

The *Arabidopsis* Copper Transporter COPT1 Functions in Root Elongation and Pollen Development*

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Copper plays a dual role in aerobic organisms, as both an essential and a potentially toxic element. To ensure copper availability while avoiding its toxic effects, organisms have developed complex homeostatic networks to control copper uptake, distribution, and utilization. In eukaryotes, including yeasts and mammals, high affinity copper uptake is mediated by the Ctr family of copper transporters. This work is the first report on the physiological function of copper transport in *Arabidopsis thaliana*. We have studied the expression pattern of COPT1 in transgenic plants expressing a reporter gene under the control of the COPT1 promoter. The reporter gene is highly expressed in embryos, trichomes, stomata, pollen, and root tips. The involvement of COPT1 in copper acquisition was investigated in CaMV35S::COPT1 antisense transgenic plants. Consistent with a decrease in COPT1 expression and the associated copper deprivation, these plants exhibit increased mRNA levels of genes that are down-regulated by copper, decreased rates of ⁶⁴Cu uptake by seedlings and reduced steady state levels of copper as measured by atomic absorption spectroscopy in mature leaves. Interestingly, COPT1 antisense plants also display dramatically increased root length, which is completely and specifically reversed by copper addition, and an increased sensitivity to growth inhibition by the copper-specific chelator bathocuproine disulfonic acid. Furthermore, COPT1 antisense plants exhibit pollen development defects that are specifically reversed by copper. Taken together, these studies reveal striking plant growth and development roles for copper acquisition by high affinity copper transporters.

Plants use copper as a cofactor for a wide variety of physiological processes such as photosynthesis, mitochondrial respiration, superoxide scavenging, cell wall metabolism, and ethylene sensing. In the absence of this essential micronutrient, plants display diverse symptoms, most of which affect young leaves and reproductive organs (1). Typical symptoms of copper

deficiency appear first at the tips of young leaves and then extend downward along the leaf margins. The leaves may also be twisted or malformed and show chlorosis or even necrosis (1, 2).

In addition to its essentiality, copper is a potentially toxic agent at supraoptimal levels. Copper reactivity can lead to the generation of harmful reactive oxygen species that cause severe oxidative damage to cells (3). To deal with this dual nature of copper, plants, as well as other organisms, have evolved sophisticated homeostatic networks controlling copper uptake, utilization, and detoxification (for reviews see Refs. 4–6). The copper homeostasis network has been primarily described in the yeast *Saccharomyces cerevisiae* (for reviews see Refs. 7–9), and its components are widely conserved among eukaryotes.

Intracellular copper distribution is performed by metallochaperones, a set of soluble copper-binding proteins that direct the metal to its final destination (for a review see Refs. 10 and 11). The copper chaperone CCH was the first metallochaperone described in plants (12). The amino-terminal half of CCH displays homology to yeast Atx1 (13), and it possesses a plant-specific carboxyl-terminal extension (14), which has been proposed to participate in intracellular copper transport through the plant symplasmic connections or plasmodesmata (15). Similarly to the task performed by homologues in other organisms, CCH is predicted to deliver copper to a P-type copper-transporting ATPase, denoted RAN1, that is putatively localized at a post-Golgi compartment (16). The RAN1 (responsive to antagonist 1) gene from *Arabidopsis thaliana* codes for a homologue of the yeast Ccc2 and human Menkes (ATP7A) and Wilson (ATP7B) proteins (17–20). This copper homeostasis-related gene was isolated in a screen for plants with an atypical response to the ethylene antagonist *trans*-cyclooctene, underscoring the critical role of copper in the ethylene-signaling pathway (16, 21). This role is explained by the fact that the ethylene receptor requires copper for high affinity ethylene binding (22).

A main step in the control of copper homeostasis is its uptake through the plasma membrane. This function is accomplished by the Ctr family of eukaryotic high affinity copper transporters. Ctr family members have been identified in yeasts and mammals (for a review see Ref. 9). The importance of copper transport into cells has been underscored by recent observations demonstrating that a murine knockout of the gene encoding Ctr1 exhibits severely defective embryonic development and prenatal lethality (23, 24). Ctr proteins contain three predicted transmembrane domains, a variable number of putative metal-binding motifs at their extracellular amino-terminal domain, and a conserved and absolutely essential MXXXM motif within the putative second transmembrane domain (25). A cDNA from *Arabidopsis* encoding a Ctr-related copper trans-

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porter (COPT1) was isolated in a functional complementation screen performed in yeast cells defective in high affinity copper uptake (26). Surprisingly, a role for COPT1 in copper uptake from the soil has not been supported because RNA blotting analysis indicated that the level of *COPT1* in roots was undetectable (26).

In this work, we focus on the function of copper transport in *Arabidopsis* by investigating both the COPT1 expression pattern and the phenotypes obtained in COPT1 antisense plants. COPT1 tissue-specific expression was monitored using COPT1::GUS transgenic plants, and the phenotype of COPT1 antisense transgenic plants is described on the basis of the main tissues expressing the transporter, which include root apical zones and pollen. Our results demonstrate the important role of COPT1 in copper uptake, root growth, and pollen development.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions and Treatments—Seeds of *A. thaliana*, ecotype Columbia (Col 0), were grown in pots and covered with a clear plastic dome. The covered pots were placed in a cold room at 4 °C for 2 days to synchronize germination and then moved to a growth cabinet at 23 °C with a 16-h photoperiod (65 mmol/m² of cool white fluorescent light). The plants were watered regularly with standard commercial greenhouse nutritive solution.

The ethylene response in seedlings was analyzed as described (27). Briefly, seeds from the indicated lines were germinated in MS medium (28) and grown under hydrocarbon-free or ethylene (10 ppm) atmospheres and complete darkness. The triple response was determined after 3 days as a decrease in hypocotyl length and an increase in hook curvature and hypocotyl width.

To study the sensitivity of seedlings to copper availability, the seeds were germinated in either standard MS medium plates including 1% sucrose (except for the root length experiments) (28) or supplemented with the indicated concentrations of bathocuproine disulfonic acid (BCS),¹ metal ions or both. The plates were photographed 2 weeks after germination, and seedling fresh weight and root length was determined from five seedlings with the standard deviation.

Plasmid Constructs and Plant Transformation—For the promoter studies a genomic library (λGEM-11; Promega) was screened, and a positive clone containing the COPT1 5' region was isolated. EcoRV/BamHI (−1334 to +102, where +1 indicates the position of the starting codon) and a XhoI/MnII (−359 to −15) restriction fragments were fused in frame to the GUS gene in the pBI121 plasmid, replacing the *CaMV35S* promoter. For functional studies, the COPT1 complementary strand was placed under the control of the *CaMV35S* promoter. The COPT1 cDNA was recovered from previous work (29) and cloned into the SacI/SmaI sites of pBI121 vector. The C58 strain of *Agrobacterium tumefaciens* was used to transform *Arabidopsis* by following the floral dip protocol (30).

Gene Expression by Semiquantitative RT-PCR—Total RNA was isolated from *Arabidopsis* tissues as described by Prescott and Martin (31). RNA was quantified by UV spectrophotometry, and its integrity was visually assessed on ethidium bromide-stained agarose gels. Total RNA (1–5 µg) isolated from the indicated tissues was first converted to cDNA by reverse transcription using SuperScript II reverse transcriptase (Invitrogen) and the anchored oligo(dT)₁₅ (Roche Applied Science) and 18 S primer (reverse, 5'-CTGGATCCAATTACCAGACTCAA). To quantify endogenous COPT1 mRNA in the antisense lines, primer COPT1 (reverse, 5'-CCTGAGGGAGGAACATAGTTAG) was added instead of oligo(dT)₁₅. For PCRs, 1.5–10 µl of the diluted (1:5) cDNA products were used as templates. The primers were designed to specifically anneal with COPT1 (forward, 5'-CAATGGATCCATGAACGAAGG; reverse, see above), COPT2 (forward, 5'-ACGTGTCAGTGGCTCAACC; reverse, 5'-GACGGCGGAAGAAGCTCGGCGG) and CCH (forward, 5'-ATGGCTCAGACCGTGTCTCT-3'; reverse, 5'-GCTCAGCTTCAGCTCCACT-3'). The reactions were run at 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s) for 25 (COPT2), 26 (CCH), or 27 (COPT1) cycles, which was within the linear reaction range. 18 S rRNA was also measured in the same reaction as an internal control with 18 S primers (forward, 5'-TGGGATACCTGCCAGTAGTCAT; reverse, see above) together with the compe-

timers 18 S (having the same sequence as their counterparts, but a 3'-terminal dideoxy nucleotide). To balance the great excess of 18 S rRNA with respect to the message of COPT2 and CCH, a series of PCRs were performed varying the ratio of competitors/(primers + competitors) from 0 to 1. The ratio that yielded the best relation of COPT to 18 S intensities, without saturating the employed detection method (see below), was selected for further analysis. PCR products were run on a 1.8% agarose gel and stained with ethidium bromide. The relative values of COPT message levels in each sample were normalized with respect to 18 S. The band intensities were analyzed and quantified with the applications ImageStore 5000 and GelBlot (Ultra Violet Products Ltd.).

GUS Expression Analyses—The assays were performed as described by Jefferson *et al.* (32). Seedlings or organs from adult plants were recovered and vacuum-infiltrated with the substrate solution (50 mM NaPO₄, pH 7.2, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.1% Triton X-100, 0.5 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Research Organics Inc.)). The reactions were developed at 37 °C for times ranging from 20 min to several hours and stopped with an ethanol series (30–70%).

Fresh GUS-stained seedlings or anthers were fixed in 4% formaldehyde for 4 h, followed by several washes in 0.11 M NaH₂PO₄, pH 7.0. The samples were dehydrated in ethanol series (70–100%), cleared with xylene, and embedded in low melting temperature paraplast (Sigma). Sections of 10 µm width were obtained in a rotating microtome and mounted in glass slides. The preparations were photographed in a Zeiss optical microscope.

Copper Uptake and Accumulation Measurements—For the metal transport experiments, 10 mg of *A. thaliana* seeds ecotype Columbia (Col 0) and COPT1 antisense A 2–1, A 3–4, and A 3–6 transgenic lines were sterilized and incubated at 4 °C for 1–2 days to synchronize germination. The seeds were germinated at 23 °C for 60 h in water with white fluorescent light. One ml of phosphate-buffered saline was added to the seedlings derived from 2 mg of dry seeds. The seedlings were incubated either at 23 °C or on ice for 1 h. ⁶⁴CuCl₂ (Mallinckrodt Institute of Radiology, Washington University, St. Louis, MO), specific activity 15–30 mCi/µg of copper, was added to a final concentration of 5 µM, and the seedlings were incubated at 23 or at 4 °C for 30 min with continuous agitation. The samples were quenched by adding ice-cold EDTA (final concentration, 10 mM), filtered, and washed with quenching buffer (10 mM EDTA in 0.1 M Tris-succinate, pH 6.0). ⁶⁴Cu was quantified in a γ-counter (Packard Cobra II) by using a standard curve. The values obtained at 0–4 °C were subtracted from 23 °C values and normalized to dry weight. The experiments were independently repeated at least in triplicate, and standard deviation were represented. To determine the copper content in wild type and transgenic plants, leaves from 4-week-old adult plants were dried and frozen in liquid N₂, ground in a mortar, and lyophilized. 50 mg of dried tissue was digested in 1 ml of HNO₃ for 10 h at 80 °C. After adding 4 ml of H₂O, the copper content of each sample was determined by atomic absorption spectrometry (Analytical Technology, Inc., Unicam Solar System 939).

Pollen Preparations for Scanning Electron Microscopy—Pollen was mounted on standard stubs and coated with gold-palladium in a Bio-Rad E5600 ion sputter for 3 min prior to observation on a Hitachi S4100 FE scanning electron microscope. Digital images were acquired with the application EMIP.

RESULTS

COPT1 belongs to a five-member family (COPT1–5) of putative *Arabidopsis* copper transporters (26, 29). Consistent with a putative role as plasma membrane copper transporters, two members of the family, COPT1 and COPT2, robustly restore the respiratory deficiency of a yeast mutant defective in high affinity copper transport, and their corresponding mRNAs are down-regulated in response to copper excess (29). The present study is focused on the functional characterization of COPT1 and its physiological role in copper transport in *Arabidopsis*.

COPT1 Is Expressed in Root Tips and Pollen Grains—To study the COPT1 spatial expression pattern, a 1.4-kb COPT1 promoter region (−1334 to +102, where +1 indicates the position of the first nucleotide in the COPT1 cDNA) was fused to the *uidA* gene, which encodes β-glucuronidase (*GUS*). Stable homozygous transgenic *Arabidopsis* lines harboring the COPT1::GUS chimeric gene were obtained, and those that ex-

¹ The abbreviations used are: BCS, bathocuproine disulfonic acid; WT, wild type; RT, reverse transcription.

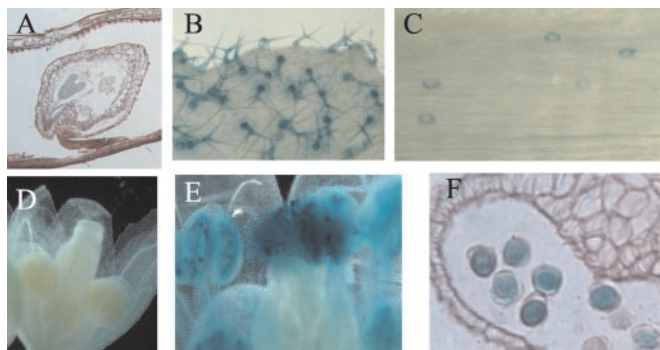


FIG. 1. **Analysis of GUS activity in *COPT1::GUS* transgenic plants.** A, cross-section of a silique showing a GUS-stained embryo at the heart stage. B and C, leaf surface GUS staining of trichomes (B) and stem stomata guard cells (C). D and E, GUS-stained flowers before (D) and after (E) anthesis, respectively. F, anther cross-section showing specific GUS staining in pollen grains.

hibited GUS activity were selected for further analysis (results not shown). Transgenic lines expressing GUS under the control of the *Cauliflower mosaic virus 35S* (*CaMV35S*) general promoter and a minimal *COPT1* promoter (−359 to −15) were used as positive and negative controls for GUS expression, respectively.

In *Arabidopsis* plants transformed with the *COPT1::GUS* construct, embryos at the heart stage are uniformly GUS-stained except in the suspensor (Fig. 1A). GUS expression was also present in the hydrated seeds of mature *Arabidopsis* embryos and the cotyledons of young seedlings (not shown). Histological analyses of adult plants showed *COPT1* promoter-driven GUS expression specifically restricted to trichomes and stomata guard cells in adult leaves (Fig. 1, B and C). Moreover, although GUS staining is not detected during the early stages of flower development (Fig. 1D), mature pollen grains are intensely stained at the pistil, and they remain so during pollination (Fig. 1E). A mature anther cross-section shows that pollen is uniformly stained (Fig. 1F), indicating that at least the vegetative cell is expressing the *COPT1::GUS* fusion. In all cases, negative controls with the *COPT1* minimal promoter remained unstained, and *CaMV35S::GUS* positive controls were uniformly stained (not shown).

Although a previous analysis of total RNA indicated the absence of *COPT1* mRNA in roots (26), we detected *COPT1* expression in roots by RT-PCR (29) and showed that GUS expression is restricted to a specific area at the root apical zone (Fig. 2). Expression in roots persisted throughout the whole life of the plant and could be observed both at the primary root (Fig. 2A) and at lateral root primordia of adult plants (Fig. 2B). GUS staining was restricted to an annular region immediately after the root tip (Fig. 2C). As a positive control, *CaMV35S::GUS* transgenic plants showed constitutive and global GUS staining in roots (Fig. 2D). To further address the cellular location of *COPT1* expression at root apical zones, histochemical detection of GUS staining was carried out in longitudinal (Fig. 2E) and transverse (Fig. 2F) root apical sections of 2-day-old *Arabidopsis* seedlings. Labeling was mainly detected at the columella, lateral root cap, epidermis, and cortex layers, whereas expression was only poorly detected at the endodermis and the stele (Fig. 2, E and F).

Copper Deficiency Responses Are Up-regulated in *COPT1* Antisense Transgenic Plants—The effects of reduced *COPT1* expression on plant phenotypes were studied in transgenic lines expressing *COPT1* in antisense orientation under the control of the *CaMV35S* promoter. Independent *Arabidopsis* transgenic lines expressing *CaMV35S::COPT1* antisense were generated and stable homozygous lines were obtained. All of

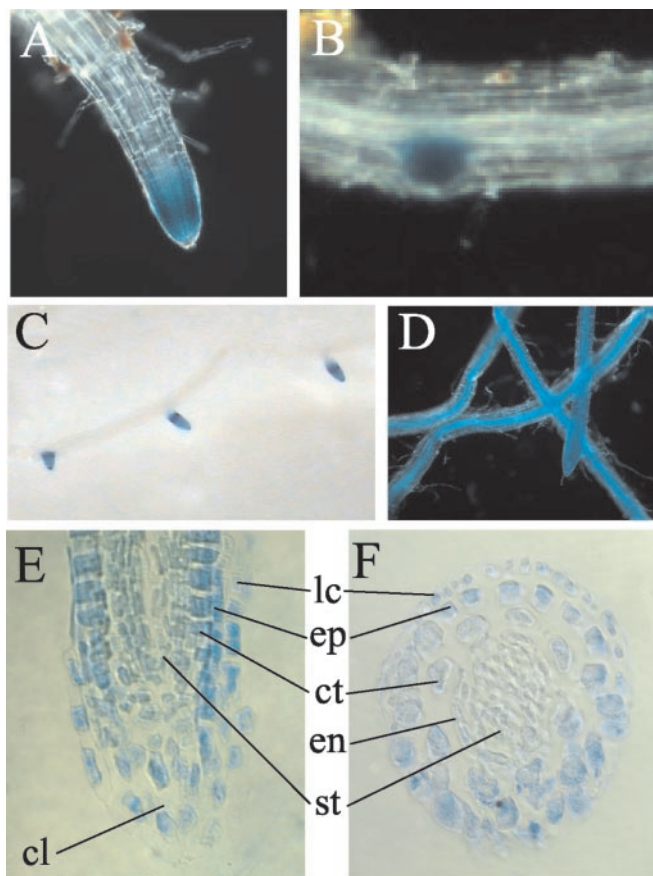


FIG. 2. **Histochemical detection of GUS activity in *COPT1::GUS* transgenic roots.** A–C, primary (A) and lateral (B and C) root tips. D, positive control from *CaMV35S::GUS* roots. E and F, longitudinal (E) and transverse (F) sections of root apical zones. cl, columella; ct, cortex; en, endodermis; ep, epidermis; lc, lateral root cap; st, stele.

the lines expressed high levels of *COPT1* antisense mRNA (not shown). To gain initial insight whether antisense *COPT1* expression causes defects in copper acquisition in transgenic lines, we accurately measured the *COPT1* mRNA endogenous levels by real time PCR in three transgenic lines. *COPT1* mRNA levels quantified by this technique turned out to be approximately the same as those found in wild type plants (not shown). Although the critical issue would be to detect *COPT1* protein stationary levels in these lines, unfortunately specific antibodies are not available. For this reason, we measured the transcript levels of *CCH* and *COPT2*, two genes that are up-regulated in response to decreased copper concentrations (12, 29). *COPT1* antisense lines (A 2–1, A 3–4, and A 3–6) displayed *CCH*, *COPT2*, or both mRNA levels up to four times higher than wild type controls (Fig. 3). The observation that genes whose expression is induced by copper limitation are induced in these lines is consistent with a reduction in bioavailable copper levels. Therefore, these lines were used for further experiments.

Copper Uptake in *Arabidopsis COPT1* Antisense Transgenic Plants—We previously demonstrated that heterologous expression of the *Arabidopsis COPT1* gene in a yeast mutant defective in high affinity copper uptake specifically stimulates ^{64}Cu uptake, and competition experiments strongly support *COPT1* specificity for Cu(I) over other transition metals (29). To further evaluate the ability of the *COPT1* protein to transport copper into *Arabidopsis*, ^{64}Cu uptake assays were carried out in wild type seedlings and compared with the *COPT1* antisense lines. *Arabidopsis* seeds were germinated for 2.5 days, and

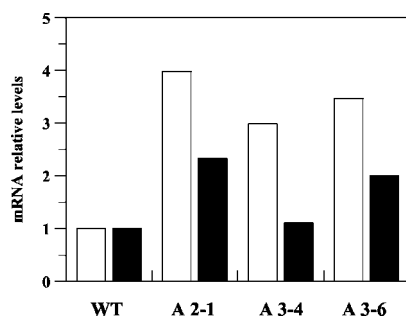


FIG. 3. Transcript levels of *CCH* and *COPT2* in wild type and *COPT1* antisense *Arabidopsis* lines. Total RNA was prepared from rosette leaves from adult plants and reverse transcribed to cDNA. The messages corresponding to each gene and to the 18 S rRNA were determined by RT-PCR amplification with specific primers. *CCH* (white bars) and *COPT2* (black bars) mRNA levels were normalized to 18 S rRNA, and the arbitrary value 1.0 was assigned to the WT.

radioactive copper accumulation in seedlings was measured after incubation with $^{64}\text{CuCl}_2$. All three *COPT1* antisense *Arabidopsis* lines (A 2-1, A 3-4, and A 3-6) showed a 40–60% reduction in the short term levels of ^{64}Cu accumulation as compared with the wild type (WT) (Fig. 4A). To evaluate whether the reduced levels of ^{64}Cu resulted in reduced steady state copper accumulation in *COPT1* antisense adult plants, copper content in plant leaves from both wild type and *COPT1* antisense transgenic lines was determined by atomic absorption spectroscopy. Consistent with the copper transport data in Fig. 4A, the average copper content in 4-week-old *Arabidopsis* rosette leaves was reduced ~40–60% in the *COPT1* antisense transgenic lines when compared with the control (WT; Fig. 4B). Taken together, these results strongly implicate the *COPT1* protein in copper uptake and accumulation in *Arabidopsis* plants.

***COPT1* Antisense Transgenic Plants Display Increased Root Length**—Despite the reduced copper uptake levels and accumulation, *COPT1* antisense plants are apparently as healthy as controls when grown under standard laboratory conditions in growth cabinets. Because of the well established requirement of copper ions for the ethylene receptor (16, 22), a more detailed study was performed to determine whether the *COPT1* antisense RNA-induced copper deficiency impacts ethylene sensing and/or signaling. For this purpose, seeds derived from the *COPT1* A 2-1, A 3-4, and A 3-6 transgenic lines were germinated in MS medium plates with 1% sucrose (28), grown for 3 days in darkness under hydrocarbon-free or ethylene (10 ppm) atmospheres, and analyzed for their triple response to ethylene (27). *Arabidopsis* seedlings from both wild type and a mutant (*ctr1-1*) for an ethylene signal transduction component conferring constitutive triple response (33) were used as controls in the assays. All of the *COPT1* antisense lines tested behaved as the wild type seedlings, indicating that ethylene sensing and signaling are not disturbed in these plants. Moreover, the addition of 30 μM of the copper chelator BCS to the growth medium did not cause any effect on the triple response, showing that ethylene action remains unaltered in these plants under these conditions (not shown).

Interestingly, seedlings from *COPT1* antisense transgenic plants exhibited roots three to six times longer than controls when grown on the sucrose-depleted medium (Fig. 5A, line A 3-6). The roots returned to wild type length when treated with 30 μM copper sulfate, whereas the addition of the same concentration of transition metals such as iron or zinc failed to restore the phenotype (Fig. 5, A and B). *COPT1* antisense roots remained 3.9 and 2.2 times longer than controls in media supplemented with iron or zinc, respectively (Fig. 5B). These results were also observed with the other two *COPT1* antisense

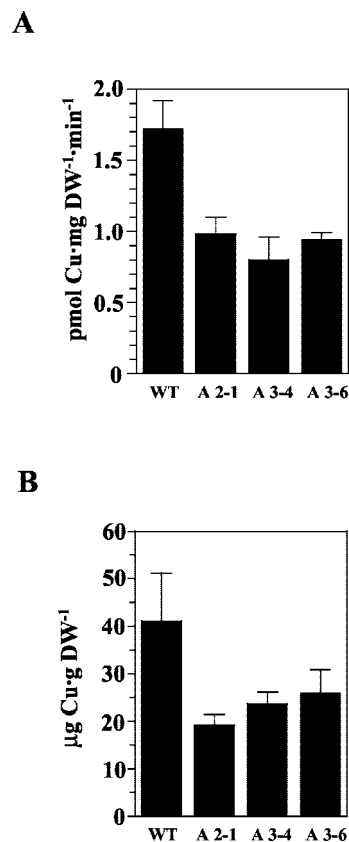


FIG. 4. Copper uptake and accumulation in wild type and *COPT1* antisense *Arabidopsis*. A, ^{64}Cu uptake rate was measured for wild type and *COPT1* antisense lines A 2-1, A 3-4, and A 3-6. Values were normalized to seed weight. B, copper content in 4-week-old control and *COPT1* antisense lines. Copper was measured from leaves by atomic absorption spectroscopy. The error bars indicate the S.D. values for triplicate measurements.

lines, A 2-1 and A 3-4, and were abolished by 1% sucrose addition (not shown). Interestingly, when wild type seedlings were supplemented with different concentrations of the copper chelator BCS, root length increased in a dose-dependent manner, whereas the *COPT1* antisense plants were not significantly affected (Fig. 5C). Therefore, the copper chelator BCS mimics the root elongation phenotype observed in the *COPT1* antisense transgenic plants in wild type plants. Taken together, these results strongly suggest that a defect in copper uptake from soil dramatically affects root elongation.

Moreover, seedlings from all three *COPT1* antisense transgenic lines grown in the presence of BCS showed a reduced rate of growth when compared with wild type; this was observed as both a decrease in cotyledon size and a delayed appearance of the first pair of leaves (Fig. 6). This growth retardation correlates with a significant reduction in the fresh weight in all three lines tested. Although the fresh weight decayed around 10% in wild type seedlings grown on BCS compared with the same seedlings grown on MS medium, a decrease of 30–40% in fresh weight was observed in the *COPT1* antisense lines (not shown). Furthermore, the addition of 30 μM copper to the growth medium completely eliminated the observed differences in development and fresh weight (Fig. 6). These results demonstrate that the growth of *COPT1* antisense plants is more sensitive than the wild type to copper limitation imposed by a copper-specific chelator.

Pollen Development Is Defective in *COPT1* Antisense Plants—Because *COPT1* is highly expressed at the final stages of pollen development (Fig. 1, E and F), we assessed potential

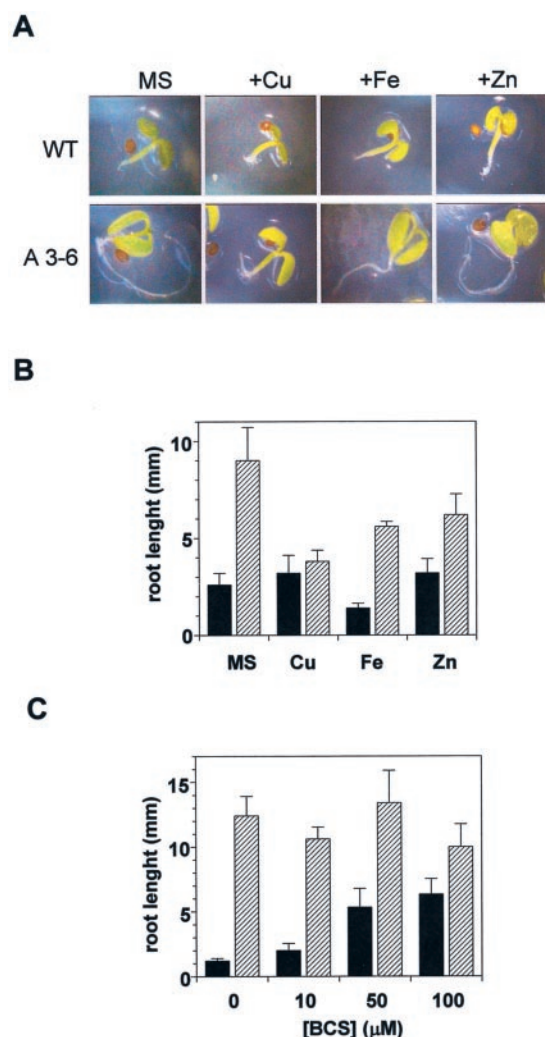


FIG. 5. Effect of the availability of different metal ions on root length of wild type and *COPT1* antisense plants. **A**, seedlings from WT (upper row) and *COPT1* antisense line A 3-6 (lower row) grown on MS medium without sucrose or on the same medium supplemented with copper sulfate ($30 \mu\text{M}$), iron sulfate ($30 \mu\text{M}$), or zinc sulfate ($30 \mu\text{M}$) and photographed after 2 weeks. **B**, the root length of five seedlings from the experiment performed in **A** were measured and are represented here as the average and corresponding S.D. **C**, average root length of five seedlings from wild type and *COPT1* antisense line A 3-6 grown on MS medium or on the same medium supplemented with 10, 50, and $100 \mu\text{M}$ BCS for a week. The black bars represent wild type, and the striped bars represent *COPT1* antisense line A 3-6 root length.

changes in pollen development in the *COPT1* antisense transgenic plants compared with the controls. Fig. 7A shows a scanning electron micrograph of pollen from *Arabidopsis* wild type plants. Interestingly, although $\sim 1\%$ of the wild type pollen possesses nutritional-induced defects related to pore development (34, 35), all three of the *COPT1* antisense transgenic lines analyzed displayed an enhanced percentage of nutritional-induced pollen abnormalities (Fig. 7, B–D), affecting ~ 10 – 13% of the pollen grains (Table I). To ascertain whether these defects were related to copper depletion during pollen grain development, wild type and antisense plants were grown under standard nutritive conditions for 2 weeks, and before the transition to reproductive phase took place, half were supplied with $10 \mu\text{M}$ copper, whereas the other half remained under standard nutrient conditions. After two more weeks, the pollen was collected from all plants and evaluated by electron microscopy. The percentage of morphological abnormalities in pollen after copper supplementation of *COPT1*

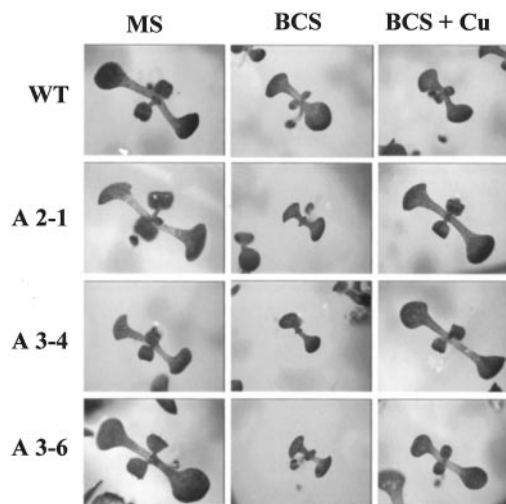


FIG. 6. Effect of copper availability on vegetative growth of wild type and *COPT1* antisense transgenic lines. Seedlings from control WT and *COPT1* antisense lines A 2-1, A 3-4, and A 3-6 were grown on MS medium with 1% sucrose or on the same medium supplemented with either $10 \mu\text{M}$ BCS or $10 \mu\text{M}$ BCS plus $30 \mu\text{M}$ copper sulfate, and a representative plant was photographed after 2 weeks at identical amplification.

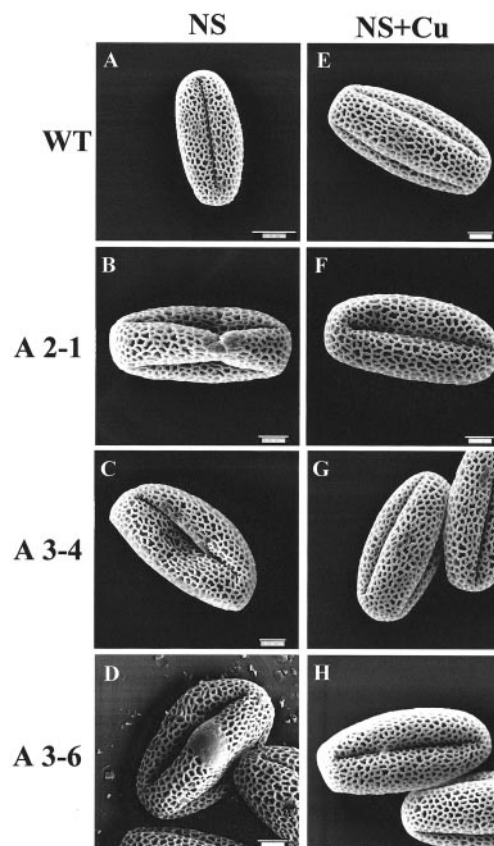


FIG. 7. Pollen grains from wild type and *COPT1* antisense transgenic *Arabidopsis* lines. Pollen was obtained from wild type (A and E) or *COPT1* antisense A 2-1 (B and F), A 3-4 (C and G), or A 3-6 (D and H) flowers at the anthesis stage from plants watered with either standard nutrient solution (NS) (A–D) or the same solution supplemented with $10 \mu\text{M}$ copper (NS+Cu) (D–H). The samples were mounted and photographed in a scanning electron microscope. Representative pictures of either normal grains (A and E–H) or the most common observed defects (B–D) are shown.

antisense plants was considerably reduced (Table I). In addition, the severity of the observed symptoms (Fig. 7, B–D, NS) was alleviated by copper supplementation (Fig. 7, F–H,

TABLE I
Percentage of nutritional-induced defects in pollen grains from wild type and *COPT1* antisense plants

NS, nutritive solution; NS + Cu, nutritive solution supplemented with 10 μ M copper. The data correspond to at least 200 pollen grains counted for each sample at different preparations.

Plants	Nutritionally induced defects	
	NS	NS + Cu
	%	
WT	1.9	1.3
A 2-1	10.4	1.2
A 3-4	11.8	5.7
A 3-6	12.9	0.8

NS+Cu), indicating that copper largely restored the observed pollen morphology defects in *COPT1* antisense plants. These observations support a role for copper delivery in normal pollen development.

DISCUSSION

Based on its characteristics as a copper transporter in a yeast mutant defective in high affinity copper uptake (26, 29) and the data in plants presented here, we conclude that *COPT1* plays an important physiological role in copper acquisition in *Arabidopsis*. Our localization data indicate that *COPT1* is expressed in embryos, trichomes, stomata, pollen, and root tips. Furthermore, our characterization of *COPT1* antisense plants strongly indicates that *COPT1* participates in copper acquisition and accumulation, is required for growth under copper-limiting conditions, and regulates root elongation and pollen development.

Inspection and *in silico* analysis of the *COPT1* promoter region shows several putative cell type-specific boxes that could account for the observed *COPT1*-driven GUS spatial expression pattern. There are two copies of the ACACNNG element (at -7 and -425) that confers embryo-specific expression and are necessary for transcriptional responses to ABA in the *Dc3* gene promoter from *Daucus carota* (36). A TAAAG element, which is sufficient to drive specific expression of the potassium transporter KST1 to potato guard cells (37), is present at position -309 in the *COPT1* promoter. Moreover, the element AAATGA is repeated four times in the *COPT1* promoter at positions -89, -452, -810, and -886. This element was identified in the promoter of the tobacco *NTP303* and *Arabidopsis* *AVP1* genes specifically expressed in pollen (38, 39).

Regarding *COPT1* function in these cell types, copper has been shown to optimize plant regeneration yield during *in vitro* culture of several explants (40, 41). Although the molecular mechanisms underlying these observations remain unclear, copper has been postulated to function in extracellular oxidation processes (42), which may play a key role in the early stages of development and cell proliferation. *COPT1* expression in embryos supports this hypothesis and may also explain the difficulty of symplasmic copper transport perhaps because of the immature plasmodesmata at this stage. On the other hand, the *COPT1* expression in trichomes observed here could indicate a role for these structures in copper detoxification, as suggested for other metals (43-45). Moreover, *COPT1* expression within specific cells along the plant, such as guard cells and pollen, suggests a symplasmic pathway for copper transport throughout the adult plant. These cells, excluded from the plant symplasmic continuum, could be forced to acquire copper from the surrounding apoplast. This expression pattern and the yeast complementation data (29) support a role for *COPT1* as a high affinity copper transporter localized to the plasma membrane, and experiments are underway to test this hypothesis. Furthermore, abundant *COPT1* expression may reflect the impaired ability to acquire copper from the surrounding

media, perhaps because of a tenacious complex formed by copper with cell wall chelators. In this sense, cellulose has been shown to efficiently adsorb copper (46), suggesting that the plant apoplast may not be an efficient pathway for copper transport in plants.

Our results showing pollen morphological abnormalities in *COPT1* antisense plants indicate that pollen development is one of the most sensitive processes to copper depletion in the whole plant, and they are in agreement with the classically described symptoms of male sterility provoked by pollen defects in plants grown in copper deficient soils (34, 35, 47). Furthermore, these results, and our observation that *COPT1* is expressed in pollen, point to *COPT1* as a copper transporter involved in pollen development.

Pollen wall development takes place from both the sporophyte and gametophyte during anther development (reviewed in Ref. 48). In this sense, anthers from plants grown under copper-deficient conditions have been shown to develop both abnormal tapetum and microspores (35). Based on our results, defective *COPT1* expression in pollen cells could be responsible for the observed defects in exine sculpturing, supporting the role of the gametophyte in this process. Defects in pollen sculpturing in the transverse axis near the aperture could be produced by defective dissolution of the callose walls surrounding the microsporogenous cells. Several cytoplasmic male sterile petunia lines have been described to occur as a consequence of defective appearance of callase activity, which is the enzyme that liberates the tetrad of microspores after the completion of meiosis. In this sense, the premature dissolution of the microsporocyte callose wall, as a result of expressing a modified β -1,3-glucanase in the tapetum, reduced male fertility in transgenic tobacco (49). However, we cannot eliminate the possibility that these defects could be due to the general low copper levels in the *COPT1* antisense plant. Thus, copper could reach anthers symplastically, because we could not detect *COPT1* expression in this tissue, affecting exine pattern through the sporophytic tissues. If this were the case, high *COPT1* expression at the pollen grain would be independent of the observed phenotype and could reflect the low copper availability in the anther locule surrounding the pollen grain. The fact that pollen-specific isoforms of cuproproteins, such as ascorbate oxidase and a ragweed allergen (Ra3, homologue to blue proteins), completely lack the otherwise conserved copper-binding boxes of their respective families (50, 51), suggests that mature pollen grains could be copper-deprived cells. The low copper content of commercial pollen further points to this possibility (52). The fact that copper supplementation largely re-established normal pollen morphology in *COPT1* antisense plants further underscores the role of copper in pollen development.

Because it was previously reported that *COPT1* mRNA is not detected in roots, the possibility of it being involved in copper uptake from the soil was not considered (26). RT-PCR demonstrated that, although at low levels, *COPT1* is indeed expressed in roots (29). In this work, *COPT1* expression in roots is not only corroborated in transgenic plants where the *COPT1* promoter drives GUS expression, but it is also assigned to specific peripheral cells in a limited narrow root apical zone. This restricted expression in roots could explain undetectable *COPT1* levels in Northern blotting analysis of total mRNA from complete roots (26). The fact that *COPT1* is expressed in root tips prompted the question of whether *COPT1* in this region plays a role in copper uptake from the soil. To address this, we constructed and analyzed several independent antisense *COPT1* transgenic plants. The results shown in this report that demonstrate reduced ^{64}Cu transport rates in seedlings from three independent *COPT1* antisense transgenic

lines compared with the controls strongly suggest that COPT1 functions as a copper transporter in *Arabidopsis* and participates in copper uptake from the surrounding medium. Furthermore, the lower copper content in the antisense plants, as measured by atomic absorption spectroscopy, further supports the role of COPT1 in copper accumulation and in global copper distribution within the plant.

The partial reduction of copper uptake by ~40% (Fig. 4A) can be explained both as an effect of the incomplete repression of COPT1 in the antisense plants and/or the participation of other metal transporter(s) in copper uptake. With respect to other Ctr-like transporters being involved in copper uptake, COPT1 and COPT2 share a similar expression pattern and copper responsiveness (29), raising the possibility of their partial functional redundancy. The observed up-regulation of COPT2 in our COPT1 antisense plants may partially restore copper uptake, thereby alleviating the reduced levels of copper transport in these plants and consequently the resulting phenotype. Further experiments on the expression and function of the other *Arabidopsis* COPT family members will be important to delineate their specific contributions to plant copper uptake and distribution.

The fact that GUS expression driven by the COPT1 promoter and ⁶⁴Cu uptake experiments were performed in seedlings at identical growth conditions points to the specific GUS-stained root apical zone cells as candidates for copper incorporation into the plant via COPT1. Considering the cellular COPT1 expression pattern, abundant in epidermal and cortex cells, and that at this seedling region cells are connected through plasmodesmata (53), copper incorporation through this specific area seems to be a mixed system from both symplasmic and apoplastic pathways. Both routes could act together until copper reaches the stele where it could then be distributed to the rest of the plant organs. Although the iron transporter IRT1 is extensively expressed in *Arabidopsis* roots (54), other elements, such as calcium, have been shown to use root apical zones for entrance to the plant (55). The advantages of using the root apical zone for metal absorption could be due to a better control of nutrient uptake by regulation of the expression of the corresponding transporter at this restricted area or to the absence of the Casparian band barrier at the root apical area, which may facilitate the entrance to the stele. The fact that the extraordinarily elongated root growth exhibited by COPT1 antisense plants could be partially mimicked in wild type plants grown on BCS medium and that this phenotype is specifically reverted by copper addition further supports a direct role for COPT1 in copper nutrition. Interestingly, a mutant impaired in directional root growth is affected in a protein related to the multiple copper oxidase family (56), suggesting a role of copper in root growth.

Taken together, our results fit a model where COPT1 plays a key role in copper uptake from the surrounding medium in *Arabidopsis* cells. In a previous work, we have postulated that symplasmic copper transport could take place through mature plasmodesmata where copper would be guided by the copper chaperone CCH, which possesses a plant-specific domain perhaps involved in that process (14). The COPT1 expression pattern seems to delineate cells where symplasmic copper transport could be impossible either by the absence of symplasmic connections or by the immature stage of plasmodesmata, as seen in rapidly growing tissues. However, we are aware that the observed phenotype is attributable to COPT1 antisense expression and that a direct implication of COPT1 in these processes must be ascertained in *copt1* null mutant plants. In this sense, a preliminary analysis of a *copt1* knockout line reveals a similar phenotype with regards to sensitivity to cop-

per chelators and root growth in these plants.² Further studies of the COPT family members and of plant mutants completely devoid of one or more COPT genes will shed additional light on the role of COPT proteins in copper acquisition in early developmental stages and plant growth.

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