# Amino Acid Polymorphisms in Strictly Conserved Domains of a P-Type ATPase HMA5 Are Involved in the Mechanism of Copper Tolerance Variation in Arabidopsis[1][W][OA]

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Copper (Cu) is an essential element in plant nutrition, but it inhibits the growth of roots at low concentrations. Accessions of Arabidopsis (*Arabidopsis thaliana*) vary in their tolerance to Cu. To understand the molecular mechanism of Cu tolerance in Arabidopsis, we performed quantitative trait locus (QTL) analysis and accession studies. One major QTL on chromosome 1 (QTL1) explained 52% of the phenotypic variation in Cu tolerance in roots in a Landsberg *erecta*/Cape Verde Islands (L*er*/Cvi) recombinant inbred population. This QTL regulates Cu translocation capacity and involves a Cu-transporting $P_{1B-1}$-type ATPase, HMA5. The Cvi allele carries two amino acid substitutions in comparison with the L*er* allele and is less functional than the L*er* allele in Cu tolerance when judged by complementation assays using a T-DNA insertion mutant. Complementation assays of the *ccc2* mutant of yeast using chimeric HMA5 proteins revealed that N923T of the Cvi allele, which was identified in the tightly conserved domain $N(x)_6YN(x)_4P$ (where the former asparagine was substituted by threonine), is a cause of dysfunction of the Cvi HMA5 allele. Another dysfunctional HMA5 allele was identified in Chisdra-2, which showed Cu sensitivity and low capacity of Cu translocation from roots to shoots. A unique amino acid substitution of Chisdra-2 was identified in another strictly conserved domain, $CPC(x)_6P$, where the latter proline was replaced with leucine. These results indicate that a portion of the variation in Cu tolerance of Arabidopsis is regulated by the functional integrity of the Cu-translocating ATPase, HMA5, and in particular the amino acid sequence in several strictly conserved motifs.

Copper (Cu) is an essential element for higher plants and plays key roles in a series of major biological systems, such as respiration, photosynthesis, and ethylene signaling (Capaldi, 1990; Maksymiec, 1997; Clemens, 2001). In most of these systems, Cu is involved in electron transfer reactions that are mediated by Cu-containing proteins such as plastocyanin, which functions in the electron transfer system of PSI, and cytochrome *c* oxidase (EC 1.9.3.1), which catalyzes terminal oxidation in the mitochondrial electron transfer chain. The ability of Cu to mediate high rates of electron transfer is also a potential cause of toxicity for plant cells (especially in growing roots). Less than 5 $\mu$M Cu inhibits the growth of roots in wheat (*Triticum aestivum*; Taylor et al., 1991; Parker et al., 1998) and Arabidopsis (*Arabidopsis thaliana*; Toda et al., 1999). This sensitivity of growing roots is a potential risk in agriculture that arises from the use of Cu-containing fungicides and fertilizers. For example, bordeaux mixture, which contains Cu (without other synthesized organic chemicals) is used in both conventional and organic agriculture (Semu and Singh, 1995). In addition, Cu accumulation in soil is expected to arise from continuous application of organic fertilizers such as pig manure, since supplemental Cu is used to improve growth rates of pigs (Coffey et al., 1994). Thus, establishment of breeding programs to develop Cu-tolerant germplasm could be important for use in sustainable agriculture systems. Molecular breeding (e.g. marker-assisted selection) is a promising approach that could be used if mechanisms of variation in Cu tolerance are clarified at the molecular level.

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Mutant studies in yeast have revealed that free Cu concentrations in the cytosol are strictly regulated by a complex Cu homeostasis system, consisting of Cu transporters (e.g. Ccc2p; Fu et al., 1995), Cu-binding proteins (e.g. Cox17p; Glerum et al., 1996), and Cu chaperone (Ccsp; Culotta et al., 1997). Functional analyses of Arabidopsis homologs suggest that plants possess similar homeostasis mechanisms (e.g. COPT1 [Kampfenkel et al., 1995], CCH [Himelblau et al., 1998], RAN1 [Hirayama et al., 1999], CCS [Chu et al., 2005], and HMA5 [Andrés-Colás et al., 2006]). Because free Cu in the cytoplasm is regulated by these systems (Clemens, 2001), variation in Cu tolerance among varieties might be regulated by the differential capacity of each component of the homeostatic system. In fact, delivery of Cu to plastocyanin and Cu/zinc (Zn) superoxide dismutase via P-type ATPases that regulate free Cu in the cytosol (Abdel-Ghany et al., 2005) has been suggested as one mechanism of variation in Cu tolerance in shoots of Arabidopsis. Although several genes that regulate Cu homeostasis in roots have been identified (e.g. methollothionine synthase in the metal-tolerant plant *Silene vulgaris* [van Hoof et al., 2001] and HMA5 [Andrés-Colás et al., 2006]), their involvement in determining the variation in Cu tolerance among varieties has not been verified.

Studies of natural variation within Arabidopsis provide a useful approach to understand the mechanisms of variation in target traits (for review, see Koornneef et al., 2004). Several critical genes regulating traits such as freezing tolerance (Alonso-Blanco et al., 2005), growth, and flowering (Balasubramanian et al., 2006) have been successfully identified by this approach. We have applied this approach to identify critical genes regulating variation in Cu tolerance in Arabidopsis. Using an experimental system optimized to study natural variation in rhizotoxic factors (e.g. tolerance to aluminum [Al; Kobayashi et al., 2005] and protons [Ikka et al., 2007; Iuchi et al., 2007]), we identified a major quantitative trait locus (QTL) in one recombinant inbred population that is associated with Cu translocation capacity. Together with a candidate gene approach based on gene function and natural allelic variation, we identified a gene, HMA5 which plays an important role in Cu homeostasis and determines the natural variation in Cu tolerance among accessions of Arabidopsis.

## RESULTS

### Cu Tolerance QTLs and Epistasis Detected from a Landsberg *erecta*/Cape Verde Islands Recombinant Inbred Population

We obtained high broad-sense heritability ($h_b^2$ = 0.98) when recombinant inbred lines (RILs) were scored for Cu tolerance with relative root length (RRL; Fig. 1). The distribution of phenotypes across RILs did not fit a normal distribution ($\chi^2$ = 46.8, $P$ = 0.004), showing signs of bimodality around the contrasting Cu tolerance parental accessions Cape Verde Islands (Cvi; mean RRL for 5 d = 30.3%) and Landsberg *erecta* (L*er*; mean RRL for 5 d = 77.9%). This suggests that a small number of genetic factors regulate Cu tolerance across the RILs. As expected, one large-effect QTL (logarithm of the odds [LOD] score = 21.0; additive effect of L*er* allele = 18.7) was identified from the L*er*/Cvi recombinant inbred (RI) population, located on the lower arm of chromosome 1 flanked by genetic markers GD.160C and HH.375L (designated QTL1), and with positive additive effect of the L*er* allele (Fig. 2). Although another QTL carrying a positive allelic effect of L*er* (additive effect = 15.2) was detected on chromosome 3 with a LOD score of 3.2 (designated QTL3), its contribution to the variation in phenotypic Cu tolerance was smaller than that of QTL1. QTL1 explained 52% of the variation in phenotypic Cu tolerance, while 15% of the variation was explained by QTL3. These QTLs could possibly account for the observed bimodal distribution of Cu tolerance across the RILs.

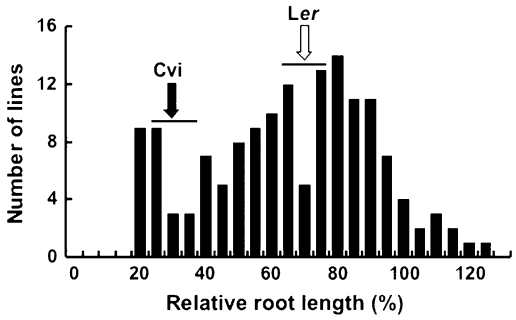


**Figure 1.** Frequency distribution of relative root lengths in the L*er*/Cvi RI population. A total of 149 L*er*/Cvi RILs were grown in the absence (control) or presence of 1.3 $\mu$M Cu solution for 5 d. Arrows and horizontal bars indicate means and SD values of RRL in the parental lines, respectively.

To further characterize the genetic architecture of Cu tolerance in the L*er*/Cvi RI population, epistatic interacting loci pairs were searched by a complete pairwise search using the Epistat program (Chase et al., 1997), which allows the detection of epistatic interacting loci pairs even if each locus was not detected significantly by the composite interval mapping (CIM) method. The QTL1 region (linked to GD.160C) interacted with two other chromosome regions, namely, chromosome 1 (linked to DF.93C; epistasis B in Fig. 2A) and chromosome 5 (linked to CC.262C; epistasis C in Fig. 2A), while the QTL3 region (linked to GD.296-Col) interacted with another chromosome 1 region (linked to DF.73L; epistasis A in Fig. 2A); Table I). The mean of allele combinations indicates that the negative additive effect of the Cvi allele at QTL1 is partially canceled by the L*er* allele on chromosome 5 (Fig. 2; Table I). On the other hand, there were six RILs that showed transgressive segregation (zero sensitive, six tolerant; LSD = 29, $P < 0.05$). This might be partially

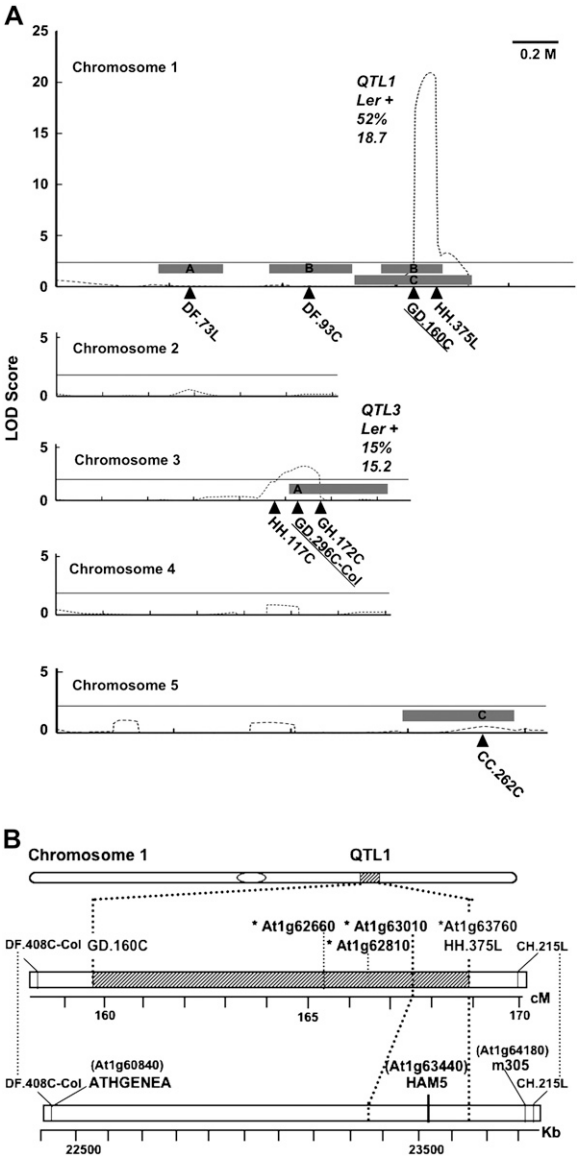


**Figure 2.** A, Genetic architecture of the Cu tolerance QTL in the L*er*/Cvi RI population. The dotted line on each chromosome indicates LOD score curves obtained by the CIM method. The percentage of phenotypic variance can be explained, and the additive effect and positive genotype of Cu tolerance are indicated for each QTL detected by the CIM method. Significant thresholds for LOD ($\alpha = 0.05$) are shown for each chromosome by a horizontal bar. Gray box pairs (A, B, and C; see values in Table I) indicate epistasis-interacting positions detected by the complete pair-wise search method ($P < 0.0005$). For QTLs detected by CIM, flanking genetic markers and closely linked genetic markers (underlined) are shown under the chromosomes. Genetic markers that gave the smallest *P* value by the complete pair-wise search are also indicated for the epistasis-interacting chromosomal region. B, Predicted physical position of QTL1. Selected genetic markers around QTL1 were indicated on the genetic linkage map of the L*er*/Cvi RI population and the physical genomic position of Col. Shared genetic markers are connected by dotted lines. The shaded region indicates the position of QTL1. Asterisks show added dCAPS markers for the reperformed CIM analysis.

explained by one of the combined allelic combinations of epistasis C (Table I).

### Characterization of QTL1 in Cu Tolerance

QTL1 showed the largest $r^2$ value (0.52), indicating that this locus contains the most important genetic factor determining variation in Cu tolerance among the L*er*/Cvi RILs. To identify the gene(s) accounting for QTL1, a candidate gene search and physiological characterization were performed using RILs contrasting in Cu tolerance and with different alleles (i.e. Cvi and L*er* alleles at QTL1).

QTL1 was flanked by two genetic markers, GD.160C and HH.375L (Fig. 2A). Although the physical positions of these genetic markers in the L*er*/Cvi RILs have not been reported, the markers can be positioned using the genetic markers shared by L*er*/Columbia (Col), whose physical position is assigned to the genomic DNA sequence of Col (Alonso-Blanco et al., 1998). Genetic markers DF.408C-Col and CH.215L flank QTL1 and are also closely flanked by genetic markers GD.160C and HH.375L, which are shared with the L*er*/Col mapping population. These genetic markers are closely related to other genetic markers, namely, ATHGENEA and m305, which correspond to two genes on chromosome 1, At1g60840 and At1g64180, respectively (TAIR SeqViewer Whole Genome View at The Arabidopsis Information Resource; http://www.arabidopsis.org/servlets/sv; Fig. 2B). From these estimations, we believe that the major gene(s) regulating QTL1 locate(s) between the At1g60840 and At1g64180 regions. To further characterize the QTL1 locus, we developed four dCAPS markers at At1g62660, At1g62810, At1g63010, and At1g63760, which were used to evaluate homozygous Cvi and L*er* alleles at given positions, and reperformed the CIM analysis. By this analysis, QTL1 is flanked by At1g60840 and At1g63760 (which correspond with HH.375L; Fig. 2B) and is closely linked to At1g63010 with a 0.50 $r^2$ value. This indicates that the major gene causing QTL1 in the L*er*/Cvi RILs is localized in this physical position. This region contains a gene previously reported to regulate Cu homeostasis in roots, namely, HMA5, which functions as a Cu translocator (At1g63440; Andrés-Colás et al., 2006). To test the possibility that this gene could be the cause of QTL1, we analyzed the metal translocation capacity of Cvi and L*er* alleles of QTL1. RILs with the Cvi allele for QTL1 showed lower Cu translocation capacity (i.e. lower shoot to total ratio of Cu content) than those carrying the L*er* allele. These differences in Cu translocation capacity were related to Cu tolerance as assessed by RRL (Fig. 3; $r^2 = 0.82$). There were no significant differences in Zn and manganese (Mn) translocation capacity between RILs (Fig. 3). This profile of metal translocation was similar to that of the knockout (KO) line of HMA5 (Supplemental Fig. S5). From these results, we inferred that the Cu sensitivity of the Cvi allele at QTL1 was correlated with low Cu translocation capacity.

**Table I.** *Epistatic interactions between two loci for Cu tolerance detected by a complete pair-wise search at $P < 0.0005$*

Different letters indicate significant differences at $P < 0.05$ by LSD test.

| Marker Interaction[a] | Interacting Chromosome Nos. | LLR | RRL Mean with Combined Alleles[b] | | | | $r^{2c}$ |
|---|---|---|---|---|---|---|---|
| | | | CC | CL | LC | LL | |
| | | | % | | | | % |
| (A) DF.73L×GD.296C-Col | 1 × 3 | 10.2 | 65.1b | 61.4b | 46.8c | 77.3a | 0.03 |
| (B) GD.160C×DF.93C | 1 × 1 | 23.5 | 46.5b | 32.1c | 75.9a | 77.5a | 1.00 |
| (C) GD.160C×CC.262C | 1 × 5 | 13.2 | 28.4c | 50.4b | 81.0a | 74.5a | 1.71 |

[a]Letters in parentheses represent positions in Figure 2. [b]C, Cvi genotype; L, L*er* genotype. [c]Proportion of phenotypic variation additively explained by epistatic interaction compared with the single-gene effect of the first marker [(gene effect of the first marker conditioned by the second marker) − (single-gene effect of the first marker)].

### Evaluation of Cvi and L*er* HMA5 Alleles by in Planta and Yeast Complementation Assays

The homozygous HMA5-KO line SALK_040252, carrying a T-DNA insertion at the first intron, showed hypersensitivity to Cu while showing similar growth to the wild type (Col-0) in control solution (Fig. 4). A 1:3 segregation ratio of Cu tolerance (sensitive:tolerant = 8:26; $\chi^2 = 0.03$, $P = 0.84$) in the F2 generation derived from a heterozygous plant of this KO line (Supplemental Fig. S1) indicated that Cvi and L*er* alleles can be evaluated by complementation test with this KO line. To minimize the potential effects of epistatic loci-interacting QTL1, we selected two RILs (L*er*-type QTL1, tolerant RI line [CVL153]; Cvi-type QTL1, sensitive RI line [CVL183]) that shared the same alleles at epistatic interacting loci but different alleles at QTL1 (i.e. Cvi or L*er*). As shown in Figure 4, the parental accession Cvi grew poorly in Cu toxic solutions, similar to HMA5-KO. Both F1 plants derived from crosses between HMA5-KO and RILs carry Cvi or L*er* alleles at QTL1 and grew similarly in control solution, whereas HMA5-KO×Cvi F1 showed short roots like Cvi (RRL of F1, 25.6%; RRL of Cvi, 20.5%) and HMA5-KO×L*er* F1 was better than Cvi (RRL of F1, 72.3%; RRL of L*er*, 92.7%) in Cu toxic solution (Fig. 4). These results indicate that the differences in the HMA5 allele are a cause of QTL1. *HMA5* expression was similar between the parental lines or two RILs used for the complementation test (Supplemental Fig. S2), suggesting that differential Cu tolerance between Cvi and L*er* alleles was not a result of differential gene expression but might instead be due to a decrease in the ability of the Cvi protein to function.

Sequence analysis identified two amino acid substitutions in the deduced amino acid sequences of Cvi-HMA5 and L*er*-HMA5 (Figs. 5A and 6A). The first substitution was at the 178th amino acid residue (L*er*: Ser [TCG] to Cvi:Lys [TTG]), while the second substitution was found at the 923rd amino acid residue (L*er*:Asn [AAC] to Cvi:Thr [ACC]; Fig. 5A). To test the effect of these substitutions, we conducted yeast (*Saccharomyces cerevisiae* Δ*ccc2* mutant) complementation assays with chimeric and authentic HMA5 proteins. As described previously, the *ccc2* mutant (Δ*ccc2*) cannot grow under iron (Fe)-limited conditions, because in the absence of the Ccc2 protein (a Cu-transporting P-type ATPase), Cu cannot be delivered to the multicopper oxidase Fet3p, which is required for high-

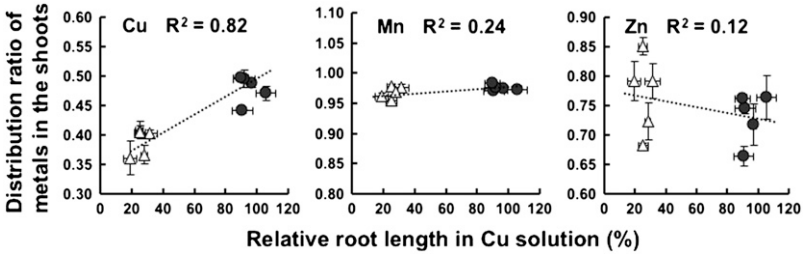


**Figure 3.** Effects of genotypes of the QTL1 region on the translocation capacities of Cu, Mn, and Zn from roots to shoots. Five Cu-tolerant RILs carrying the L*er* genotype at QTL1 (black circles) and five sensitive RILs carrying the Cvi genotype at QTL1 (white triangles) were grown in control solution for 10 d and then incubated in a Cu toxic solution containing 1.3 μM Cu, 20.6 μM Mn, and 2.0 μM Zn for 2 d. Concentrations of the metals in the shoot and root were quantified by inductively coupled plasma-mass spectrometry. Values shown are means ± SE of the translocation ratio (shoot to total; $n = 3$ from biologically independent replications) and relative root lengths in Cu toxic solution in the QTL condition for Cu tolerance ($n = 3$).

affinity Fe uptake at the plasma membrane. We have previously shown that this mutant can be complemented by the plant Cu-translocating protein BnRAN1 (*Brassica napus* homolog of Arabidopsis RAN1, which is a homolog of HMA5; Southron et al., 2004). Introduction of authentic L*er*-HMA5 cDNA (L*er*L*er*) partially complemented the *ccc2* mutation on agar plates, while introduction of Cvi-HMA5 (CviCvi) did not (Fig. 5B). Growth of *ccc2* mutant carrying L*er*-HMA5 (L*er*L*er*) in liquid medium was about half that of the parental strain (BJ2168) but significantly greater than with Cvi-HMA5 (CviCvi; Fig. 5C). In this condition, replacement of 178S with 178L (designated CviL*er* in Fig. 5) caused no significant change compared with L*er*-HMA5. On the other hand, replacement of 923T with 923N gave complementation in the Cvi-HMA5-type HMA5 protein (Fig. 5). These results indicate that the change at the 923rd amino acid residue (N to T) could account for the phenotypic differences associated with the L*er*-HMA5 and Cvi-HMA5 alleles and was in turn detected by the major QTL in the L*er*/Cvi RI population.

### HMA5 Polymorphism and Cu Tolerance among Accessions of Arabidopsis

We assessed the Cu tolerance of 103 Arabidopsis accessions (JA series obtained from the RIKEN BioResource Center and parental accessions of RILs) and found that Cu tolerance among RRL ranged between 16.7% and 88.6% (RRL). To determine whether variation in HMA5 is involved in the mechanism of Cu tolerance variation among these accessions, we compared the deduced amino acid sequence of HMA5 among Cu-tolerant and Cu-sensitive accessions. Sequence analysis of DNA from selected Cu-tolerant and Cu-sensitive accessions (total of 40) identified seven polymorphic sites at various positions, which can be grouped into seven haplotypes (Fig. 6A).

Haplotype 1 was shared by 18 accessions, including the tolerant parent of the L*er* and the background

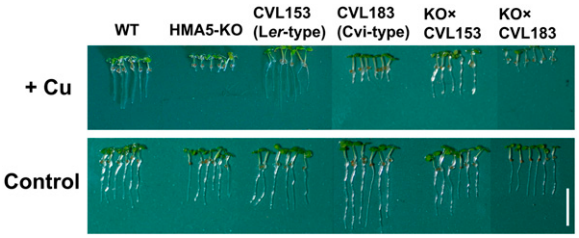


**Figure 4.** Complementation assay of the Arabidopsis HMA5-KO allele in planta by the L*er* or Cvi allele. F1 progeny were obtained by crossing HMA5-KO (female parent) and RILs (male parent) carrying L*er* (RI number CVL153) or Cvi (RI number CVL183) chromosome at QTL1. These RILs carry the same genotypes at other major QTL regions (i.e. QTL3 and epistasis-interacting region of chromosome 5). Root growth in the control and Cu toxic solutions (1.3 $\mu$M) at day 5 are shown. WT, Wild type. Bar = 10 mm.

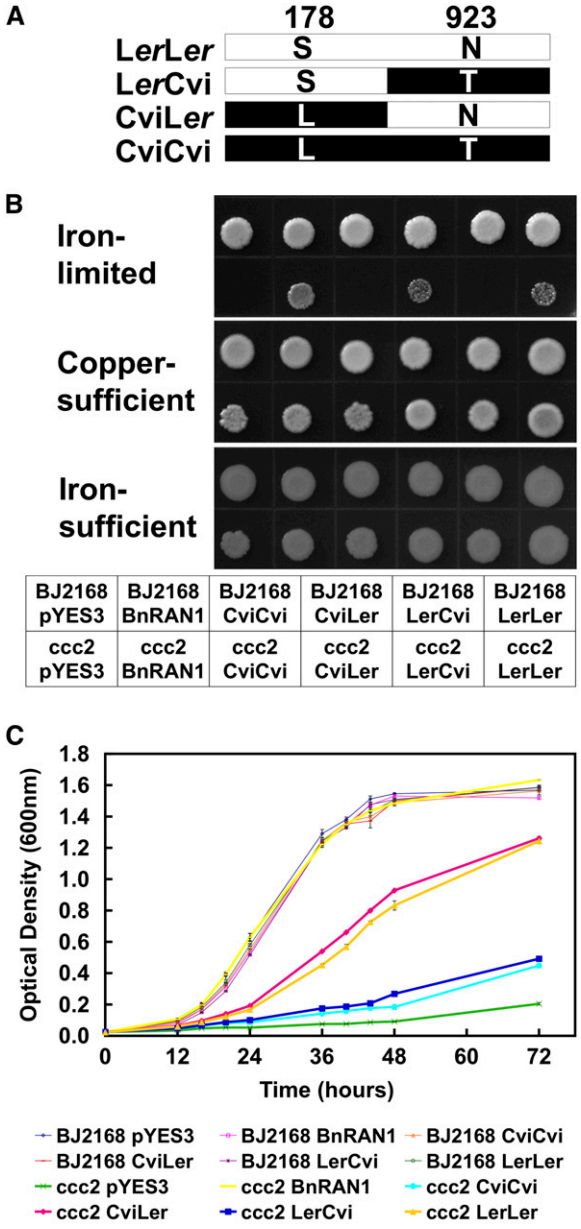


**Figure 5.** Impact of the amino acid substitutions in the Cvi allele on the ability of the HMA5 protein to complement a yeast *ccc2* mutant. The Cu-sensitive Cvi allele contains two amino acid substitutions, S178L and N923T, compared with the Cu-tolerant L*er* allele. cDNAs encoding authentic L*er* (L*er*L*er*) and Cvi (CviCvi) alleles and synthetic chimeric alleles (CviL*er* and L*er*Cvi; A) were introduced into the yeast expression vector pYES3 and then transformed into BJ2168 (parental strain) and the *ccc2* mutant. A known plant Cu-exporting ATPase, *BnRAN1*, was used as a control for all complementation experiments. The Fe-limited medium (plates [B] and liquid [C]) was used to detect complementation of the *ccc2* mutant. Iron-sufficient and Cu-sufficient plates (B), which allow growth of the *ccc2* mutant, demonstrate that all of the yeast strains were viable. All of the medium contained Gal and raffinose as a carbon source, and the plates (B) were incubated for 14 d. Data represent optical density at 600 nm (means ± SE; $n = 3$; C).

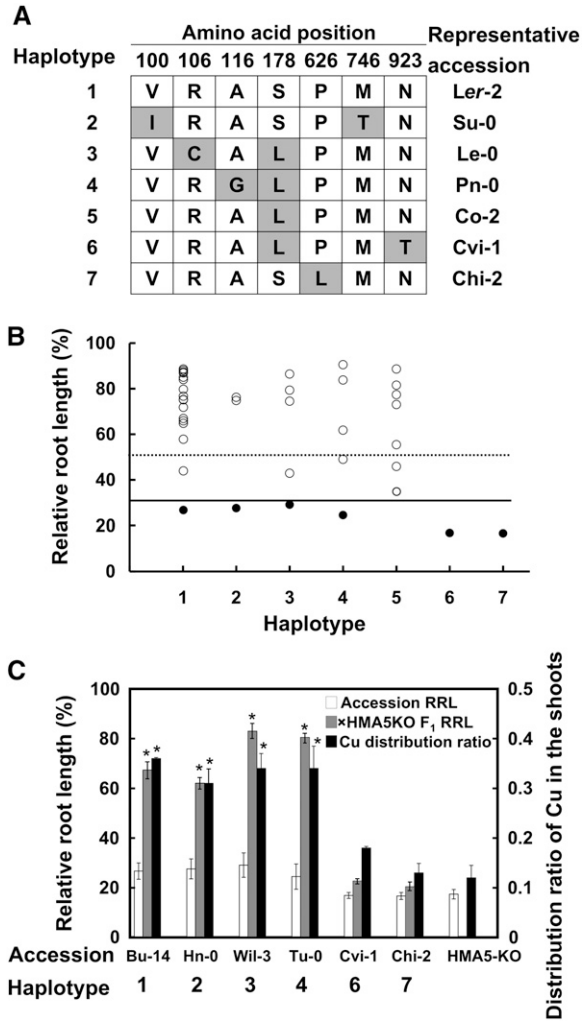


**Figure 6.** Variation in Cu tolerance among accessions of Arabidopsis with different haplotypes of HMA5. A, Amino acid polymorphism of HMA5 detected among 40 accessions. Positions of polymorphism in the HMA5 protein are shown. Each row shows a haplotype and a representative accession. Gray shading represents polymorphic amino acid residues compared with haplotype 1. B, Distribution of Cu-tolerant variation within each haplotype. Black circles represent typical sensitive accessions (RRL in 1.3 $\mu$M Cu solution $< 30\%$) that were used for other experiments shown in C, while white circles show other accessions. The dotted line shows the mean value of RRL in Cu solution of 103 accessions, while the solid line shows 30% RRL. C, Complementation assay of HMA5-KO haplotypes in planta. White bars show the RRL of each accession in Cu solution, while gray bars show those of F1 plants obtained by crosses between the accessions and HMA5-KO. Asterisks represent significant difference from the RRL of each accession (*t* test; $P < 0.05$). Black bars show the distribution ratio of Cu in the shoot. Translocation capacities were defined as the ratio of ion content in the shoot to total ion content in the plant and are shown as means ± SE ($n = 3$).

accession of HMA5-KO, Col-0 (Supplemental Table S1). Almost all of accessions that belonged to haplotype 1 showed Cu tolerance (i.e. greater than the average of 103 accessions; Fig. 6A) and greater Cu translocation capacity than HMA5-KO (Supplemental Fig. S5). One Cu-sensitive accession (Bu-14) belonged to haplotype 1, but its sensitivity was complemented by HMA5-KO, and the shoot to total ratio of Cu in Bu-14 was significantly higher than that of HMA5-KO (Fig. 6C). This observation further supports the notion that the L*er* HMA5 allele, haplotype 1, is a Cu-tolerant allele in Arabidopsis.

The remaining 22 accessions consisted of nine sensitive (including Cvi-1) and 13 tolerant accessions. These accessions showed various amino acid polymorphisms at seven different positions, which were grouped into six other haplotypes (Fig. 6, A and B). Haplotypes 2 to 5 were shared by both tolerant and sensitive accessions, suggesting that these substitution (s) may not alter HMA5 functional capacity and Cu tolerance (Fig. 6B). In fact, the phenotype of the most nsitive accession of haplotypes 2 (Hn-0), 3 Wil-3), and 4 (Tu-0) were complemented by HMA5-KO in the F1 generation (Fig. 6C). Also, their Cu translocation capacities were significantly greater than that of HMA5-KO (Fig. 6C). This indicates that the Cu sensitivity of these accessions is not caused by decreased HAM5 function and is not associated with reduced Cu translocation. On the other hand, haplotype 6 and 7 were specific to sensitive accessions, Cvi-1 and Chisdra-2 (Chi-2), respectively (Fig. 6, A and B; Supplemental Table S1). Chi-2 was not complemented by HMA5-KO (F1 progeny, 20.4%; Fig. 6C) but was complemented by Col-0 (F1 progeny, 98.3%; Supplemental Fig. S4), indicating that the Chi-2 allele is another Cu-sensitive allele of HMA5. In this case, the Cu translocation capacity of Chi-2 was significantly lower than that of the other accessions and was the same as that of the HMA5-KO (Fig. 6C), while Zn and Mn translocation capacities were the same among all accessions (Supplemental Fig. S5).

### Characterization of Amino Acid Substitutions on a Motif and Domain Map of HMA5

Previous studies of mutations in Cu-transporting ATPases indicated that substitution(s) in or adjacent to essential motifs in conserved domains caused disruption of protein function and in turn a mutant phenotype (e.g. *ran1* and *paa1* mutations in Arabidopsis; Hirayama et al., 1999; Shikanai et al., 2003; Supplemental Fig. S3). These amino acids are usually highly conserved in Cu-transporting ATPases and other P-type ATPases (Williams and Mills, 2005) among various eukaryotes. To estimate the impact of HMA5 polymorphisms on Cu tolerance, we characterized each substitution by comparing its position relative to functional domains and by determining the degree to which these residues are conserved among homologs from eukaryotes and prokaryotes.

A large proportion of substitutions found in the functionally normal HMA5 alleles (haplotypes 1–5) were located in regions that do not contain a conserved domain, such as the HMA domain, transmembrane region, and phosphorylation domain (Figs. 6A and 7A;

Supplemental Fig. S3). Similarly, a large proportion of substitutions were found in regions where the particular amino acid residue was not highly conserved among the $P_{1B-1}$-ATPases of a variety of organisms (see 20 organisms in Fig. 7, B and C, in which conserved percentages ranged from 5%–45%; Fig. 7A). On the other hand, both of the less functional HMA5 alleles (Cvi-1 and Chi-2; haplotypes 6 and 7) carried a substitution in an amino acid that is 100% conserved among reported $P_{1B-1}$-ATPases (Fig. 7, B and C; Argüello, 2003; Williams and Mills, 2005). The P626L substitution (unique to Chi-2) and the N923T substitution (unique to Cvi-1) are located in the amino acid motifs [$CPC(x)_6P$; the latter P to L] and [$N(x)_6YN(x)_4P$; the former N to T], which are located in the sixth and seventh transmembrane domains, respectively (Fig. 7).

### Impact of Dysfunctional HMA5 on the Growth of Arabidopsis in Cu-Contaminated Soil

We have shown that HMA5 is a critical factor in the Cu tolerance of roots grown in hydroponic culture. To test whether this has impact on other growth conditions, we grew contrasting Cu tolerance RILs and a set of HMA5-KO (Col background) and Col plants on soil artificially contaminated with Cu. In control and lower contaminated Cu soil, no differences were observed among all tested accessions. However, the growth of sensitive RILs (i.e. Cvi HMA5 allele) and HMA5-KO was more affected by Cu on highly contaminated Cu soil than tolerant RILs or Col (Fig. 8). These results indicated that *HMA5* is a critical gene for Cu tolerance in Cu-contaminated soil.

## DISCUSSION

Molecular biological studies, mainly in yeast, have led to the development of hypotheses concerning the mechanisms for Cu sensitivity in relation to Cu homeostasis (Puig et al., 2007). The roles of similar mechanisms that might contribute to natural variation in plant tolerance to Cu, especially for root growth, have remained unclear. We have performed QTL and accession analyses using RRL as a specific index of Cu tolerance and found that amino acid polymorphisms in a single major gene can account for the Cu sensi-

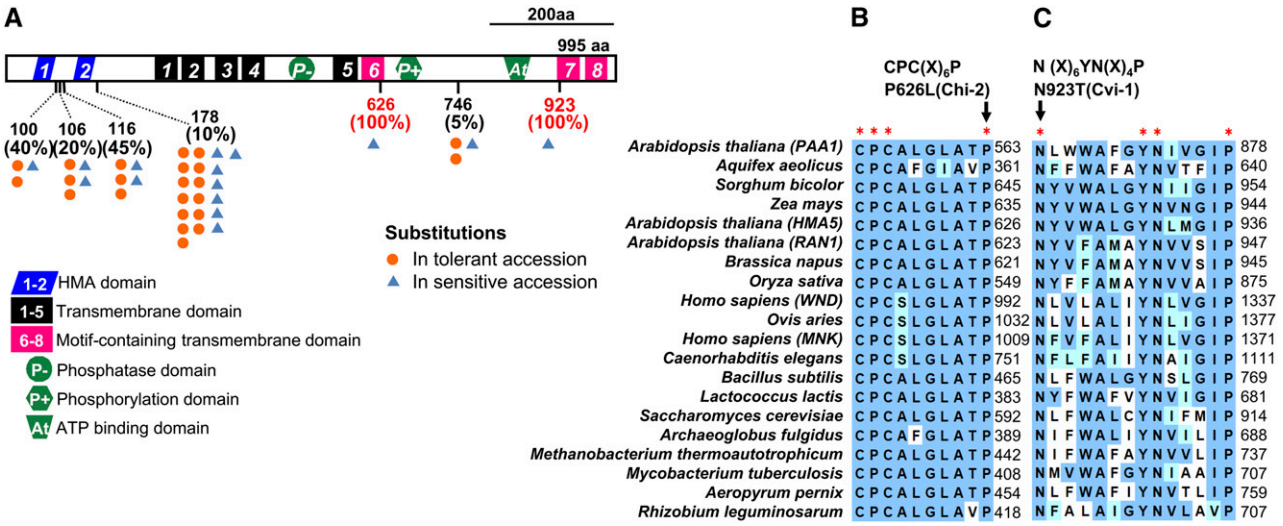


**Figure 7.** Amino acid polymorphisms of HMA5 among Arabidopsis accessions. A, Using Col-0 HMA5 as a reference, substituted amino acids were identified from various accessions (see Fig. 6). Dots indicate substitutions in the tolerant (orange circles) or sensitive (blue triangles) accessions, and numbers indicate substituted amino acid positions. The conservation percentage of each residue among $P_{1B-1}$-ATPase of various eukaryotes and prokaryotes shown in B is indicated under the substitution position number. B and C, Substitutions identified in the dysfunctional HMA5 alleles of Cu-sensitive accessions, Cvi-1 and Chi-2, respectively, which are located in the highly conserved motifs among known $P_{1B-1}$-ATPases of various eukaryotes and prokaryotes as follows: *Arabidopsis thaliana* (PAA1), Q9SZC9; *Aquifex aeolicus* (AQ1445), O67432; *Sorghum bicolor*, Q6JAG2; *Zea mays*, Q6JAH7; *Arabidopsis thaliana* (AtHMA5), Q9SH30; *Arabidopsis thaliana* (AtRAN1), Q9S7J8; *Brassica napus* (BnRAN1), Q941L1; *Oryza sativa* (OsHMA9), Os06g45500; *Homo sapiens* (ATP7B/WND), P35670; *Ovis aries* (ATP7B), Q9XT50; *Homo sapiens* (ATP7A/MNK), Q04656; *Caenorhabditis elegans* (CUA-1), O17737; *Bacillus subtilis* (YvgX), O32220; *Lactococcus lactis* (COPA), Q9CHA4; *Saccharomyces cerevisiae* (CCC2), P38995; *Archaeoglobus fulgidus* (AF0473), O29777; *Methanobacterium thermoautotrophicum* (MTH1535), O27578; *Mycobacterium tuberculosis* (CTPA), Q10876; *Aeropyrum pernix* (APE1454), Q9YBZ6; *Rhizobium leguminosarum* (FIXI), O33533. Asterisks in B and C show $CPC(X)_6P$ and $N(X)_6YN(X)_4P$ motifs, respectively. Arrows indicate polymorphic positions of Chi-2 and Cvi-1; strictly conserved amino acids are shaded dark blue, while residues with a positive Blosum62 score are shaded light blue.

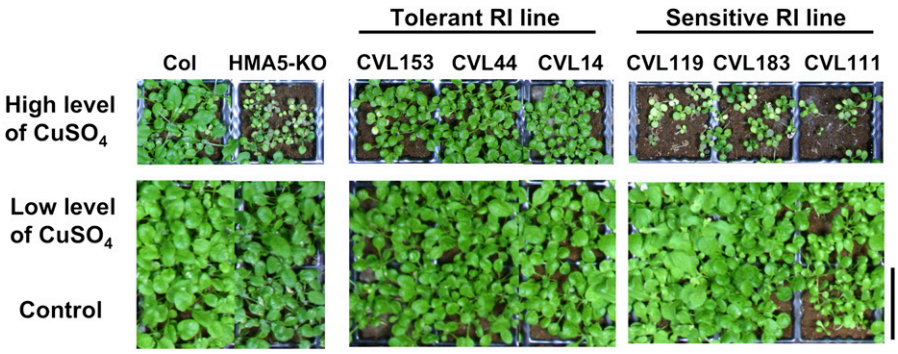


**Figure 8.** Growth of HMA5-KO, Col-0, and Cu-tolerant and Cu-sensitive RI lines on the artificial Cu-contaminated soil. Plants were grown for 3 weeks with different levels of Cu (control, no additional Cu; low level, +20 mg of Cu to 1 kg of control soil; high level, +100 mg of Cu to 1 kg of control soil). Bar = 3 cm.

tivity of some accessions. We found that the L*er* genotype at QTL1 confers Cu tolerance (greater than Cvi) due to its higher capacity for Cu translocation from roots to shoots. This capacity for metal translocation was not observed for Mn and Zn (Fig. 3). This is consistent with the proposed function of HMA5 (our candidate gene colocating in QTL1), which detoxifies Cu in the root by translocation of Cu from roots to shoots (Andrés-Colás et al., 2006). The conclusion that *HMA5* plays a critical role in QTL1 was supported by a series of complementation assays that utilized the yeast mutant *ccc2* (Fig. 5) and the Arabidopsis HMA5-KO (Fig. 4).

*HMA5* belongs to a large family of genes encoding $P_{1B}$-type cation-transporting ATPases that transport a variety of cations. Several amino acid residues are highly conserved among or within subfamily members (Argüello, 2003). Interestingly, previous mutant studies with Cu and other metals indicate that substitutions in highly conserved amino acids negatively affect the transport capacity of these proteins. In fact, Arabidopsis mutants with defects in Cu-transporting ATPases, namely, *ran1-1* (Hirayama et al., 1999; *RAN1* is homolog of *HMA5*), *ran1-3* (Woeste and Kieber, 2000), and *paa1-2*, *-5*, and *-6* (Shikanai et al., 2003; Cu transport into plastid), all show substitutions at amino acid residues that are conserved in Cu-transporting ATPases. In our study, the less functional HMA5 alleles, Cvi-1 and Chi-2, which have lower capacity for Cu translocation (Fig. 6C), carry amino acid substitutions in the consensus amino acid residues shared by all $P_{1B-1}$-type cation-transporting ATPases (Argüello, 2003; Fig. 7, B and C). Similar dysfunctional mutations have been identified in human Menkes and Wilson diseases (de Bie et al., 2007). A substitution near the $N(x)_6YN(x)_4P$ motif (A1362D [strictly conserved A to D]; Fig. 7C) in the protein responsible for Menkes disease (MNK; ATP7A; Donsante et al., 2007) was identified. This mutated allele showed a reduced capacity to complement a yeast *ccc2* mutant. Our yeast complementation tests (Fig. 5) suggest that the amino acid substitution located in the $N(x)_6YN(x)_4P$ motif (N923T, found in CviCvi and L*er*Cvi proteins) was responsible for the Cvi phenotype (Figs. 4 and 6C). This is further supported by the observation that the other substitution, S178L, is also found in tolerant accessions (Fig. 7A). Substitutions in strictly conserved amino acids in the $CPC(x)_6P$ motif (Fig. 7B), including C1000R of the protein for Menkes disease (MNK; ATP7A; Petris et al., 2002) and P992L of the protein for Wilson disease (WND; ATP7B; Forbes and Cox, 2000), were identified as molecular causes of these diseases. The former was found to reduce the rescue of the *ccc2* yeast mutant. Thus, we propose that the P626L substitution in Chi-2 would have the greatest impact on protein function. These results suggest that human pathogenesis caused by Cu-exporting ATPases and natural phenotypic variation of Cu tolerance in Arabidopsis is driven by similar mechanisms. This raises an interesting question for future research: whether the same mechanism can account for variation in Cu tolerance of other crop species.

Previous studies of natural variation in Arabidopsis identified several genes controlling important traits, such as *CRY2* for flowering time (El-Din El-Assal et al., 2001), *PHYA* for hypocotyl length (Maloof et al., 2001), and *APR2* for sulfate content (Loudet et al., 2007). Using a similar approach, we have identified one natural null allele due to a nonsense mutation in a critical gene for Al tolerance, *AtALMT1*, from Warshou-0 (Kobayashi et al., 2007a). These results demonstrate that studies of natural variation in Arabidopsis provide a powerful tool to understand the molecular mechanism of phenotypic variation to various traits.

In this study, we have applied an association analysis between *HMA5*, a candidate gene for QTL1, and Cu tolerance among natural accessions. We sequenced 40 accessions that contained almost all of the sensitive accessions identified from a larger population (103 accessions). This allowed us to identify seven haplotypes in the amino acid polymorphism of HMA5, including two sensitive alleles from Cu-hypersensitive accessions (i.e. Cvi and Chi-2; haplotypes 6 and 7). Because these two alleles were both unique among sequenced accessions, we could not identify the cause of Cu hypersensitivity of these dysfunctional alleles directly by this approach. As described above, however, the negative impact of polymorphisms that occurred in these sensitive alleles could have been predicted from the similarity of polymorphism in human disease research (for review, see de Bie et al., 2007; Thompson et al., 2008) and other molecular genetics studies in $P_{1B-1}$-ATPase mutants (e.g. RAN1; Hirayama et al., 1999). On the other hand, we could

reject a large impact of the other five haplotypes (i.e. haplotypes 1–5) by the association mapping approach because of the segregation of haplotypes among the tolerant accessions (Fig. 6B). Our results suggest that the combination of association mapping analysis and the biochemical approach could be used to identify key gene(s) regulating variations of target traits among accessions.

Using a complete pair-wise search (Chase et al., 1997), we identified epistatic interactions between QTL1 and other loci (Fig. 2; Table I), suggesting that other factors that affect Cu homeostasis with HMA5 may be involved in these epistatic loci. A search for candidate genes revealed that *COPT1* (a copper uptake transporter of the root; Sancenón et al., 2003, 2004) and *RAN1* (a homolog of *HMA5*; Hirayama et al., 1999; Woeste and Kieber, 2000), which are known as major members involved in Cu homeostasis in plants, are located in the epistasis-interacting region of chromosome 5. Some of these candidate genes may account for epistatic interactions. In addition, we have identified several Cu-sensitive accessions that have functional HMA5 alleles, as demonstrated by the observation that their Cu-sensitive phenotype can be complemented by HMA5-KO (Fig. 6C). These accessions, namely, Bu-14, Hn-0, Wil-3, and Tu-0, would also be useful genetic material to clarify other molecular mechanisms of Cu tolerance variation (other than HMA5) that may not affect Cu translocation from the roots to the shoots.

Our results suggest that part of the tolerance to Cu in Arabidopsis is controlled by Cu translocation capacity from roots to shoots. This is similar to the mechanisms of tolerance to other metal such as Zn and cadmium (Cd; AtHMA2 and AtHMA4 [Hussain et al., 2004; Mills et al., 2005]; TcHMA4 [Papoyan and Kochian, 2004]) or Al (ALS3; Larsen et al., 2005). Increased translocation capacity of Cd, regulated by *HMA4* expression, was recently identified as part of the molecular mechanism that results in greater Cd accumulation capacity of *Arabidopsis halleri* than of Arabidopsis (Hanikenne et al., 2008). Other mechanisms in metal homeostasis, such as detoxification of ions in the cytosol or decreased uptake rates, may enhance the tolerant phenotype. In fact, a Cu accumulator plant, *Elsholtzia haichowensis*, carries a greater capacity for Cu detoxification in the roots by increased production of a Cu-binding protein (Lou et al., 2004) and increased scavenging of reactive oxygen species (Zhang et al., 2008). Such capacity would be another target phenotype for clarifying the molecular mechanisms of Cu tolerance in Arabidopsis.

## MATERIALS AND METHODS

### Arabidopsis Accessions

A recombinant Arabidopsis (*Arabidopsis thaliana*) inbred population (accession no. N22000) derived from a cross between L*er*-2 (N8581) and Cvi-1 (N8580; Alonso-Blanco et al., 1998) was obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/). A set of 149 RILs for L*er*/Cvi was used in this study. A T-DNA insertion line of HMA5 (designated HMA5-KO; SALK_040252) was obtained from the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/index.html). Homozygous T-DNA insertion lines of HMA5-KO and the wild type (Col-0) were isolated from the next generation of original seeds using protocols released from SALK (http://signal.salk.edu/cgi-bin/tdnaexpress) using T-DNA-specific or gene-specific primers (5′-CCAGTACATTAAAAACGTC-CGCAATGTGTT-3′ in T-DNA, 5′-GATTCGCGCACAAGACGATCGATTA-CATGG-3′ and 5′-GCATCCTCAATAGTCTCACGAATTGTCTCC-3′ in gene). Arabidopsis accessions used in this study (Supplemental Table S1) were derived from the RIKEN BioResource Center (http://www.brc.riken.jp/lab/epd/Eng/species/arabidopsis.shtml). Additionally, N933 (Col-4) and NW20 (L*er*-0), parental lines of L*er*/Col RILs, were obtained from the Nottingham Arabidopsis Stock Centre.

### Hydroponic Culture and Phenotyping of Cu Tolerance

Hydroponic culture of Arabidopsis was carried out as described in a previous study of lanthanum tolerance (Kobayashi et al., 2007b) with minor modifications. Culture solutions were prepared by adding aliquots of Japanese Industrial Standard Cu solution for atomic absorption spectrometry [10 mg $L^{-1}$ $Cu(NO_3)_2$ in 0.1 N $HNO_3$; Wako Pure Chemical] to basal test solution consisting of 200 $\mu$M $CaCl_2$ and 2% other components of MGRL nutrients (Fujiwara et al., 1992) without phosphate at an initial pH of 5.0. Seedlings were grown on nylon mesh (about 40 $\mu$m span) supported by a plastic photo slide mount (Fuji Film; Toda et al., 1999). All solutions were renewed every 2 d, and the seedlings were grown for 5 d (QTL analysis) or 7 d (JA series) under controlled-environment conditions (12-h day/night cycle, 30 $\mu$mol quanta $m^{-1}$ $s^{-1}$ at 25°C). For QTL analysis and phenotyping of accessions, all lines were grown in 8 L of the same solution (in plastic containers) to minimize environmental differences.

Ten seedlings were grown in both Cu toxic and control solutions, and root length was measured using a video microscope as described by Toda et al. (1999). Average root length values were calculated using the top three root lengths in the each treatment. RRL was then calculated using the formula RRL = mean root length in Cu solution/means of the root length in control solution (%). This experiment set was repeated three times, and the averages of those three RRL measurements were used for QTL analyses and the accession study.

### QTL Analyses

QTL analyses, consisting of the CIM and complete pair-wise search for epistasis, were conducted as described by Kobayashi et al. (2007b). Briefly, genetic linkage maps for each population were constructed with Mapmaker/EXP version 3.0b (Lander et al., 1987; obtained from http://www.broad.mit.edu/genome_software/) using segregation data for each RIL obtained from databases (167 markers in the L*er*/Cvi RILs; obtained from the Nottingham Arabidopsis Stock Centre; http://arabidopsis.info/). In addition to these public markers, we constructed and used a new dCAPS marker in QTL1 (http://helix.wustl.edu/dcaps/dcaps.html; Fig. 2B; primer sequences are listed in Supplemental Table S2). Using phenotype (RRL) and genotype data sets for the RI population, QTL parameters (likelihood, location, additive effect, and percentage of variance explained by each QTL) were calculated with model 6 based on the CIM method (Zeng, 1993, 1994) using QTL Cartographer version 1.13 (Basten et al., 1994; obtained from http://statgen.ncsu.edu/qtlcart/). Thresholds of LOD for each linkage group were calculated by a permutation test method (Churchill and Doerge, 1994) with 1,000 permutations at the permutation significance level of $\alpha = 0.05$. Epistatic interactions between any two molecular markers were determined using a complete pair-wise search method with a significance threshold of $P < 0.0005$ using EPISTAT (Chase et al., 1997). This method can identify significant epistasis-interacting marker pairs even if the marker has not been detected as a significant QTL by the CIM method. Broad-sense heritability ($h_b^2$) was estimated using RRL values derived from repeated experiments by the following formula: $h_b^2 = \sigma^2_g/\{(\sigma^2_e/r) + \sigma^2_g\}$, where $\sigma^2_g$ is the genetic variance, $\sigma^2_e$ is the environmental variance, and r is the number of data employed ($n = 3$).

### Determination of Translocation of Cu and Other Ions

Three sets of 50 seedlings of each line were pregrown for 10 d in control solution containing inorganic phosphate, then transferred to a solution

containing 1.3 $\mu$M Cu for 2 d. Seedlings were then harvested and rinsed in distilled water, and roots and shoots were separated using a blade. The contents of $^{63}Cu$, $^{55}Mn$, and $^{66}Zn$ were measured by inductively coupled plasma-mass spectrometry (ELAN 6000; PerkinElmer Japan) as described by Kobayashi et al. (2007b). Translocation capacity was defined as metal content in the shoot divided by total metal content (shoot + root) and determined for the parental accessions in the L*er*/Cvi RI population (L*er*-2 and Cvi-1), selected RILs carrying different genotypes of HMA5 at the QTL1 locus (L*er* genotype of QTL1, RI line nos. CVL14, CVL44, CVL101, CVL153, and CVL163; Cvi genotype, RI line nos. CVL26, CVL111, CVL119, CVL135, and CVL183), HMA5-KO (Col-0 background), and several accessions.

## DNA Sequencing and Protein Sequence Analyses

Genomic DNA was isolated from leaves as described by Kobayashi et al. (2007b). The HMA5 region was sequenced by a direct sequencing procedure using the ABI BigDye Terminater System (version 3.1) and an ABI PRISM3100 DNA sequencer according to the manufacturer's manual. Primers used for this process are shown in Supplemental Table S3. Using the Col-0 sequence as a reference, the complete coding sequence of HMA5 was obtained for each accession. Amino acid sequences of previously characterized $P_{1B-1}$-ATPase genes were obtained from a P-type ATPase database (http://www.patbase.kvl.dk./) and sequences reported by Argüello (2003) and Williams and Mills (2005). Multiple amino acid alignment was performed using the ClustalW and Jalview 2.2 programs. The BLOCKS program (http://blocks.fhcrc.org/, http://motif.genome.jp/) and information described in a recent review (Williams and Mills, 2005) were used to predict functional domains and motifs in the HMA5 protein. Transmembrane-spanning domains of HMA5 were predicted by ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/).

## Yeast Complementation Test for Cvi and L*er* HMA5 Proteins

There are two amino acid substitutions between L*er*-2 and Cvi-1 at positions 178 and 923 (L*er*-2, S and N; Cvi-1; L and T). cDNAs encoding authentic and chimeric protein, designated L*er*L*er*, L*er*Cvi, CviL*er*, and CviCvi, by amino acid polymorphism at positions 178 and 923 were packaged in the yeast expression vector pYES3 (Invitrogen) downstream of the GAL1 promoter (i.e. driven by Gal but not by Glc). Chimeric cDNAs were made from cDNA of each accession using appropriate sites for the restriction endonucleases. The vectors were transformed to the *ccc2* mutant and BJ2168 wild-type yeast, respectively, which we used previously to characterize *BnRAN1* (a kind of Cu-transporting ATPase in *Brassica napus*; Southron et al., 2004). Using pYES3-*BnRAN1* as a control, growth capability on Fe-limited medium (which reflects CCC2 complementation capacity) was estimated as described by Southron et al. (2004). Each experiment was performed twice, and representative results are shown.

## Complementation Test of HMA5 Alleles in Planta

F1 seeds were obtained from crosses between individual accessions and HMA5-KO or Col-0. Both HMA5-KO and Col-0 were used as the female parent, and the other accessions were used as the male parent. F1 seeds from these crosses were used in root elongation assays as described above and then rescued for genotyping to confirm cross-fertilization.

## Soil Culture

Cu-tolerant and Cu-sensitive RILs carrying different genotypes of HMA5 at the QTL1 locus (L*er* genotype of QTL1, RI line nos. CVL14, CVL44, and CVL153; Cvi genotype, RI line nos. CVL111, CVL119, and CVL183), HMA5-KO (Col-0 background), and Col-0 were grown in a plastic chamber (3 cm wide $\times$ 3 cm long $\times$ 3.5 cm deep) using a brown forest soil (Zao). Basal soil was prepared by adding 2.5 g of $CaCO_3$, 2.5 g of $NaH_2PO_4$, 0.48 g of KCl, 0.36 g of $MgSO_4$, and 1.32 g of $(NH_4)_2SO_4$ in 1 kg of soil. Cu-contaminated soil was prepared by adding 20 or 100 mg of Cu ($CuSO_4$) to 1 kg of the basal soil. After germination treatment (4°C in the dark for 5 d), 20 seeds per each line were planted in soil. Seedlings were grown under the 12-h day (30 $\mu$mol quanta $m^{-2}$ $s^{-1}$)/night cycle at 25°C for 3 weeks.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU877765 to EU877803.

## Supplemental Data

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

**Supplemental Figure S1.** Segregation of Cu tolerance among seed progeny derived from a heterozygous HMA5-KO plant.

**Supplemental Figure S2.** *HMA5* expression patterns in roots of selected RILs and parental accessions.

**Supplemental Figure S3.** Functional domains and amino acid polymorphisms of HMA5.

**Supplemental Figure S4.** Complementation assays of the Arabidopsis HMA5-KO mutant by Cu-sensitive accessions.

**Supplemental Figure S5.** Translocation capacities of Zn, Mn, and Cu among Arabidopsis accessions.

**Supplemental Table S1.** List of accessions used in the current study and their Cu tolerance phenotype and haplotype.

**Supplemental Table S2.** Primer sequences for dCAPS marker analysis in the QTL1 region.

**Supplemental Table S3.** Primer sequences for sequencing analysis of *HMA5*.

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