Atx1 [5].

In addition to these similarities between AtATX1 and CCH, both metallochaperones exhibit important functional differences. First, we show here that AtATX1 interacts *in vivo* with the MBDs of the RAN1 Cu-transporting P-type ATPase, suggesting that the AtATX1 metallochap-

erone functions in Cu delivery to Arabidopsis Cu-transporting P-type ATPases. However, CCH yeast two-hybrid interaction with RAN1, as well as HMA5 Cu-transporting P-type ATPase [18], is abolished in the presence of its CTD. Second, only CCH but not AtATX1 mRNA levels are down-regulated upon Cu treatment ([13], and Fig. S2). And third, CCH protein is strikingly found in plant vascular tissues and in cells that lack nuclei [14]. These differences are probably a consequence of CCH specific CTD function in plants. In this report, we show that CCH CTD from Arabidopsis, rice and soybean show multiple common structural properties including increased β -sheet, the high tendency to self-aggregation forming amyloid-like filaments, and the insolubility in the presence of detergents, which are shared with prion protein pathological conformations [20].

We propose that higher plants possess at least two different types of ATX1-like Cu chaperones: (1) the short-type Cu chaperones, which may play a predominant role in Cu delivery to P-type ATPases within the cell; and (2) the CCH-type Cu chaperones, which could mediate a plant-specific function such as Cu transport through the plasmodesmata and, therefore, Cu mobilization during senescence. Although further *in vivo* studies in plants are necessary to clarify the exact function of the additional CCH CTD, results shown in this report suggest that it plays an important and plant-conserved role in regulating CCH function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2006.12.215](https://doi.org/10.1016/j.bbrc.2006.12.215).

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