Copper Chaperone Antioxidant Protein1 Is Essential for Copper Homeostasis[1][W][OA]

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Copper (Cu) is essential for plant growth but toxic in excess. Specific molecular mechanisms maintain Cu homeostasis to facilitate its use and avoid the toxicity. Cu chaperones, proteins containing a Cu-binding domain(s), are thought to assist Cu intracellular homeostasis by their Cu-chelating ability. In Arabidopsis (*Arabidopsis thaliana*), two Cu chaperones, Antioxidant Protein1 (ATX1) and ATX1-Like Copper Chaperone (CCH), share high sequence homology. Previously, their Cu-binding capabilities were demonstrated and interacting molecules were identified. To understand the physiological functions of these two chaperones, we characterized the phenotype of *atx1* and *cch* mutants and the *cchatx1* double mutant in Arabidopsis. The shoot and root growth of *atx1* and *cchatx1* but not *cch* was specifically hypersensitive to excess Cu but not excess iron, zinc, or cadmium. The activities of antioxidant enzymes in *atx1* and *cchatx1* were markedly regulated in response to excess Cu, which confirms the phenotype of Cu hypersensitivity. Interestingly, *atx1* and *cchatx1* were sensitive to Cu deficiency. Overexpression of *ATX1* not only enhanced Cu tolerance and accumulation in excess Cu conditions but also tolerance to Cu deficiency. In addition, the Cu-binding motif MXCXXC of ATX1 was required for these physiological functions. ATX1 was previously proposed to be involved in Cu homeostasis by its Cu-binding activity and interaction with the Cu transporter Heavy metal-transporting P-type ATPase5. In this study, we demonstrate that *ATX1* plays an essential role in Cu homeostasis in conferring tolerance to excess Cu and Cu deficiency. The possible mechanism is discussed.

Copper (Cu) is a transition metal involved in widespread physiological activity, including photosynthesis, mitochondrial respiration, antioxidant activity, and ethylene signaling (Puig et al., 2007a). Intracellular Cu must be accurately utilized to avoid toxicity caused by the free Cu ion with generated reactive oxygen species such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical that damage proteins, lipids, and DNA (Brewer, 2010). Studies of yeast (*Saccharomyces cerevisiae*) indicate that free Cu ion is restricted to less than one molecule in a cell and is not available for metalloenzymes in physiological activity (Rae et al., 1999). Therefore, Cu uptake must be tightly controlled and Cu must be chelated intracellularly to maintain homeostasis and delivery.

Plants with a vascular transport system have developed a proficient homeostatic mechanism to manage Cu homeostasis (Puig and Thiele, 2002). A fundamental step in maintaining Cu homeostasis is to control suitable uptake and efflux through the plasma membrane. In yeast and mammals, the transporters responsible for Cu uptake are mainly members of the high-affinity Cu transporter family (Puig and Thiele, 2002). In Arabidopsis (*Arabidopsis thaliana*), the high-affinity Cu transporters are members of the copper-transporter protein (COPT) family (Sancenón et al., 2003). *COPT1* was the first to be characterized and identified as a Cu uptake transporter (Kampfenkel et al., 1995). *COPT1* mRNA accumulates mainly in root tips and is positively regulated by Cu deficiency. Cu acquisition and accumulation in *COPT1* knockdown lines is decreased to 50% that of the wild type, and such lines showed a Cu-deficient phenotype (Sancenón et al., 2004). In addition, pollen development is defective in these lines (Sancenón et al., 2004). Thus, COPT1 functions as a major Cu uptake transporter in Arabidopsis and is important for plant growth and development. Recently, COPT5 was identified as a prevacuolar compartment/vacuolar Cu exporter. Cu must be released from the root vacuole for long-distance transport to aerial organs (Garcia-Molina et al., 2011; Klaumann et al., 2011; Pilon, 2011).

Heavy metal-transporting P-type ATPases (HMAs) 5 to 8 are also associated with Cu homeostasis (Burkhead et al., 2009). Among the four HMAs, HMA5 was reported to be crucial for Cu efflux and vascular translocation (Andrés-Colás et al., 2006). *HMA5* is mainly expressed in the root and flower and is up-regulated by excess Cu. The phenotype of the *hma5* mutant is root Cu hypersensitive, and Cu remains in

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roots under excess Cu conditions (Andrés-Colás et al., 2006). The *COPT1* knockdown line is sensitive to Cu deficiency, and the *hma5* mutant is sensitive to excess Cu. This contrasting Cu-sensitive phenotype between the *COPT1* knockdown lines and the *hma5* mutant supports COPT1 and HMA5 as being responsible for Cu uptake and efflux, respectively. Thus, the balance among the environment, roots, and translocation in maintaining suitable intracellular Cu concentration relies on a coordinated expression of *COPT1* and *HMA5* (Burkhead et al., 2009).

Intracellularly, free Cu must be chelated and delivered to its physiological partner proteins by Cu chaperones after uptake. These Cu chaperones show open-faced β-sandwich global folding with a conserved MXCXXC Cu-binding motif (Harrison et al., 1999). Arabidopsis has at least three Cu chaperones, including the Cu chaperone for superoxide dismutase (SOD; *CCS*) and two homologs of yeast Antioxidant Protein1 (*ATX1*), the Copper Chaperone (*CCH*) and *ATX1* (Casareno et al., 1998; Chu et al., 2005; Puig et al., 2007b). In yeast, CCS is required to transfer Cu to Cu/zinc (Zn)-SOD for the activity (Rae et al., 1999). Arabidopsis has three isoforms of Cu/Zn-SOD, cytosolic (CSD1), chloroplastic (CSD2), and peroxisomal (CSD3) forms, and only one CCS (Chu et al., 2005). In the *ccs* mutant, the activities of all three Cu/Zn-SOD isoforms are sharply reduced, which indicates that CCS could deliver Cu to CSD2 in the plastid and to CSD1 and CSD3 in the cytosol in Arabidopsis.

CCH was the first Cu chaperone gene identified as a functional homolog of yeast *ATX1* and later *ATX1* in Arabidopsis (Himelblau et al., 1998; Puig et al., 2007b). Both *CCH* and *ATX1* can complement the yeast *atx1* mutant (Puig et al., 2007b). The analysis of amino acid alignment revealed the conserved Cu-binding motif in these two Cu chaperones. However, CCH has a unique C-terminal extension, whereas ATX1 has a probable N-terminal signal peptide (Mira et al., 2001b). The C-terminal extension of CCH was proposed to be involved in the translocation of proteins through plasmodesmata to nonnucleated cells, such as sieve elements, to provide a symplastic pathway for Cu redistribution and reutilization (Mira et al., 2001a). The mRNA expression of *CCH* is induced in the absence of Cu and reduced with excess Cu, whereas *ATX1* expression is induced by excess Cu. Opposite Cu-regulated expression of *CCH* and *ATX1* suggests that they may function differently in Cu homeostasis in higher plants (Puig et al., 2007a). Therefore, more complicated or divergent functions could have evolved for handling different compartmentalization and translocation in higher plants than in yeast.

Previous yeast two-hybrid experiments suggested that full-length ATX1 and C-terminal extension-deleted CCH interact with Responsive to Antogonist1 (RAN1)/HMA7 and HMA5 (Andrés-Colás et al., 2006; Puig et al., 2007b). RAN1 possesses Cu-transporting P-type ATPase activity and is required for ethylene signaling in Arabidopsis (Hirayama et al., 1999), whereas HMA5 contributes to Cu efflux (Andrés-Colás et al., 2006). Thus, *CCH* and *ATX1* could be involved in Cu homeostasis and ethylene signaling. However, no phenotype related to these functions has been reported. Therefore, the biological importance of *CCH* and *ATX1* in plants remains unknown.

In this study, we investigated the role of *ATX1* and *CCH* and found a requirement of *ATX1* but not *CCH* for tolerance to excess Cu and Cu deficiency in the vegetative stage of Arabidopsis. Furthermore, high Cu accumulation and tolerance of *ATX1* overexpression lines grown in high-Cu soil were also observed. The phenotype of enhanced growth with *ATX1* overexpression suggests its positive roles in Cu homeostasis.

RESULTS

Isolation of Cu Chaperone Mutants

To examine the biological function of CCH and ATX1, we used Arabidopsis mutants with transfer-DNAs (T-DNAs) inserted in *CCH* (SALK_138593) and *ATX1* (SALK_026221; Supplemental Fig. S1, A and B). Reverse transcription (RT)-PCR used to analyze the expression of *CCH* and *ATX1* revealed no signals in *cch* or *atx1* mutants (Supplemental Fig. S1C), so the T-DNA insertions resulted in complete loss of gene expression in these mutants. To confirm the null function of both genes, we generated antibodies against CCH and ATX1 and found neither CCH nor ATX1 accumulated in the *cch* or *atx1* mutant, respectively (Fig. 1A). The *cchatx1* double mutant, created by crossing the *cch* and *atx1* mutants, showed no CCH or ATX1 protein accumulation (Fig. 1B). We used these Cu chaperone mutants for phenotypic characterization.

The *atx1* and *cchatx1* Mutants Are Highly Sensitive to Excess Cu

To study the biological roles of *CCH* and *ATX1* in plant development, we analyzed tolerance to Cu, iron (Fe), Zn, and cadmium (Cd) stresses; triple responses to ethylene treatment; and responses to paraquat, heat, and cold shock in the wild type and Cu chaperone mutants (Lin and Culotta, 1995; Woeste and Kieber, 2000; Shibasaki et al., 2009; Liu et al., 2011) in terms of plant biomass and root length (Marschner, 1995; Lequeux et al., 2010). The *atx1* and *cchatx1* mutants were hypersensitive to excess Cu among the heavy metals in root length and growth (Fig. 1C; Supplemental Fig. S2). The other treatments produced no obvious phenotype (data not shown). Fresh weight and root length were lower for *atx1* and *cchatx1* than for the wild type and the *cch* mutant with excess Cu (Fig. 1, D and E). The degrees of growth reduction for both *atx1* and *cchatx1* were almost identical, which suggests no added effects with the *cch* defect. With 25 and 35 μM Cu, the fresh weight for both *atx1* and *cchatx1* was 49% and 51%, respectively, that of the wild type. Additionally, with 25,

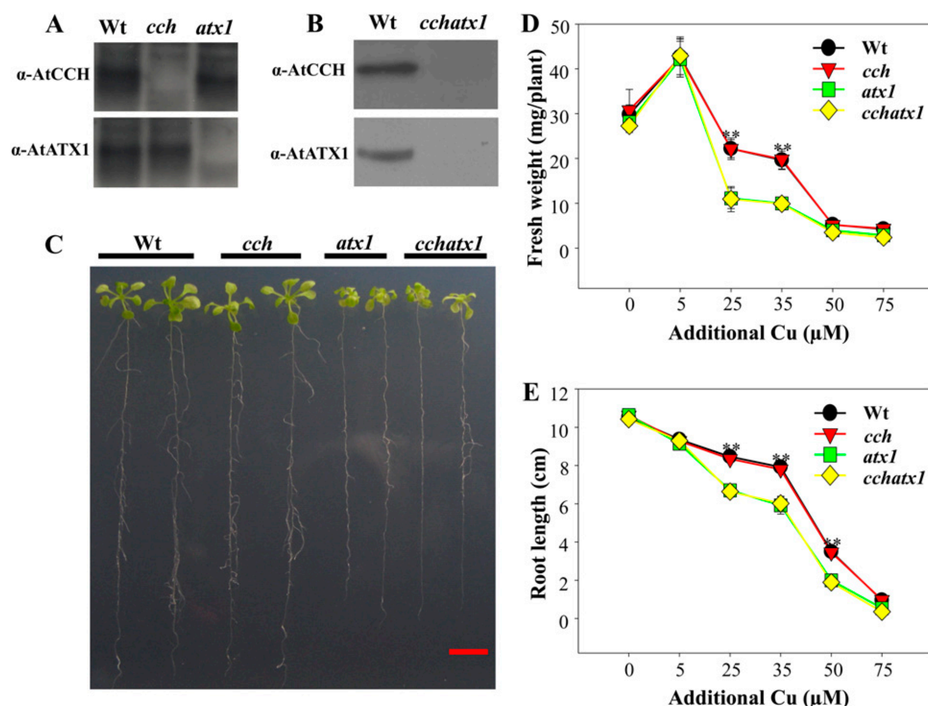

Figure 1. Growth of wild-type (Wt) and Cu chaperone mutant plants under Cu stress. A and B, Plants were grown on one-half-strength MS agar plates for 14 d. Western-blot analysis is shown for protein levels of CCH and ATX1 in *cch* and *atx1* mutants (A) and the *cchatx1* double mutant (B) detected by CCH (α-AtCCH) and ATX1 (α-AtATX1) antibodies. C, Seeds of the wild type and mutants were grown vertically on one-half-strength MS agar plates and treated with 35 μM $CuSO_4$ for 17 d. Bar = 1 cm. D and E, Wild-type, *cch*, *atx1*, and *cchatx1* plants were grown in one-half-strength MS medium and treated with additional Cu as indicated for 17 d, and fresh weight (D) and root length (E) were measured. Data are means ± SD of four replicates with 40 seedlings each. ** $P < 0.01$ compared with *atx1* and *cchatx1* in the same condition.

35, and 50 μM Cu, the root length was about 80%, 76%, and 57%, respectively, that of the wild type. Of note, shoot Cu accumulation was similar in the wild type and mutants grown in one-half-strength Murashige and Skoog (MS) medium with excess Cu or other heavy metals (Supplemental Fig. S2D). Additionally, the wild type and mutants did not differ in shoot Fe, Zn, manganese (Mn), magnesium (Mg), or calcium (Ca) accumulation with excess Cu (Supplemental Fig. S3). In summary, *atx1* and *cchatx1* mutants were specifically sensitive to Cu stress under our tested conditions. The response of *cch* to excess Cu was similar to that of the wild type. Therefore, ATX1 but not CCH is involved in Cu tolerance in Arabidopsis.

Expression of *CCH* and *ATX1* Is Independent of Each Other

Both *CCH* and *ATX1* are predicted to contribute to Cu homeostasis, and their expression is influenced by Cu availability (Mira et al., 2001a; Puig et al., 2007b). However, whether they affect each other's expression is not known. We examined the protein accumulation of ATX1 and CCH in *cch* and *atx1* mutants, respectively, under different Cu conditions. CCH expression was induced by Cu deficiency and reduced with excess Cu, whereas ATX1 expression was induced with excess Cu (Fig. 2). These data support previous mRNA accumulation results (Himelblau et al., 1998; Puig et al., 2007b). In addition, the ATX1 and CCH accumulation patterns in *cch* and *atx1* were identical to those in the wild type (Fig. 2). Thus, the expression of *CCH* and *ATX1* is independent in response to Cu excess or deficiency.

Excess Cu Negatively Affects Chlorophyll Content, Lipid Peroxidation, and Antioxidant Enzymes in *atx1* and *cchatx1*

Cu toxicity initiates a loss of chloroplast integrity, inhibited photosynthetic electron transport, increased lipid peroxidation, and influences antioxidant enzymes

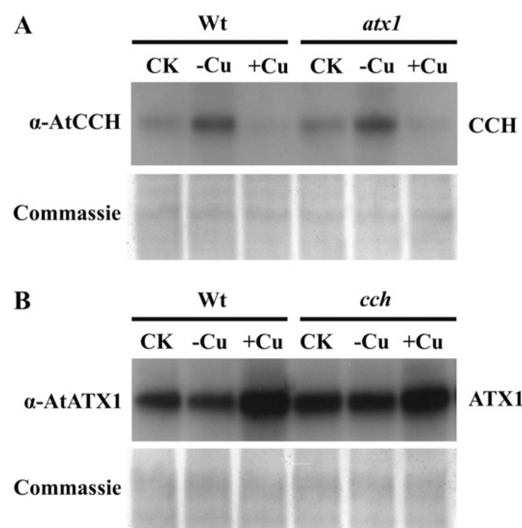

Figure 2. Accumulation of CCH and ATX1 protein under excess Cu and Cu deficiency. Plants were grown on one-half-strength MS phytagel plates for 11 d and transferred to one-half-strength MS agar plates with 35 μM $CuSO_4$ for 3 d. Western blot analysis (20 μg of total protein per lane) is shown for protein levels of CCH (A) and ATX1 (B) detected by CCH (α-AtCCH) and ATX1 (α-AtATX1) antibodies. Coomassie blue staining of protein was used to verify the loadings of total protein. Wt, Wild type.

(Pätsikkä et al., 2002; Drazkiewicz et al., 2004; Sun et al., 2010). The most common symptom to judge the loss of chloroplast integrity is chlorosis, which results from reduced chlorophyll and carotenoid contents in vegetative tissue. With leaf chlorosis in seedlings with excess Cu for 3 d, total chlorophyll content in *atx1* and *cchatx1* mutants was 73% of the wild-type content (Fig. 3A). Furthermore, carotenoid content was similarly reduced with excess Cu (Supplemental Fig. S4A).

PSII is a primary target for Cu toxicity (Kupper et al., 2003). With excess Cu, low-efficient PSII exhibits photooxidative damage, which results in an inhibited electron transport chain. We used the potential quantum yield of PSII (F_v/F_m) as an indicator of photooxidative damage. With excess Cu, the F_v/F_m ratio was significantly lower for *atx1* and *cchatx1* than for the wild type and the *cch* mutant (Supplemental Fig. S4B). Therefore, excess Cu induces high damage to plastids in *atx1* and *cchatx1* mutants.

As a redox-active metal, Cu can catalyze the formation of superoxide anion and result in the production of H_2O_2 and hydroxyl radical by the Fenton reaction (Schützendübel and Polle, 2002). These excess reactive oxygen species remove electrons from the lipids of cell membranes and cause lipid peroxidation, thereby damaging cells. Malondialdehyde (MDA) is one of the final products of lipid peroxidation. MDA content has been used to estimate the degree of oxidative stress in plants with excess Cu (Cho and Sohn, 2004; Skorzynska-Polit et al., 2010). We found that with excess Cu, leaf MDA content in *atx1* and *cchatx1* was 175% of the wild-type content (Fig. 3B). Root MDA content was also increased in the mutants (Supplemental Fig. S4C). Therefore, excess Cu induces high lipid peroxidation in *atx1* and *cchatx1* mutants.

According to a previous study, Cu toxicity induced the activity of peroxidase (POX) and reduced that of catalase (CAT) in Arabidopsis (Drazkiewicz et al., 2004). We further examined the activation of POX and CAT and found a significant increase in POX activity in shoots and roots of Arabidopsis and especially *atx1* and *cchatx1* with Cu treatment (Fig. 3C; Supplemental Fig. S4D). With excess Cu, the activity of POX in *atx1* and *cchatx1* was about 156% and 152%, respectively, of the wild-type activity in shoots and 156% and 164%, respectively, of the wild-type activity in roots (Fig. 3C; Supplemental Fig. S4D). However, with excess Cu, CAT activity in mutants was 67% of the wild-type activity in shoots and about 83% of the wild-type activity in roots (Fig. 3D; Supplemental Fig. S4E). Thus, *atx1* and *cchatx1* mutants experienced higher oxidative stress with excess Cu than the wild type and the *cch* mutant. ATX1 may play a crucial role in Cu tolerance by suppressing the negative effects of excess Cu.

Expression of *HMA5* and *COPT1* in Mutants

The Cu-sensitive phenotype of *atx1* and *cchatx1* mutants was enhanced with increased Cu concentration in the medium. Increased Cu may disrupt the homeotic regulation of Cu. The balance between Cu uptake and transport mainly relies on the expression of COPT1 and HMA5 in the root, which are regulated by Cu content in Arabidopsis (Sancenón et al., 2004; Andrés-Colás et al., 2006). We used quantitative

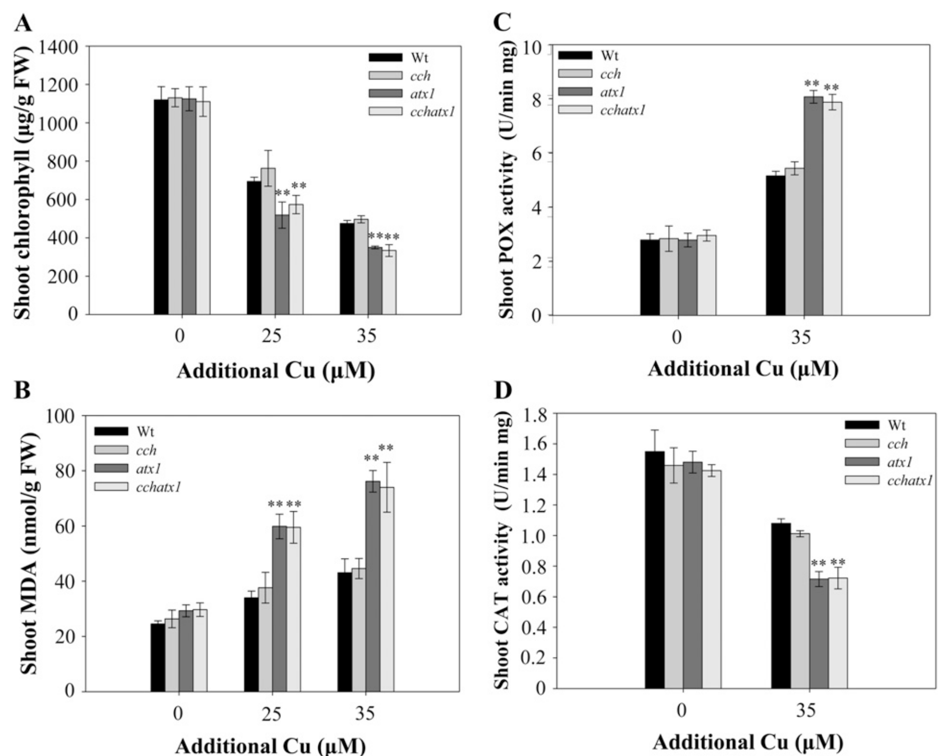

Figure 3. Chlorophyll content, lipid peroxidation, and antioxidant enzyme activities of Cu chaperone mutants under excess Cu. Plants were grown on one-half-strength MS phytagel plates for 11 d and transferred to one-half-strength MS agar plates with additional $CuSO_4$ as indicated for 3 d. Total chlorophyll (A) and MDA (B) contents and POX (C) and CAT (D) activities in shoots are shown. Data are means ± SD of four replicates with 10 seedlings each. ** $P < 0.01$ compared with the wild type (Wt) in the same condition. FW, Fresh weight.

RT-PCR to determine whether excess Cu leads to the misregulation of *COPT1* and *HMA5* in *atx1* and *cchatx1*. In the 3-d treatment, we found that excess Cu induced the *HMA5* level in roots of the wild type and *cch* about 144% and 152%, respectively ($P = 0.02$; Fig. 4A). The induction in the wild type was also observed previously in a prolonged treatment (Andrés-Colás et al., 2006). With excess Cu, *HMA5* level was much higher in *atx1* and *cchatx1* than in wild-type roots (Fig. 4A), but *COPT1* level was similar among wild-type and mutant roots (Fig. 4B). The up-regulation of *HMA5* with excess Cu was thought to participate in reducing the Cu toxicity in the root (Burkhead et al., 2009). Therefore, excess Cu could induce the expression of *HMA5* in *atx1* and *cchatx1*, which confirmed that *atx1* and *cchatx1* mutants were adversely affected by the Cu stress.

ATX1-Overexpressed Arabidopsis Exhibits Tolerance to Excess Cu

We generated Arabidopsis transgenic plants overexpressing *ATX1* in wild-type and *atx1* mutant backgrounds (Wt-ATX1 and *atx1*-ATX1, respectively) and used immunoblotting with total proteins extracted from 14-d-old T3 homozygous plants to examine the accumulation of ATX1 protein in both Wt-ATX1 and *atx1*-ATX1 (Fig. 5A). To determine Cu tolerance in these transgenic lines, we measured fresh weight and root length. Overexpression of *ATX1* restored the tolerance to excess Cu in the *atx1* mutant (Fig. 5B). The fresh weight of transgenic plants was about 136% to 139% with one-half-strength MS and about 145% to 300% with excess Cu as compared with the wild type and *cch* (Fig. 5C). Additionally, with one-half-strength MS and excess Cu, root lengths were longer for Wt-ATX1-1, Wt-ATX1-2, *atx1*-ATX1-1, and *atx1*-ATX1-2 than for the wild type and *cch* (Fig. 5D). Therefore, overexpression of ATX1 rescued the Cu-hypersensitive phenotype of *atx1* and *cchatx1* mutants and stimulated growth under both one-half-strength MS and excess Cu conditions.

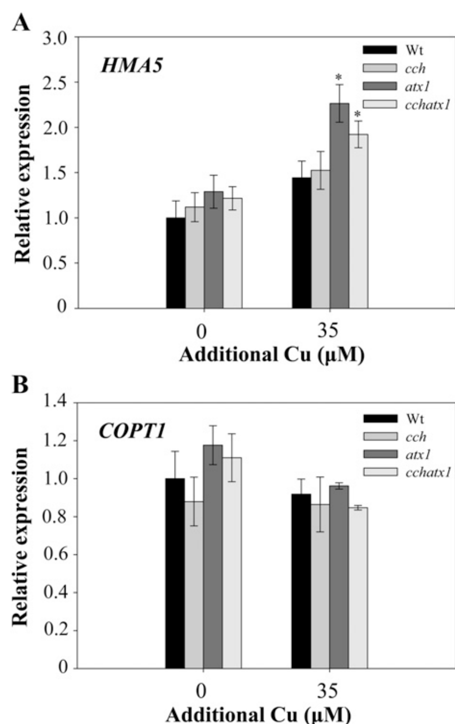

Figure 4. Expression of *HMA5* and *COPT1* with excess Cu. Plants were grown on one-half-strength MS phytagel plates for 11 d and transferred to one-half-strength MS agar plates with additional $CuSO_4$ as indicated for 3 d. Quantitative PCR analysis of mRNA expression of *HMA5* (A) and *COPT1* (B) in roots relative to *Actin2* is shown. Data are means ± SD of three replicates with 10 roots each. * $P < 0.05$ compared with the wild type (Wt) in the same condition.

ATX1-Overexpressed Arabidopsis Shows Tolerance to Cu Deficiency

The expression of CCH was induced with Cu deficiency and reduced with excess Cu (Fig. 2A). To test the importance of CCH in Cu deficiency, we examined the phenotype of the *cch* mutant and *CCH*-overexpressing lines in both the wild-type and *cch* backgrounds. Arabidopsis transgenic plants overexpressing the *CCH* gene were generated in the wild-type and *cch* mutant backgrounds (Wt-CCH and *cch*-CCH, respectively). Figure 6A shows the accumulation of CCH protein in selected transgenic lines of Wt-CCH and *cch*-CCH. The *cch* mutant and CCH-overexpressing lines showed no obvious changes in phenotype with Cu deficiency and excess Cu (Fig. 6B; data not shown). Interestingly, the *atx1* mutant and *ATX1*-overexpressing lines showed a phenotype under Cu-deficient conditions. The *atx1* and *cchatx1* mutants were more sensitive to Cu deficiency, whereas *ATX1*-overexpressing lines were more tolerant of Cu deficiency (Fig. 6C). With Cu deficiency, the biomass and root length of ATX1-overexpressing lines were about 170% and 120%, respectively, those of the wild type (Fig. 6, D and E). Thus, *ATX1* is required for tolerance to Cu deficiency. This finding implies that ATX1 increases Cu use efficiency, which results in enhanced growth on one-half-strength MS medium, considered a Cu-insufficient condition.

The MXCXXC Motif Is Required for the Function of ATX1

To elucidate whether the only conserved MXCXXC Cu-binding motif of ATX1 is essential for the function of ATX1 (Supplemental Fig. S5), we mutated the two Cys residues to Gly residues in the motif to create MXGXXG in mutated ATX1 for producing overexpressing lines in an *atx1* background (*atx1*-CG). We detected mutated ATX1 protein accumulated in the two independent *atx1*-CG lines (Fig. 7A) but observed no rescued phenotype under Cu-excess or Cu-deficient conditions in both lines (Fig. 7B). Sensitivity to excess

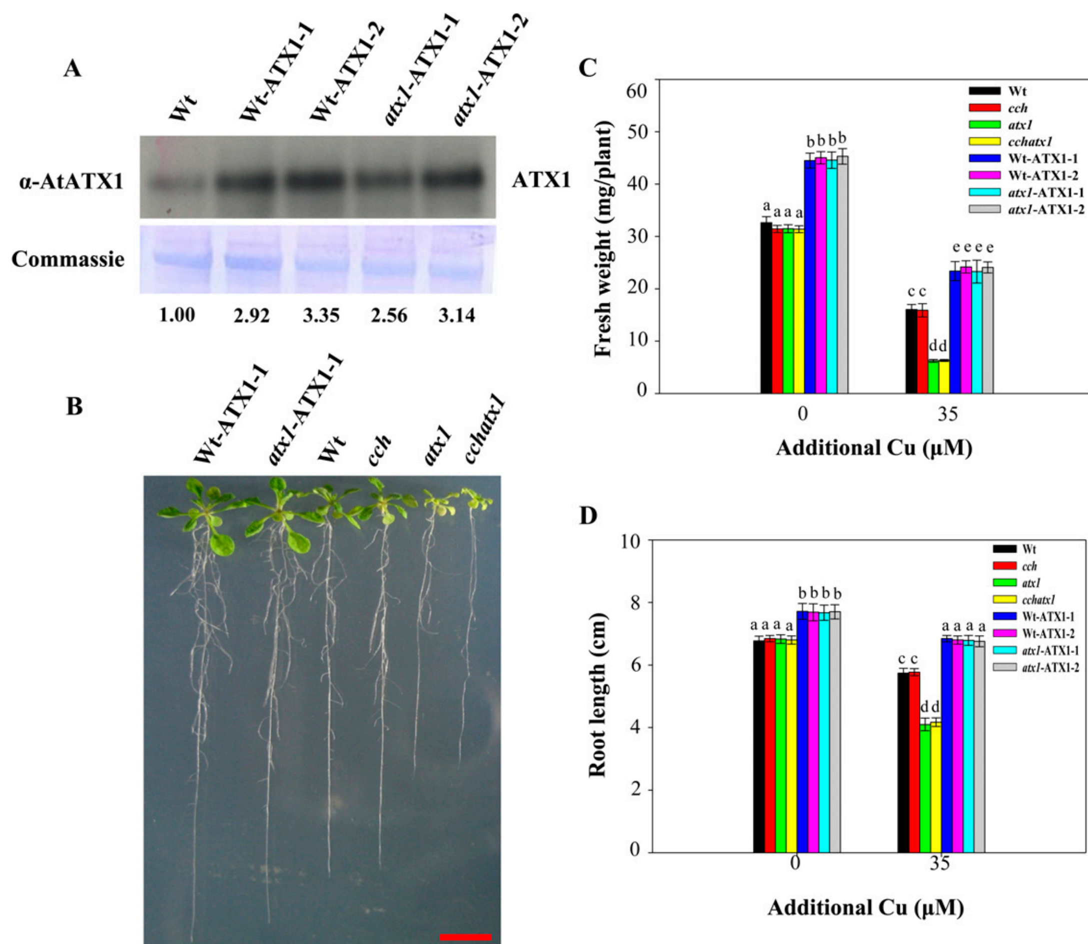

Figure 5. Phenotypes of *ATX1* transgenic lines, the wild type (Wt), and Cu chaperone mutants with excess Cu. A, Protein level of ATX1 detected by ATX1 antibody (α-AtATX1) in total protein (20 μg) isolated from each line. Coomassie blue staining of protein was used to verify the loadings. Numbers indicate the relative intensity of immunoblotting by normalization to the wild type. B, Plants were grown on one-half-strength MS agar plates with 35 μM $CuSO_4$ for 17 d. Bar = 1 cm. C and D, Fresh weight (C) and root length (D) of plants (13 d of growth on one-half-strength MS agar plates with additional $CuSO_4$ as indicated). Data are means ± SD of seven replicates with 10 seedlings (C) and 40 seedlings (D). Different lowercase letters represent statistical differences by Student's *t* test. Data are shown for at least three representative lines of each transgenic construct characterized.

Cu was similar for the *atx1*-CG-1 and *atx1*-CG-2 transgenic lines and the *atx1* mutant (Fig. 7B). With excess Cu, the biomass and root length for *atx1*, *atx1*-CG-1, and *atx1*-CG-2 was about 60% and 50%, respectively, those of the wild type (Fig. 7, C and D). Therefore, ATX1-mediated tolerance to excess Cu may have depended on the MXCXXC motif. Furthermore, the *atx1*-CG-1 and *atx1*-CG-2 transgenic lines, similar to *atx1*, showed a loss of tolerance to Cu deficiency (Fig. 7B). Thus, the MXCXXC Cu-binding motif is required for ATX1 function in response to both excess Cu and Cu deficiency. Additionally, Cu chelating is the crucial action of ATX1 in conducting its biological function.

ATX1 Overexpression Enhances Cu Accumulation

Our finding of the overexpression of *ATX1* enhancing Cu tolerance implies the potential use of *ATX1* for phytoremediation in Cu-contaminated soil. To mimic the natural condition, we challenged plants with Cu-grouted soil. Grouting continuously with excess Cu elevates Cu stress in soil to an explicit Cu-sensitive phenotype. *ATX1* overexpression lines showed high Cu tolerance as compared with the wild type (Fig. 8A). The relative fresh weight was higher (170%–180% increase) for ATX1 overexpression lines in both the wild-type and *atx1* backgrounds than in the wild type and was higher (320%–340% increase) than for the *atx1* and *cchatx1* mutants in Cu-grouted soil (Fig. 8B). Although shoot Cu accumulation was similar for the medium-grown wild type and the *atx1* mutant (Supplemental Fig. S3), to further investigate the ATX1 function in Cu accumulation, we analyzed Cu content in these transgenic plants grown in high-Cu-content soil. After sowing in high-Cu soil, plants were grouted with water only, which reduced the influence of the growth

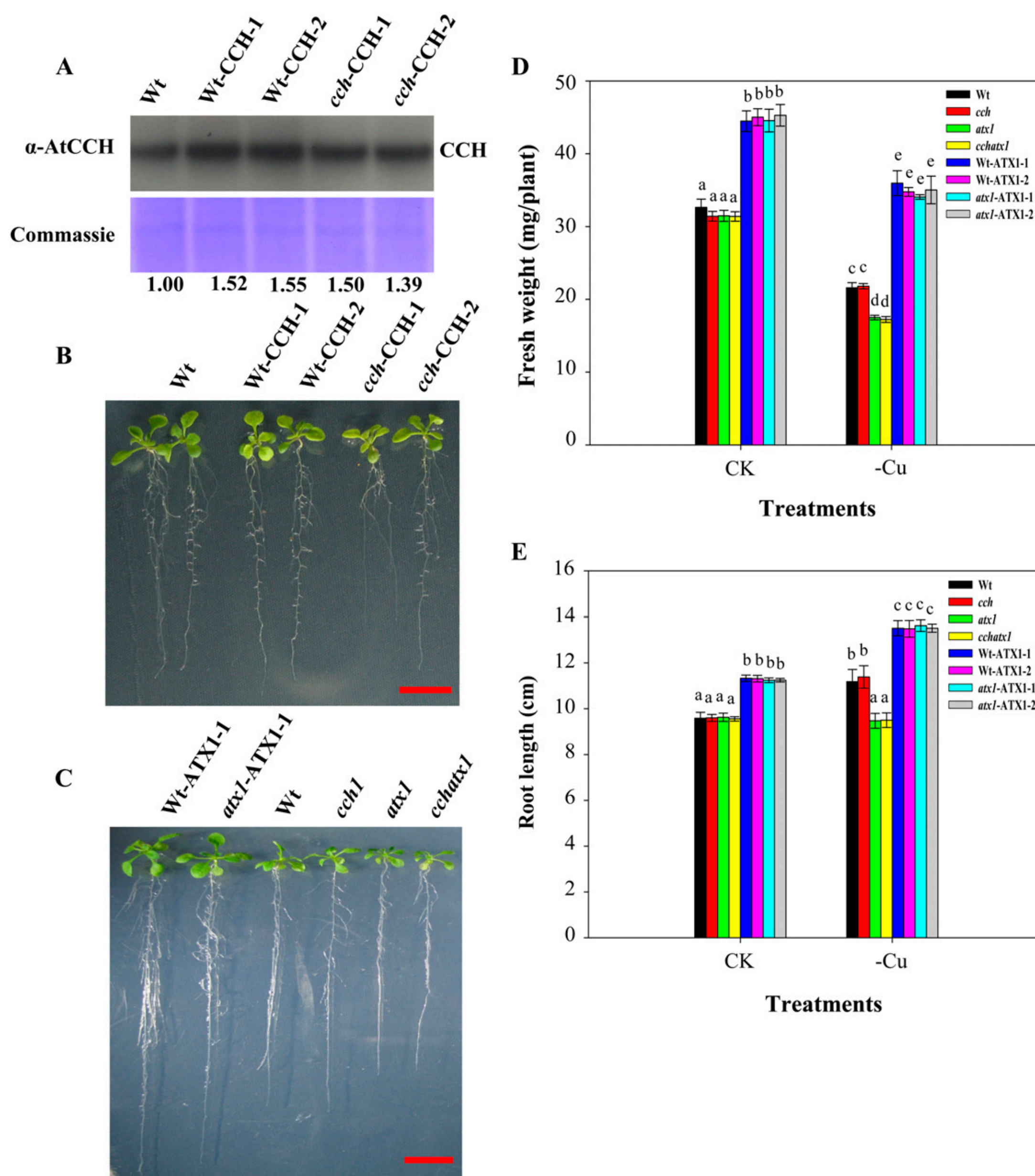

Figure 6. Phenotypes of *CCH* and *ATX1* transgenic lines, the wild type (Wt), and Cu chaperone mutants with Cu deficiency. A, Protein level of CCH detected by CCH antibody (α-AtCCH) in total protein (20 μg) isolated from each line. Coomassie blue staining was used to verify the loadings. Numbers indicate the relative intensity of immunoblotting by normalization to the wild type. B and C, Plant seeds were grown vertically on one-half-strength MS agar plates and treated with 10 μM Cu chelator bathocuproine disulfonate for 17 d. Bars = 1 cm. D and E, Fresh weight (D) and root length (E) of bathocuproine disulfonate-treated plants. Data are shown for representatives of at least three lines of each transgenic construct characterized. Data are means ± SD of four replicates with 10 seedlings each. Different lowercase letters represent statistical differences by Student's *t* test.

defect in high-Cu toxicity. The Cu concentration was surprisingly higher, by about 200%, in shoots of Wt-ATX1-1, Wt-ATX1-2, *atx1*-ATX1-1, and *atx1*-ATX1-2 lines than in shoots of the wild type and mutants (Fig. 8C). By contrast, *atx1* and *cchatx1* mutants accumulated less Cu (80%) under excess Cu in soil (Fig. 8C). However, the contents of Fe, Zn, and Mn remained unchanged (Supplemental Fig. S6). These data again support that ATX1 plays an important role in Cu tolerance and accumulation in planta.

The overexpression of ATX1 enhances Cu accumulation and elevates the tolerance threshold to Cu toxicity. By multiplying the effects on biomass and accumulation, overexpressing ATX1 enhances Cu extraction by

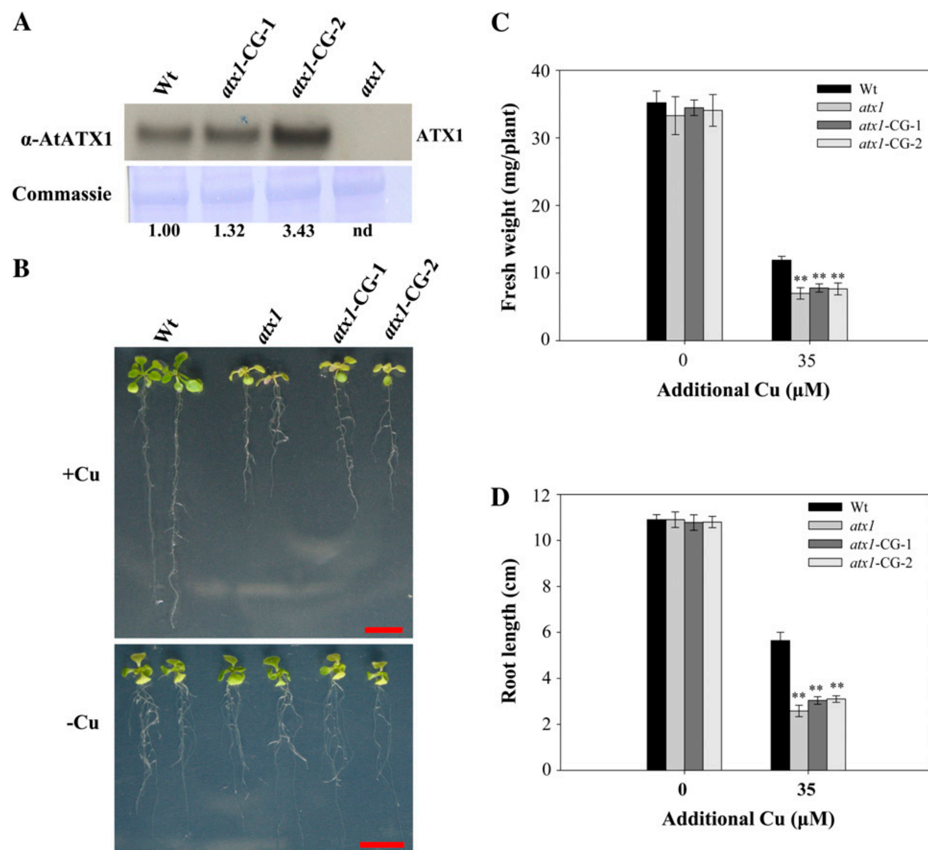

Figure 7. Phenotypes of CG-ATX1 transgenic lines, the wild type (Wt), and *atx1* mutants with Cu stress. A, Protein level of ATX1 detected by ATX1 antibody (α-AtATX1) in total protein (20 μg) isolated from each line. Coomassie blue staining was used to verify the loadings. Numbers indicate the relative intensity of immunoblotting by normalization to the wild type. nd, Not detected. B, Plant seeds were grown vertically on one-half-strength MS agar plates for 4 d and then transferred to one-half-strength MS agar plates with 35 μM $CuSO_4$ (+Cu) or 10 μM bathocuproine disulfonate (−Cu) for 13 d. Bars = 1 cm. C and D, Fresh weight (C) and root length (D) of treated plants. Data are means ± SD of four replicates with 10 seedlings each. ** $P < 0.01$ compared with the wild type in the same condition.

about 400% of the wild-type extraction. Therefore, overexpression of ATX1 leads to an overaccumulation of Cu and then tolerance to excess Cu.

DISCUSSION

The homeostasis of metal ions, including macronutrients and micronutrients, is regulated by mechanisms of uptake, compartmentalization, and translocation to support plant growth and development. Cu is one of the least-abundant micronutrients and is essential for many biochemical reactions in plant tissues (Marschner, 1995; Burkhead et al., 2009). An amount of 6 mg L^{-1} Cu was considered an adequate concentration, and 20 mg L^{-1} or greater can induce toxicity in shoot tissues (Marschner, 1995; Burkhead et al., 2009). To prevent Cu deficiency or excess, the homeostasis of Cu must be strictly fine-tuned as compared with that of other metals. Cu chaperones were thought to perform the fine-tuning by the deduced dual functions of Cu trafficking and detoxification (Harrison et al., 1999). Despite the hypothetical functions of Cu chaperones, little is known about their physiological significance in plants.

In this study, we found that *ATX1* but not *CCH* chaperones are required for tolerance to Cu excess and deficiency in Arabidopsis, which suggests that the two chaperones possess different homeostatic properties and distinct functions in planta. The *atx1* but not *cch* mutant showed increased Cu sensitivity. The phenotype of the *cchatx1* double mutant was similar to that of *atx1* (Figs. 1 and 3). Thus, we demonstrate the importance of *ATX1* in homeostasis for tolerance to excess Cu, and its induced expression by excess Cu also supports a role in Cu tolerance (Fig. 2).

Yeast ATX1 was reported to chelate Cu with excellent affinity (Pufahl et al., 1997; Shoshan and Tshuva, 2011). Additionally, the MXCXXC motif of yeast ATX1 acts as a high-affinity Cu-binding site and is important for Cu-dependent protein-protein interaction (Pufahl et al., 1997; Shoshan and Tshuva, 2011). The alignment of protein sequences revealed that ATX1 in Arabidopsis contains only one MXCXXC motif and the only known metal-binding motif (Supplemental Fig. S5). We showed that this motif is required for ATX1 function. CG-ATX1, containing a mutated MXCXXC motif with two Cys residues replaced by two Gly residues, could neither rescue Cu hypersensitivity nor enhance tolerance to Cu deficiency (Fig. 7). Additionally, transgenic lines with different CG-ATX1 levels showed complete loss of function of both excess Cu and Cu deficiency but no dominant-negative effect or intermediate phenotype. These data clearly demonstrate the specific role of the MXCXXC motif in the biological function of ATX1. Together with previous results (Pufahl et al., 1997; Hara et al., 2010), our results show that the biological function of ATX1 requires Cu chelation on the MXCXXC motif. Although CCH also

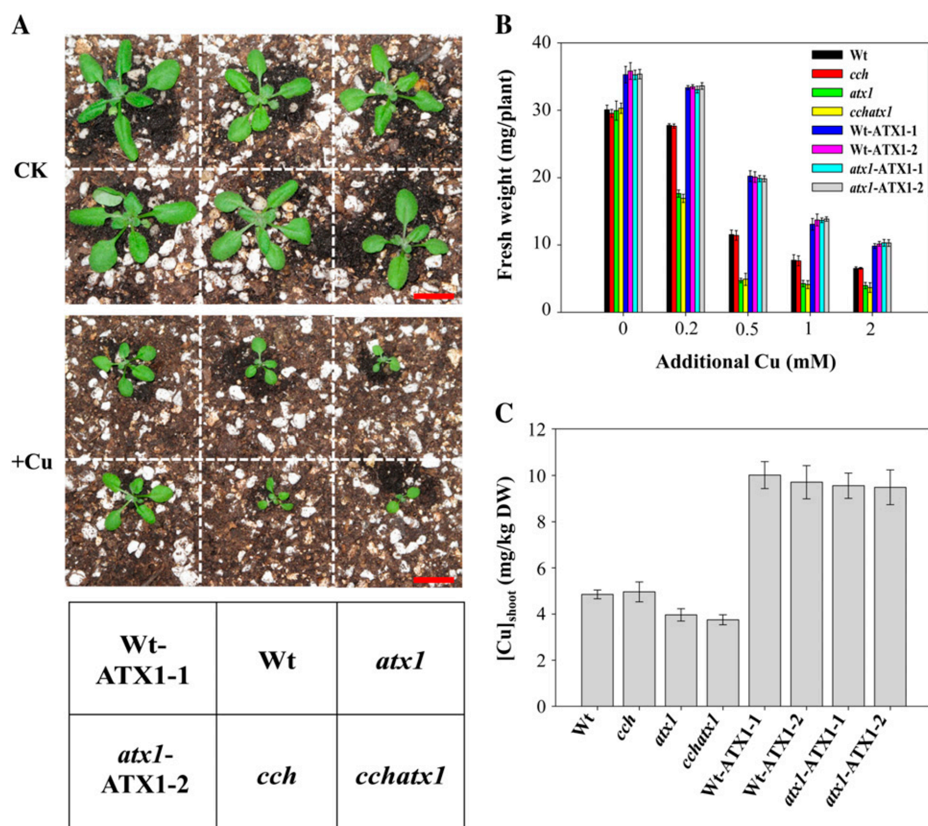

Figure 8. Phenotypes of *ATX1* transgenic lines, the wild type (Wt), and *cch* mutants in soil with Cu grouting. A, The seeds of plants were directly grown in soil (CK) and 500 μM $CuSO_4$-presoaked soil (+Cu) for 21 d and then grouted with water (CK) or 500 μM $CuSO_4$ solution (+Cu) two times every week, respectively. Bars = 1 cm. The bottom panel shows the arrangement of plants in soil. B, Fresh weights of 21-d-old plants with different concentrations of $CuSO_4$ grouting. C, The seeds of plants were directly grown in 500 μM $CuSO_4$-presoaked soil for 21 d and then grouted with water two times every week. Cu content in shoots was determined by inductively coupled plasma-optical emission spectrometry. Data are means ± SD of four replicates with 20 seedlings each. DW, Dry weight.

possesses an MXCXXC motif, we did not observe the phenotype in the knockout mutant *cch* or in overexpression lines under the conditions we tested. The CCH function could be compensated by redundancy of the genome's other metal-binding proteins, whose functions are currently not known (Hara et al., 2010; Shoshan and Tshuva, 2011).

Metallothioneins (MTs) are proteins of low molecular mass (4–14 kD) with rich Cys residues that chelate Cu, Zn, and Cd via Cys residues by forming sulfhydryl ligands (Hara et al., 2010). The arrangement of Cys residues is crucial in determining the metal-binding properties of MT proteins and their functions (Guo et al., 2008). Cys residues in MTs are arranged in metal-binding motifs, C-C, C-X-C, or C-X-X-C. These defined protein motifs explain MTs conferring tolerance to excess Cu, Zn, and Cd. All MTs possess different affinity to various metals. For example, most MTs can bind to Cu effectively, and type 4 MTs have high affinity to Zn (Guo et al., 2008). By contrast, ATX1 contains one MXCXXC motif but no C-C, C-X-C, or C-X-X-C motifs. Therefore, ATX1 more effectively and specifically binds Cu than other metals (Badarau and Dennison, 2011). The difference in the composition of metal motifs implies that MTs and ATX1 function differentially. However, ATX1 is specifically involved in Cu homeostasis in plants. This hypothesis is further supported by our finding of Cu-specific tolerance and accumulation in *ATX1*-overexpressing plants and Cu-specific hypersensitivity in the *atx1* mutant (Figs. 1, 3, 5, and 8; Supplemental Fig. S4).

In addition, the regulation of MT expression is important in tolerance to Cu toxicity (Cobbett and Goldsbrough, 2002). MTs are deduced to mobilize metal ions from senescing leaves and sequester excess metal ions (Guo, 2003). However, ATX1 and MTs differ in that the expression patterns of MTs in Arabidopsis are tissue specific (Cobbett and Goldsbrough, 2002), whereas ATX1 is ubiquitously expressed in many Arabidopsis vegetative tissues (Puig et al., 2007a). MTs also show redundancy in tissues. The Arabidopsis *mt1a-2mt2b-1* double mutants are not sensitive to excess Cu (Guo et al., 2008), but the Arabidopsis *mt1a-2mt2b-1cad1-3* triple mutant is sensitive to excess Cu (Guo et al., 2008). Therefore, MTs involved in Cu tolerance require a synergy with phytochelatin. By contrast, we found the ATX1-defective mutants *atx1* and *cchatx1* sensitive to excess Cu (Fig. 1). Thus, ATX1 expression may be a first-line response against excess Cu stress. ATX1 could be primarily responsible for tolerance to excess Cu, and then MTs could be responsible for the escaped Cu and the process of Cu redistribution and detoxification (Guo et al., 2008).

Previous studies indicated that the transcription factor *SQUAMOSA Promoter Binding Protein-Like7* (*SPL7*) was essential in the response to Cu deficiency (Yamasaki et al., 2009). The *spl7* mutant was hypersensitive to Cu deficiency, but the expression of *ATX1*

was not affected in the mutant (Yamasaki et al., 2009). Therefore, the roles of *SPL7* and *ATX1* in Cu deficiency are independent.

The expression of ATX1 is universal, and the accumulation of CCH is mostly in phloem-enucleated sieve elements (Mira et al., 2001a; Puig et al., 2007a). The expression of CCH is induced by Cu deficiency, and that of ATX1 increases under excess Cu, which again supports the hypothesis of differential functions between ATX1 and CCH (Fig. 2). Furthermore, the unique C-terminal domain of CCH blocks the interaction of RAN1 and HMA5 (Andrés-Colás et al., 2006; Puig et al., 2007b). These observations suggest that CCH has a specific function that differs from that of ATX1 regulated by its unique C-terminal domain.

Yeast two-hybrid screening revealed that two transporters, RAN1 and HMA5, interact with ATX1 (Andrés-Colás et al., 2006; Puig et al., 2007b), which may suggest the Cu delivery role of ATX1. The phenotype of *ran1* can be suppressed by additional Cu supply, but it is not Cu hypersensitive. We did not observe any deficiency in ethylene-related responses in the *atx1* mutant. Arabidopsis may have alternative pathways to compensate ATX1 function in the ethylene response.

The closest homolog of *RAN1* in Arabidopsis is *HMA5* (Williams and Mills, 2005). HMA5 is an efflux transporter of Cu. The expression of *HMA5* is induced by Cu and is mainly in roots and flowers (Andrés-Colás et al., 2006). The *hma5* mutant is Cu hypersensitive in the root and is accompanied by wave-like root growth. Therefore, HMA5 was proposed to have a role in Cu translocation from root to shoot (Andrés-Colás et al., 2006). On the basis of the interaction between ATX1 and HMA5, ATX1 was proposed to deliver Cu to HMA5 for Cu detoxification in roots and translocation to shoots. We observed root hypersensitivity and high expression of *HMA5* (Fig. 4) with low shoot Cu accumulation in the *atx1* mutant (Fig. 8C), which supports that ATX1 is involved in Cu detoxification with HMA5. In addition, *ATX1* also expresses in the shoot and *atx1* shows hypersensitivity in the shoot, which suggest its additional role in the shoot. Although only RAN1 and HMA5 have been found to interact with ATX1, ATX1 may also interact with other proteins, at least in the shoot, for Cu homeostasis. Besides, the universal expression of *ATX1* was suggested (Puig et al., 2007a), but the tissue/organ-specific expression had not been clarified under various Cu conditions. Further studies to elucidate the detailed mechanism in different tissues are warranted.

Cu chaperone mutants and the wild type showed similar growth under one-half-strength MS medium. However, the *atx1* mutant showed sensitivity to both excess Cu and Cu deficiency, whereas *ATX1* overexpression conferred tolerance to excess Cu and Cu deficiency (Figs. 5 and 6). ATX1 may be involved in chelating Cu under Cu overload and facilitate Cu usage under deficiency. Recently, the tonoplast Cu transporter COPT5 was shown to act as an exporter and was required for tolerance to Cu deficiency; COPT5 may transport Cu from the vacuole or prevacuolar compartment to the cytosol to redistribute Cu in cells during Cu deficiency (Garcia-Molina et al., 2011; Klaumann et al., 2011; Pilon, 2011). ATX1 may have a role in adapting Cu released from the vacuole via COPT5 for use under Cu deficiency.

Although one-half-strength MS medium is a Cu-sufficient condition, growth medium with about 3 to 5 μM Cu is considered abundant and makes better vegetative growth than in one-half-strength MS medium (Yamasaki et al., 2009; Kopittke et al., 2010). Our finding that the wild type grew best in one-half-strength MS with 5 μM $CuSO_4$ (Fig. 1D) supports previous observations and explains the enhanced growth of ATX-overexpressing lines with one-half-strength MS. Therefore, ATX1 overexpression increases growth fitness under Cu-deficient and -excess conditions by facilitating Cu usage and arresting unchelated Cu from causing toxicity, respectively. It is worth mentioning here that low-Cu conditions could be more biologically relevant. Reduced growth was observed in the *atx1* and *cchatx1* mutants under Cu-deficient treatment. This indicates that Cu deficiency imposes a positive selection advantage on *ATX1*.

In summary, we demonstrate the biological function of *ATX1* in Arabidopsis in response to excess and deficient Cu. *ATX1* contributes to tolerance to excess Cu and tolerance to Cu deficiency. Its function requires the Cu-binding MXCXXC motif. *ATX1* may have an important role in Cu homeostasis in Arabidopsis. On the other hand, the biological role of *CCH* has not been defined in this study. Further efforts are required not only to understand the roles of both Cu chaperons, *CCH* and *ATX1*, in the specific developmental stage or tissues but also for understanding the molecular mechanism(s) involved in the Cu homeostasis process.

MATERIALS AND METHODS

Plant Growth Conditions

The procedure was modified from a previous study (Chen et al., 2011). Seeds of wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-0), the *cch* T-DNA insertion line (SALK_138593), the *atx1* T-DNA insertion line (SALK_026221), all from the Arabidopsis Biological Resource Center, and the *cchatx1* double mutant cross from SALK_138593 and SALK_026221 were surface sterilized with 70% ethanol for 5 min, then treated with 1.2% bleach containing 0.02% SDS for 15 min, rinsed five times with sterilized water, and kept in darkness at 4°C for 3 d for seed stratification. Sterilized seeds were grown on one-half-strength MS medium salt (Sigma-Aldrich), 1% Suc (J.T. Baker), 0.5 g L^{-1} MES (J.T. Baker), and 0.7% agar (Sigma-Aldrich; A.7002) at pH 5.7 for the designated times. Chemical treatment is described in the figure legends. Seeds were grown (after 3 d of stratification) in pots containing organic substrate, vermiculite, and mica at a ratio of 9:1:1 at a light intensity of 100 μmol m^{-2} s^{-1} under a 16-h-light/8-h-dark cycle at 22°C.

Overexpression of *CCH* and *ATX1*

Agrobacterium tumefaciens strain GV3101, harboring the plasmids 35S:*AtCCH*/pCAMBIA1305.1 or 35S:*AtATX1*/pCAMBIA1305.1 to overexpress the coding sequence including *CCH* or *ATX1* of Arabidopsis driven by a cauliflower mosaic virus 35S promoter, was transformed into plants with a *cch* or *atx1* mutant background. For overexpressing *CCH* or *ATX1* in the wild type, the same constructs were transformed into a wild-type background. For PCR amplification of the coding sequence, the following primers were used:

FP-CCH-*Nco*I (5′-AACCATGGGGATGGCTCAGACCGTTGTCCTCA-3′), RP-CCH-*Pml*I (5′-AACACGTGTTAAACTTGTGATGGCTTAGTCT-3′), FP-ATX1-*Nco*I (5′-AACCATGGATGCTTAAAGACTTGTTCCAAG-3′), and RP-ATX1-*Pml*I (5′-AACACGTGTTAAGCCTTAGCAGTTTCACCTTC-3′).

Protein Extraction and Immunoblot Analysis of CCH and ATX1

The procedure was described previously (Chen et al., 2011). Plant samples were extracted with the extraction buffer (2× SDS sample buffer containing 20 mM *N*-ethylmaleimide, 100 mM $Na_2S_2O_5$, and one tablet of protease inhibitor cocktail [Roche Applied Science] per 50 mL). Samples were centrifuged at 12,000*g* for 10 min, and the protein concentration was determined by use of the BCA Protein Assay Kit (Thermo Scientific). Total protein (20 μg) was separated on a NuPAGE 4% to 12% Bis-Tris Gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), which was blocked with 5% fat-free milk and 0.1% Tween 20 in phosphate-buffered saline (PBS) for 1 h, incubated with 1:5,000-diluted purified anti-CCH or anti-ATX1 antibody, washed with PBS buffer containing 0.1% Tween 20, and incubated for 1 h with 1:10,000-diluted secondary antibody (POX-conjugated goat anti-rabbit IgG; Millipore). The membrane was washed five times for 10 min each with PBS buffer containing 0.1% Tween 20 solution before development. Specific protein bands were visualized by use of the Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore).

Elemental Analysis

Elemental analysis was as described (Lin et al., 2009). Harvested plant samples were washed with $CaCl_2$ and water and dried for 3 d before digestion. Microwave-digested samples (CEM) were analyzed by inductively coupled plasma-optical emission spectrometry (OPTIMA 5300; Perkin-Elmer).

RNA Isolation and Quantitative Real-Time RT-PCR

The procedure was described previously (Chen et al., 2011). Frozen root tissues were ground in liquid nitrogen by use of a tissue homogenizer (SH-48; J&H Technology). Total RNA was isolated by the TRIzol method. RNA was precipitated by adding 0.5 mL of isopropanol and incubating at −80°C for 30 min. After centrifugation at 15,000*g* at 4°C for 15 min, the resulting pellet was washed twice with 75% ethanol. RNA was redissolved in 30 μL of diethyl pyrocarbonate-treated water. The concentration of the RNA was determined at 260 nm on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). Subsequently, 2 μg of RNA was treated with RQ1 RNase-free DNase (Promega), and the reaction buffer was replaced with 5× first-strand RT buffer (Invitrogen). The cDNA was synthesized by use of SuperScript III Reverse Transcriptase (Applied Biosystems). Quantitative real-time RT-PCR analyses involved the use of SYBR Green I dye (ABI). The expression of *Actin2* was used as the internal control for all tested genes. The sequences of primers are given in Supplemental Table S1.

Photosynthetic Activity Assay

The F_v/F_m was measured by use of a portable chlorophyll fluorometer (PAM-2100; Heinz Walz).

MDA Content Quantification

An amount of 0.05 g of shoot or root tissue was homogenized with 2 mL of 0.1% (w/v) cool trichlorocactic acid (TCA) on ice. The homogenates were centrifuged at 14,000*g* for 10 min at 4°C, then 250 μL of supernatant was mixed with 1.5 mL of TCA/thiobarbituric acid reagent (0.25% thiobarbituric acid containing 10% TCA). The mixture was incubated in a water heater at 95°C for 30 min, kept on ice for 5 min, and centrifuged at 3,000*g* for 10 min; then, 200 μL of supernatant containing MDA equivalents was monitored by measuring A_{532}, A_{600}, and A_{440} by spectrophotometry (BioTek). MDA content was calculated as follows: $(A_{523} - A_{560})/155$ (K mM^{-1} cm^{-1}) × 5 × 4 × 1,000/fresh weight (g).

POX and CAT Activity Assay

Shoot or root tissue was homogenized with liquid nitrogen and suspended in 0.1 mL of 10 mM PBS buffer (pH 7.0). The homogenates were centrifuged at 14,000*g* for 20 min, and the supernatant was collected for analysis. POX activity was determined by measuring the increase in A_{470} after 20 min of incubation at room temperature by spectrophotometry (BioTek). The reaction mixture was 25 μL of 50 mM H_2O_2, 5 μL of 250 mM guaiacol, 195 μL of 12.5 mM 3,3-dimethylglutaric acid (pH 6.0), and 25 μL of protein extracts. The reaction was started by adding 100 μL of protein extract to 900 μL of reaction solution. One unit of POX isoenzymes was defined as the amount of enzyme that could produce 1 nmol of tetraguaiacol per minute (extinction coefficient is 26.6 mM^{-1} cm^{-1} at 470 nm). CAT activity was determined by monitoring the decrease in A_{240} at room temperature by spectrophotometry. The reaction mixtures contained 5 mM H_2O_2 in 50 mM PBS buffer (pH 7.0). The reaction was started by adding 100 μL of protein extract to 900 μL of reaction solution. One unit of CAT was defined as the amount of enzyme able to decompose 1 μmol of H_2O_2 in 1 min at 25°C (extinction coefficient is 0.039 mM^{-1} cm^{-1} at 240 nm).

Statistical Analysis

Student's *t* test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GmATX1, AF198627; LeCCH1, AAP06757; OsATX1, AF198626; and ScATX1, CAA65485.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *CCH* and *ATX1* T-DNA insertion mutants SALK_138593 (*cch*) and SALK_026221 (*atx1*).

Supplemental Figure S2. Effect of excess Fe, Zn, and Cd on the wild type and Cu chaperone mutants.

Supplemental Figure S3. Shoot concentrations of Fe, Zn, Mn, Cu, Mg, and Ca in the wild type and mutants under Cu stress.

Supplemental Figure S4. Effect of excess Cu on oxidative stress in the wild type and Cu chaperone mutants.

Supplemental Figure S5. Sequence alignment of the MXCXXC motif of Cu chaperones.

Supplemental Figure S6. Shoot Fe, Zn, and Mn concentrations of soil-grown plants.

Supplemental Table S1. Primers used for quantitative real-time RT-PCR in determining *HMA5* and *COPT1* expression.

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