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Identification of a Functional Homolog of the Yeast Copper Homeostasis Gene *ATX1* from Arabidopsis[1]

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A cDNA clone encoding a homolog of the yeast (*Saccharomyces cerevisiae*) gene *Anti-oxidant 1* (*ATX1*) has been identified from Arabidopsis. This gene, referred to as *Copper CHaperone* (*CCH*), encodes a protein that is 36% identical to the amino acid sequence of ATX1 and has a 48-amino acid extension at the C-terminal end, which is absent from *ATX1* homologs identified in animals. *ATX1*-deficient yeast (*atx1*) displayed a loss of high-affinity iron uptake. Expression of *CCH* in the *atx1* strain restored high-affinity iron uptake, demonstrating that *CCH* is a functional homolog of *ATX1*. When overexpressed in yeast lacking the superoxide dismutase gene *SOD1*, both *ATX1* and *CCH* protected the cell from the reactive oxygen toxicity that results from superoxide dismutase deficiency. *CCH* was unable to rescue the *sod1* phenotype in the absence of copper, indicating that *CCH* function is copper dependent. In Arabidopsis *CCH* mRNA is present in the root, leaf, and inflorescence and is up-regulated 7-fold in leaves undergoing senescence. In plants treated with 800 nL/L ozone for 30 min, *CCH* mRNA levels increased by 30%. In excised leaves and whole plants treated with high levels of exogenous $CuSO_4$, *CCH* mRNA levels decreased, indicating that *CCH* is regulated differently than characterized metallothionein proteins in Arabidopsis.

Copper, a plant micronutrient, acts as an effective electron acceptor and donor in the active sites of many proteins involved in oxidation and reduction reactions (for review, see Marschner, 1995). These include electron-transport proteins such as Cyt oxidase and proteins involved in the detoxification of oxygen radicals such as copper/zinc SOD. A variety of enzymes with oxidase function, including ascorbate oxidase, diamine oxidase, and phenol oxidase, require copper for their activity.

Despite its importance to plant metabolism, copper is toxic at high concentrations. Copper toxicity can be oxygen dependent through the Haber-Weiss reaction, which generates reactive oxygen intermediates (Halliwell and Gutteridge, 1989), or oxygen independent by inappropriate binding to biomolecules (Kalstrom and Levine, 1991). Organisms have evolved various metal homeostasis factors to control the cellular accumulation, distribution, and sequestration of the metal (Vulpe and Packman, 1995; Koch et al., 1997). In the yeast *Saccharomyces cerevisiae* there is an overlap between systems controlling copper-ion homeostasis and oxygen radical metabolism. In yeast, copper-binding metallothioneins protect not only against copper toxicity but also detoxify the superoxide anion (Tamai et al., 1993). Similarly, the copper/zinc SOD1 contributes to both the maintenance of copper homeostasis and to superoxide scavenging (Culotta et al., 1995).

The product of the yeast gene *Anti-oxidant 1* (*ATX1*) shows a similar overlap between copper homeostasis and oxygen radical metabolism. The *ATX1* gene was originally isolated in strains lacking *SOD1* by its ability to suppress oxygen toxicity in a copper-dependent manner (Lin and Culotta, 1995). *ATX1* encodes a soluble copper chaperone. ATX1 binds Cu(I) in the cytoplasm and delivers it to a copper transporter in the membrane of a post-Golgi vesicle. In the vesicle, the copper is inserted into a multicopper oxidase essential for high-affinity iron uptake (Lin et al., 1997; Pufahl et al., 1997). Thus, ATX1 may be involved in both copper transport and defense against oxidative stress. HAH1, the human homolog of ATX1, demonstrates both of these functions, with the amino acid residues involved in antioxidant function separate from the copper-binding region (Hung et al., 1998).

Here we describe the identification of the gene *Copper CHaperone* (*CCH*) from Arabidopsis encoding a 121-amino acid protein with sequence similarity to *ATX1*. By expressing *CCH* in yeast, we show that *CCH*, like *ATX1*, can protect *SOD1*-deficient yeast from active oxygen tox-

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Abbreviations: BCS, bathocuproinedisulfonic acid; DAG, days after germination; SOD, superoxide dismutase.

icity. Moreover, *CCH* expression can also restore high-affinity iron uptake to *ATX1*-deficient yeast, indicating that *CCH* and *ATX1* are functional homologs. Thus, the gene product of *CCH* may be involved in both the detoxification of active oxygen and the delivery of copper. Whereas there is basal *CCH* mRNA expression in many Arabidopsis tissues, *CCH* mRNA levels increase during leaf senescence, suggesting a role for the *CCH* gene product in that process.

MATERIALS AND METHODS

Plant Growth and Determination of Nutrient Content

Seedlings of Arabidopsis ecotype Columbia (Col) and Landsberg *erecta* (Ler) were transplanted to a commercial potting mix (Fafard Germination Mix, Agawam, MA) and grown at 24°C. Ler plants were grown with continuous illumination of approximately 100 μmol/m^2 of cool-white fluorescent light. Col plants were grown with a photoperiod of 16 h of light/8 h of dark in growth cabinets with 65 μmol/m^2 of cool-white fluorescent light.

Sequence Analysis

A partial amino acid sequence of SAM45 (Crowell and Amasino, 1991) was entered into the database search program BLASTN (National Institutes of Health, Bethesda, MD). The Arabidopsis cDNA 31B12T7 (DBEST accession no. T04721; isolated by the Arabidopsis Expressed Sequence Tag Project Group [Newman et al., 1994]) revealed high homology to SAM45 and was ultimately named *Copper CHaperone* (*CCH*). The nucleotide sequence of the *CCH* cDNA clone was determined by cycle sequencing on an automated sequencer (Applied Biosystems). The clone was sequenced completely from both the 5′ and 3′ ends. Sequences related to *CCH* were identified using the program BLASTN. Alignments were generated by the Genetics Computer Group software package (program BESTFIT, Madison, WI). Predictions of secondary structure were determined using the EMBL-Heidelberg data bank and the Genetics Computer Group software.

Yeast Strains and Vectors

The yeast (*Saccharomyces cerevisiae*) strains used in this study were: KS107 (*sod1*Δ) (Culotta et al., 1995) and SL215 (*atx1*Δ derivative of YPH250) (Lin et al., 1997). SL201 (*sod1*Δ,*ctr1*Δ) was constructed by introducing a *ctr1*Δ::*LEU2* deletion (Dancis et al., 1994) into KS107.

To create a vector to express *CCH* in yeast, the *CCH* coding sequence was amplified from expressed sequence tag cDNA clone 31B12T7 (Newman et al., 1994); the upstream primer for amplification was designed such that an *Eco*RI site was generated 3 bases upstream of the *CCH* start codon (5′-AAGAATTCGCCATGGCTCAGACCG-3′). The downstream primer for amplification was the SP6 primer (Promega). The 3′ end of the amplified fragment contained a *Bam*HI site downstream of the poly(A^+) tail of the *CCH* cDNA. The *CCH* coding region was directionally subcloned into the *Eco*RI and *Bam*HI sites of the yeast expression vector pSM703 (Culotta et al., 1995) to create vector pB-038 for *CCH* expression in yeast. Other plasmids used were p413-A1 (expression of *ATX1*) (Lin et al., 1997) and pRS413 (the expression vector used to create p413-A1 with no insert) (Sikorski and Hieter, 1989).

Complementation of Yeast by *CCH*

The *atx1*Δ strain SL215Δ was transformed with p413-A1, pRS413, pSM703, or pB-038 as described previously (Lin et al., 1997). To test for restoration of iron uptake in this strain, transformants were grown on complete synthetic dextrose medium (Rose et al., 1991) or synthetic dextrose medium buffered with 50 mM Na-Mes, pH 6.1, and 3 mM ferrozine (ferrozine(3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine); Sigma) with or without 50 μM ferrous ammonium sulfate (Sigma) for 5 d at 30°C.

pB-038 and p413-A1 were transformed separately into KS107. To test for reversal of Lys and Met auxotrophy of this SOD1-deficient yeast strain, transformants were plated on complete synthetic dextrose medium, synthetic dextrose medium without Lys, and synthetic dextrose medium without Met, and grown in air for 3 d at 30°.

To determine whether *CCH* action was copper dependent, strain KS107 was transformed with either pSM703 (an empty expression vector) or pB-038 (a CCH expression vector). These were tested for oxygen tolerance by aerobic growth in yeast extract-peptone-dextrose liquid medium as described previously (Lin and Culotta, 1995). Anaerobic growth was tested as described (Liu et al., 1992). Where indicated, cultures were supplemented with 150 μM BCS or 150 μM BCS plus 50 μM $CuSO_4$. After 18 h, A_{600} was determined for each culture.

Ozone Treatment

Three- to 4-week-old plants (ecotype Col) grown under described conditions were moved into 500-L ozone fumigation chambers located inside growth cabinets. Ozone (800 nL/L) was generated by an ozonifier (model Eco-Lab.ppm, Eco Ozono, Valencia, Spain) and monitored continuously using an ozone analyzer (model 1180, Dabisi, Environment Corp., Glendale, CA). Rosettes were harvested and frozen in liquid nitrogen at the indicated times and stored at −80°C until RNA isolation.

Metal Treatment

The fifth and sixth leaves of Arabidopsis were removed 16 DAG and placed in Murashige and Skoog liquid medium (Murashige and Skoog, 1962) with or without 50 μM $CuSO_4{\cdot}5H_2O$ (Mallinckrodt, Chesterfield, MO). Leaves were incubated in continuous light at 24°C for 18 h with gentle shaking. Total RNA was extracted from the leaves as described below.

Three- to 4-week-old Arabidopsis plants (ecotype Col) were removed from soil and their roots were submerged in 1 mM $CuSO_4$ solutions for 0.25, 0.5, 1, 2, 4, or 8 h. After treatment, shoots (primarily rosette tissue, no roots) were harvested for nutrient analysis or for mRNA extraction.

Copper uptake was determined by atomic absorption spectroscopy on 10 mg of lyophilized Arabidopsis plants treated with nitric acid at 80°C overnight and diluted one-third with water.

RNA Isolation and Gel-Blot Analyses

Total RNA was isolated from Arabidopsis plants (ecotype Col) as described by Prescott and Martin (1987). Total RNA was isolated from the fifth and sixth leaves (excluding cotyledons) of Arabidopsis (ecotype Ler). Total RNA was purified from leaf samples using an RNA isolator (Genosys Biotechnologies, The Woodlands, TX) following the manufacturer's instructions. Total RNA was quantified by spectrophotometry and by 18S rRNA abundance as visualized by ethidium bromide staining on agarose gels and by hybridization to a radiolabeled 18S rRNA probe. For RNA blots, equal amounts of RNA were separated by denaturing-agarose gel electrophoresis and transferred to a nylon membrane as described previously (Sambrook et al., 1989). RNA blots were probed with [^{32}P]ATP-labeled cDNA probes and analyzed using a phosphor imager (Molecular Dynamics, Sunnyvale, CA), a radioanalytical imaging system (InstantImager 2024, Packard, Canberra, Australia), or by exposure to x-ray film at −80°C with an intensifying screen.

RESULTS

Identification of the *CCH* Gene

We have previously isolated a gene, *SAM45*, which is up-regulated at the mRNA level in soybean cell cultures that are cytokinin depleted (Crowell and Amasino, 1991). Cytokinins are known to prevent leaf senescence, and removal of cytokinin from cell cultures causes some symptoms of senescence. We identified an Arabidopsis homolog of *SAM45* in the expressed sequence tag collection (accession no. 21536) (Newman et al., 1994) with 97% identity to SAM45 at the amino acid level. The 585-bp cDNA clone contained an open reading frame capable of encoding a 121-amino acid polypeptide with a molecular mass of 13 kD and a pI of 4.9 (Fig. 1A). The reading frame shown was the only possible open reading frame in the cDNA and had both a start and stop codon, indicating that the sequence contained the complete coding region. Similar sequences were identified from *S. cerevisiae* (*ATX1*; Lin and Culotta, 1995) and from rice (OsATX1; accession no. DBEST 70798). *ATX1* is involved in copper trafficking (Lin et al., 1997), and because the Arabidopsis gene corresponding to cDNA 31B12T7 has a similar function (see below), we refer to it as *Copper Chaperone*. DNA-blot analysis indicated that there was a single copy of *CCH* in the Arabidopsis genome (data not shown).

The alignment of ATX1 with the predicted sequence of CCH and other similar amino acid sequences is shown in Figure 1B. The amino acid sequence of CCH and ATX1 are 36% identical and 54% similar across the length of ATX1. CCH exhibits the same degree of similarity to ATX1 homologs from mouse (ATOX1) and human (HAH1). CCH is

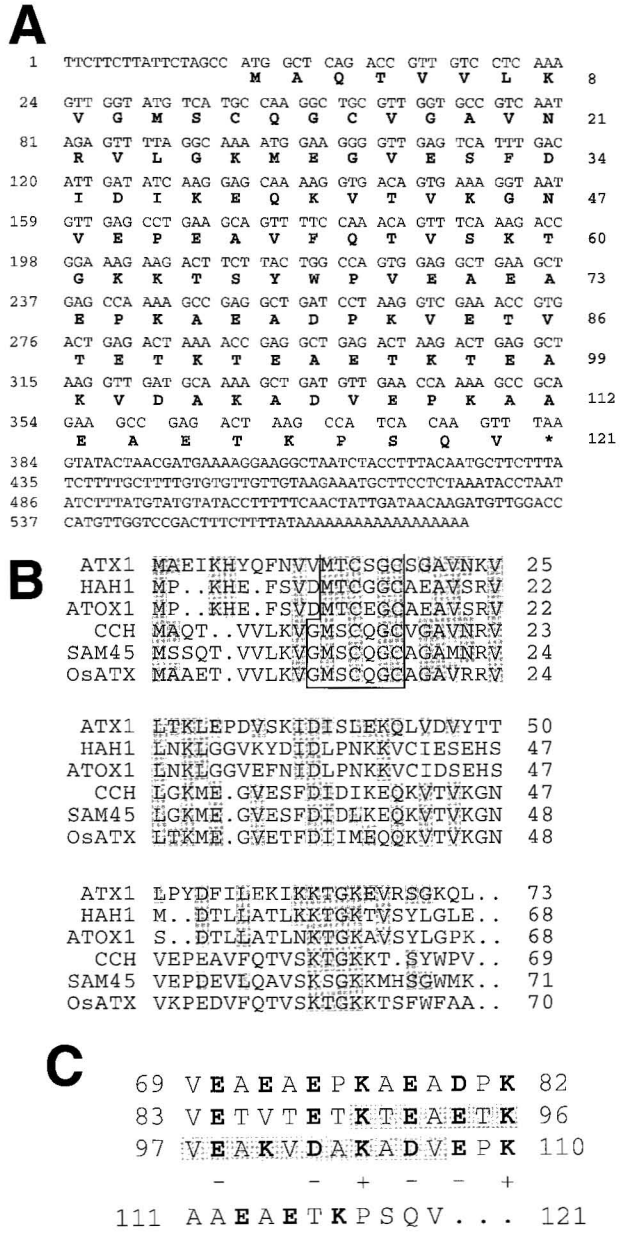

Figure 1. CCH sequence analysis. A, Nucleotide and predicted amino acid sequences for the *CCH* cDNA. B, Alignment of yeast ATX1 with the predicted amino acid sequence of HAH1 (human), ATOX1 (mouse; accession no. AF004591), and the 69 N-terminal amino acids of CCH (Arabidopsis), SAM45 (soybean), and rice ATX1 (OsATX1; accession no. DBEST 70798). Conserved residues in the alignment are shaded. The putative metal-binding domain is boxed. C, Amino acid sequence features of the C-terminal region of CCH. Charged residues are shown in boldface and their charges are indicated below them. Secondary-structure prediction suggested that an α-helix could be formed in the shaded region.

67% identical to a potential ATX1 homolog from rice (OsATX1). Both the CCH and OsATX1 polypeptides conserve the motif GMXCXXC (boxed in Fig. 1B), which is also described at the N-terminal region of copper-pumping ATPases such as CopA from *Enterococcus hirae* and the human Menkes and Wilson disease gene products (Solioz

Figure 2. CCH restored high-affinity iron uptake to *atx1* mutant yeast. The *atx1* mutant strain SL215 was transformed with vector p413-A1 (*ATX1* expression), pRS413 (expression vector, negative control), pB-038 (*CCH* expression), or pSM703 (expression vector, negative control) and streaked on plates in the positions indicated. All plates contained ferrozine, an iron chelator. Plates contained synthetic dextrose medium plus 3 mM ferrozine (SD+fer) or synthetic dextrose medium plus 3 mM ferrozine plus 50 μM ferrous ammonium sulfate (SD+fer+Fe). Plates were photographed after incubation at 30°C for 4 d.

et al., 1994). This sequence is similar to the more general heavy-metal-binding motif (MTCXXC) found in ATX1 and other metal-binding proteins (Lin and Culotta, 1995). This motif has been shown to bind copper (Pufahl et al., 1997).

CCH extended 48 amino acids beyond the C-terminal end of ATX1; this domain contained 44% charged amino acids, many of which were separated by a single nonpolar amino acid (Fig. 1C). The alternating opposite-charged amino acids observed within the CCH C-terminal region suggests that an α-helix could be formed from residues 83 to 100, with a spatial distribution of basic amino acids on one side and acidic amino acids on the other. Thus, CCH may be composed of two different domains, an N-terminal region involved in copper binding and the highly charged C-terminal region, which may be involved in interactions with other molecules.

Complementation of *atx1* and *sod1* Mutant Yeast Strains

ATX1 is involved in the transport of copper ions to the secretory pathway, where the copper is made available to a copper-dependent oxidase essential for high-affinity iron uptake at the plasma membrane (Pufahl et al., 1997; Valentine and Gralla, 1997). Thus, the *atx1* mutant strain is deficient in high-affinity iron uptake. In the presence of the iron chelator ferrozine, yeast cells acquire iron through the high-affinity uptake pathway. Thus, *atx1* mutants cannot grow on ferrozine-containing medium unless supplemented with excess iron (Lin et al., 1997). To determine if CCH could replace the function of ATX1 in high-affinity iron uptake, *CCH* was expressed in an *atx1* mutant strain. The complete open reading frame of *CCH* was inserted into a multicopy vector, allowing constitutive expression of *CCH* in yeast. Transformants were plated on medium with and without supplemental iron (Fig. 2). Strains expressing either *ATX1* or *CCH* grew in the absence of supplemental iron, demonstrating that high-affinity iron uptake was restored by expression of either *ATX1* or *CCH*. Although restored growth on ferrozine was slow, the growth rate was comparable to that of the wild type (data not shown).

ATX1 was identified by its ability to protect against oxygen toxicity when expressed in a yeast strain lacking the SOD genes *SOD1* and *SOD2* (Lin and Culotta, 1995). Phenotypes of *SOD1* deficiency in aerobically grown yeast include retarded growth rate and auxotrophy for Lys and Met (Liu et al., 1992). The synthetic pathways for these amino acids contain steps that are hypersensitive to reactive oxygen (Liu et al., 1992; Slekar et al., 1996). In the *sod1* mutant, *ATX1* expressed from a multicopy expression vector can restore Lys and Met biosynthesis, allowing the transformed strain to grow without supplemental Lys or Met (Lin and Culotta, 1995).

To determine whether CCH could restore Lys and Met synthesis to aerobically grown, SOD-deficient yeast, we expressed *CCH* in the mutant strain (Fig. 3). The untransformed SOD-deficient yeast could grow on complete me-

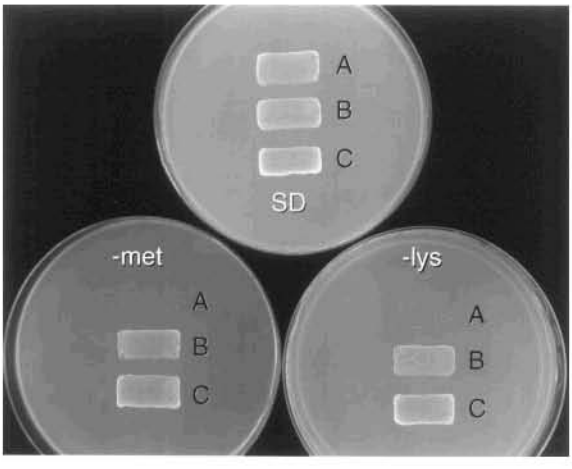

Figure 3. CCH can restore Lys and Met synthesis in aerobically grown *sod1* mutant yeast. Strain KS107 (*sod1*) was plated after transformation with no expression vector (A, negative control), pRS-A1 (B, *ATX1* expression), or pB-038 (C, *CCH* expression). Plates contained complete medium (SD) or medium lacking Met (−met) or Lys (−lys). Plates were photographed after aerobic growth at 30°C for 2 d.

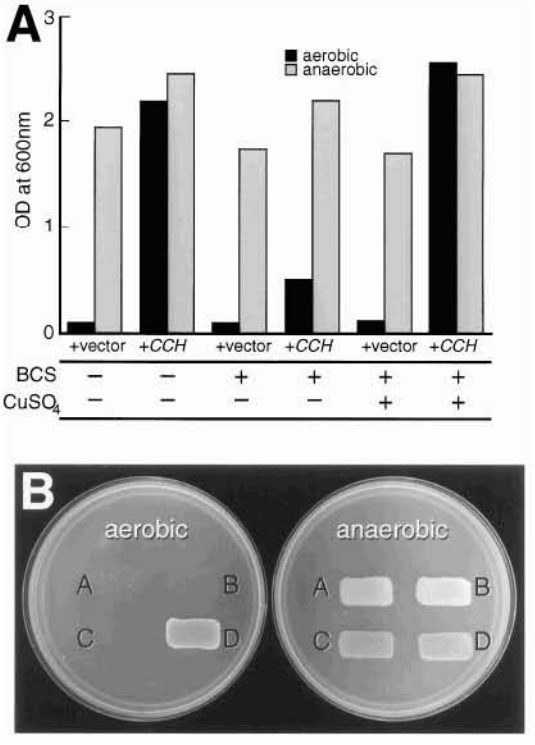

Figure 4. CCH rescued *sod1* mutant yeast in a copper-dependent manner. A, Strain KS107 (*sod1*Δ) was transformed with the vector pSM703 (+vector) as a negative control or pB-038 (+*CCH*) as the *CCH* expression vector. Yeast was grown in yeast extract-peptone-dextrose medium alone or yeast extract-peptone-dextrose plus 150 μM BCS with or without 50 μM $CuSO_4$. A_{600} was determined after yeast was grown in either aerobic or anaerobic conditions for 18 h beginning at A_{600} = 0.01. B, A, SL201 (*sod1*Δ,*ctr1*Δ) plus pSM703 (empty expression vector); B, KS107 (*sod1*Δ) plus pSM703; C, SL201 plus pB-038 (*CCH* expression); and D, KS107 plus pB-038 yeast were grown in aerobic or anaerobic conditions for 2 d on synthetic dextrose medium lacking Lys.

dium but failed to grow in the absence of Lys or Met. The same strain expressing either ATX1 or CCH could grow with Lys and Met absent from the medium, indicating that *CCH* expression in aerobically grown SOD-deficient yeast protects the cells from oxygen toxicity to the extent that Lys and Met synthesis are restored. Thus, CCH function can replace ATX1 function in this strain.

To determine whether *CCH* protects *sod1* yeast in a copper-dependent manner, a *sod1* strain transformed with the *CCH* expression construct was grown in the presence of BCS, a copper chelator (Fig. 4A). BCS chelated copper in the growth medium, making it unavailable to the growing cells. In the absence of BCS, *CCH* expression allowed *sod1* yeast to grow in aerobic conditions at a rate similar to that of *sod1* yeast grown in anaerobic conditions. When free copper was chelated by BCS, the growth of the *CCH*-expressing *sod1* cells in aerobic conditions was inhibited and was similar to that of *sod1* cells transformed with the vector only. When excess $CuSO_4$ was added to medium containing BCS, copper became available to the cells; under these conditions, growth was restored to *CCH*-expressing *sod1* yeast in aerobic conditions. As additional evidence for the requirement of copper in CCH function, *CCH* was expressed in *sod1*,*ctr1* double mutants. In these mutants the absence of *CTR1*, a copper transporter at the plasma membrane, prevents uptake of copper to the cytoplasm (Dancis et al., 1994). *CCH* expression was unable to restore Lys synthesis in *sod1*,*ctr1* double mutants (Fig. 4B). These results indicate that the ability of CCH to protect *sod1* yeast from reactive oxygen toxicity is dependent on copper availability.

CCH Gene Expression

RNA-blot analysis established that *CCH* mRNA was expressed in the root, stem, leaf, inflorescence, and silique (not shown). To determine the expression of *CCH* during leaf senescence, total RNA was isolated from the fifth and sixth leaves of Arabidopsis at 16, 23, and 28 DAG (Fig. 5). In this population, leaves at 16 DAG were not fully expanded, leaves at 23 DAG were fully expanded but showed no yellowing, and leaves at 28 DAG were, by visual estimation, 50% yellow and clearly undergoing senescence. In these experiments, *CCH* mRNA levels increased by more than 7-fold after the onset of leaf senescence. The senescence-associated gene *SAG12*, which is expressed in a highly senescence-specific manner, and the chlorophyll *a*/*b*-binding protein CAB, which is rapidly down-regulated during senescence, serve as positive and negative controls for leaf senescence (Lohman et al., 1994).

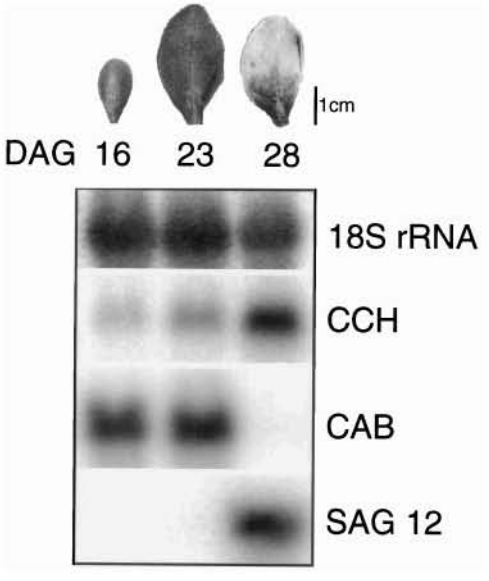

Figure 5. CCH mRNA was up-regulated during leaf senescence. The fifth and sixth leaves of Arabidopsis plants (ecotype Ler) were removed at 16, 23, or 28 DAG, and total RNA was extracted and used for RNA-blot analysis (leaf photographs are representative of leaves at each time point). RNA blots were created with equal amounts of each RNA sample, as determined by spectrophotometry and ethidium bromide staining. Equal loading was confirmed by detection of 18S rRNA with a radiolabeled probe. RNA blots were probed with [^{32}P]ATP-labeled cDNA probes for 18S rRNA (control for equal loading on the RNA blot), *CCH* cDNA, chlorophyll *a*/*b*-binding protein (CAB), and the senescence-associated gene (SAG 12).

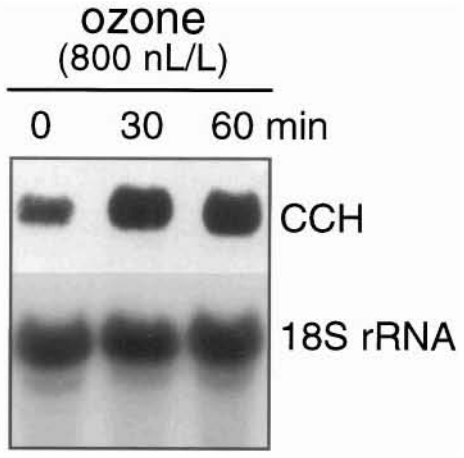

Figure 6. CCH mRNA levels increased after treatment with ozone. Arabidopsis plants (3 to 4 weeks old) were subjected to 800 nL/L ozone treatment for 0, 30, or 60 min in ozone fumigation chambers. Ten micrograms of total RNA was used for RNA-blot analysis and probed with either *CCH* cDNA or 18S rRNA.

Response to Ozone

The expression of *ATX1* is induced by oxidative stress (Lin and Culotta, 1995). To determine whether *CCH* was similarly induced, 3- to 4-week-old Arabidopsis plants were treated with high ozone concentrations (800 nL/L) for 30 or 60 min and total RNA from the aerial tissues was analyzed for *CCH* expression by RNA-blot analysis (Fig. 6). After 30 min of treatment a more than 30% increase in *CCH* mRNA was observed. Longer exposures did not increase the level of *CCH* transcripts. This modest induction of the *CCH* message by ozone treatment was observed in three separate trials.

Response to Copper Treatment

To determine whether *CCH* is regulated by copper, the fifth and sixth leaves of Arabidopsis plants at 16 DAG were removed and incubated in liquid medium with or without 50 μM $CuSO_4$ for 18 h. Total RNA isolated from these leaves was used to generate RNA blots (Fig. 7A). *CCH* mRNA levels decreased by more than 5-fold during this treatment, suggesting that *CCH* is down-regulated at the mRNA level by high levels of exogenous copper. Separately, as a control for copper treatment, mRNA levels of *MT1* increased in copper-treated leaves, as has been observed previously (Zhou and Goldsbrough, 1994). These results indicate that the *CCH* message is regulated differently than the message of a copper-binding metallothionein, at least when nonphysiologically high levels of copper are applied.

To determine if copper could down-regulate *CCH* expression in intact plants, 3- to 4-week-old Arabidopsis plants were removed from soil and their roots were submerged in 1 mM $CuSO_4$ solutions for different times. Copper transport into the shoot was followed by atomic absorption of nitric acid-digested samples (Fig. 7B). Associated with the accumulation of copper was a decrease in *CCH* mRNA (Fig. 7C). After 30 min, RNA blotting revealed that mRNA levels of *CCH* decreased by 50%. Longer copper treatment further lowered *CCH* levels.

DISCUSSION

We describe the characterization of *CCH* from Arabidopsis. CCH encodes a predicted protein of 121 amino acids

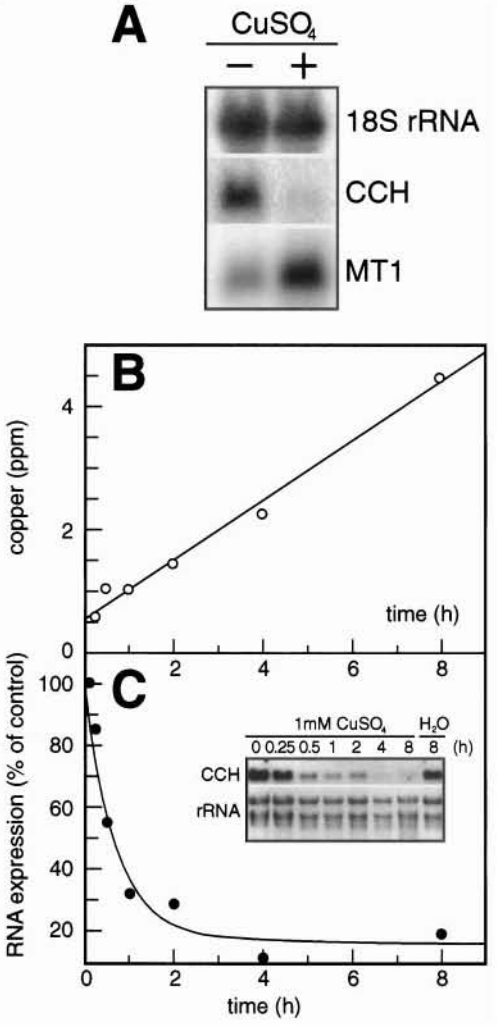

Figure 7. CCH mRNA was down-regulated in response to copper treatment. A, The fifth and sixth leaves were removed from Arabidopsis plants (ecotype Ler) at 16 DAG. Leaves were incubated in light for 18 h with gentle shaking in either Murashige and Skoog medium (−) or Murashige and Skoog medium plus 50 μM $CuSO_4$ (+). Total mRNA was extracted from leaves and used for RNA-blot analysis. Blots were probed with either 18S rRNA (control for equal loading on RNA blot), *CCH*, or *MT1* cDNA. B, Arabidopsis plants (ecotype Col) were removed from soil and their roots submerged in 1 mM $CuSO_4$. Kinetics of copper uptake to the aboveground parts of the plant were measured by atomic absorption at various time points. C, Total RNA was isolated from plants after the treatment described above, as well as from plants treated in parallel with water. RNA blots made from these samples were probed with [^{32}P]ATP-labeled *CCH* (see inset). The graph plots the change in CCH mRNA levels (expressed in cpm on the RNA blot probed with the *CCH* probe) over time of treatment. The 0-h time point was set to 100% and subsequent values were normalized to the 0-h value.

that, in the N-terminal region, is similar in sequence to *ATX1* from yeast and contains the heavy-metal-binding sequence MXCXXC that is common to metal-binding proteins in bacteria, fungi, animals, and plants. The predicted amino acid sequence of CCH includes a highly charged carboxyl domain that could interact with other molecules, perhaps in a copper-dependent manner. The homologs of *ATX1*, *HAH1* (human), and *ATOX1* (mouse) encode proteins of similar length to ATX1, i.e. they do not bear a C-terminal extension (Klomp et al., 1997).

CCH is a functional homolog of *ATX1*. Both *CCH* and *ATX1* can restore Met and Lys biosynthesis to a *SOD1*-deficient strain, indicating that *CCH* and *ATX1* share the ability to protect yeast from some effects of the oxidative damage produced by SOD deficiency. The observation that *ATX1* levels increase in yeast cells challenged with reactive oxygen intermediates is consistent with *ATX1* being involved in a response to reactive oxygen (Lin and Culotta, 1995).

It will be interesting to determine whether the *CCH* gene product has the ability to suppress oxygen toxicity in Arabidopsis mutants lacking SOD in a copper-dependent manner, as has been described for yeast (Culotta et al., 1995; Lin and Culotta, 1995). Also, in the wild type, it would be interesting to determine whether CCH overexpression might protect against the pernicious effects of ozone common in industrialized areas. Levels of *CCH* mRNA increase moderately after ozone exposure, yet it remains to be determined whether this up-regulation is part of a defense against oxidative stress. Because little information exists regarding the interactions between copper metabolism and protection from oxidative stress in plants, the study of *CCH* could clarify the relationship between these two processes. The human homolog of CCH, HAH1, has distinct regions mediating copper delivery and oxidative defense (Hung et al., 1998). CCH shows high homology to HAH in these regions and may retain the same functions.

In the yeast *S. cerevisiae*, ATX1 is part of pathway that links copper transport to iron uptake at the cell surface (Yaun et al., 1995; Stearman et al., 1996; Lin et al., 1997; Valentine and Gralla, 1997). ATX1 interacts with the membrane-bound copper transporter CCC2 to deliver copper to the interior of a post-Golgi vesicle (Pufahl et al., 1997). Ultimately, copper becomes incorporated into a complex capable of reducing iron outside the cell (Stearman et al., 1996). An extracellular mechanism for iron reduction serves to reduce Fe(III), the common extracellular form of iron, to Fe(II) to facilitate uptake. A homologous copper transport pathway has been identified in humans; defects in this pathway are the cause of Menkes and Wilson disease, the symptoms of which result from deficiencies in copper loading of copper-containing proteins such as ceruloplasmin (Yaun et al., 1995; Klomp et al., 1997).

The discovery that the *CCH* gene product can replace ATX1 in the high-affinity iron uptake pathway indicates that CCH can interact with CCC2 to deliver copper to the post-Golgi vesicles of yeast cells. It remains to be determined whether plants express genes encoding functions homologous to those of other members of the yeast high-affinity iron-uptake pathway. Nevertheless, the physiology of iron uptake in plants is consistent with the existence of such a pathway. In many plants uptake of iron requires reduction of Fe(III), the common form of iron in aerated soils, to Fe(II) in the root zone (Marschner, 1995). *CCH* mRNA is expressed at a basal level in the roots, stems, flowers, siliques, and leaves of Arabidopsis. It is not known whether iron reduction is taking place in all of these tissues or, if so, whether CCH is needed for that reduction.

Several metallothioneins that are up-regulated at the mRNA level during leaf senescence have been identified from plants (Buchanan-Wollaston, 1994; Weaver et al., 1997). These metallothioneins are believed to protect the cell by binding free copper ions in the cytoplasm. Their up-regulation during leaf senescence suggests that a copper detoxification function is important in senescing leaf cells. The *CCH* gene product may serve a similar function. However, *CCH* is regulated differently than one senescence-induced metallothionein, *MT1*, in nonsenescent leaves treated with exogenous copper; copper treatment sufficient to induce *MT1* mRNA expression eliminates *CCH* expression at the mRNA level. Thus, if CCH functions as a copper chelator, it does not appear to do so in all situations in which copper toxicity threatens the leaf cell. The yeast *ATX1* message is also not induced in cells treated with exogenous copper (S.-J. Lin and V.C. Culotta, unpublished data).

We have observed in Arabidopsis that *CCH* is up-regulated during leaf senescence, suggesting a possible role for CCH function in copper binding or transport during that process. During senescence nutrients are redistributed from senescing tissue, contributing to growth in other parts of the plant (Noodén and Leopold, 1988). An indication of the importance of copper in plant metabolism is that many plants redistribute copper from leaves before abscission, thus avoiding copper loss to the environment (Mauk and Nooden, 1992; Drossopoulos et al., 1994, 1996; Hocking, 1994). Metals exiting the leaves during senescence are likely to do so by way of the phloem. Recent studies of metal transport in castor bean indicate that copper and iron can move through phloem chelated to organic molecules, in particular the amino acid nicotianamine (Schmidke and Stephan, 1995).

As chloroplasts and their constituent proteins are broken down during senescence, copper is released. As discussed above, the metallothionein *MT1* is up-regulated at the mRNA level during leaf senescence and may be involved in the sequestration of copper released during senescence. Also, *CCH* mRNA is up-regulated during senescence and may be involved in copper sequestration during this process. Furthermore, *CCH*, as a functional homolog of *ATX1*, may be involved in the delivery of copper to the secretory system in preparation for phloem loading and transport from senescing leaves. In Arabidopsis we have observed that copper levels drop by one-half several days after the onset of senescence (E. Himelblau and R.M. Amasino, unpublished data). It will be interesting to determine if plants in which *CCH* expression is eliminated or attenuated during senescence can continue to transport copper from senescing tissue. Also, reactive oxygen intermediates accumulate in leaves undergoing senescence (Noodén and

Leopold, 1988). As described above, *CCH* may encode antioxidant function, suggesting an additional role for *CCH* expression during leaf senescence.

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