Regulation of Copper Homeostasis by Micro-RNA in Arabidopsis*

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Major copper proteins in the cytoplasm of plant cells are plastocyanin, copper/zinc superoxide dismutase, and cytochrome c oxidase. Under copper limited conditions, expression of copper/zinc superoxide dismutase is down-regulated and the protein is replaced by iron superoxide dismutase in chloroplasts. We present evidence that a micro-RNA, miR398, mediates this regulation in Arabidopsis thaliana, by directing the degradation of copper/zinc superoxide dismutase mRNA when copper is limited. Sequence analysis indicated that the transcripts encoding cytosolic copper/zinc superoxide dismutase and COX5b-1, a subunit of the mitochondrial cytochrome c oxidase, are also targeted by miR398. This regulation via miR398 takes place in response to changes in a low range of copper levels (0.2–0.5 μM), indicating that miR398 is involved in a response to copper limitation. On the other hand, another major copper protein, plastocyanin, which is involved in photosynthetic electron flow and is essential in higher plants, was not regulated via miR398. We propose that miR398 is a key factor in copper homeostasis in plants and regulates the stability of mRNAs of major copper proteins under copper-limited conditions.

In plants, copper must be delivered to a number of important enzymes that are active in various subcellular locations (1). One of the most abundant copper proteins in photosynthetic tissues is plastocyanin (PC), which localizes to the thylakoid lumen of chloroplasts and mediates electron transport between the cytochrome b6f complex and photosystem I. Although a heme-containing cytochrome c can serve as an alternative to PC under low copper conditions in certain algae (2), an ortholog that can replace PC function is not found in higher plants (3). Indeed, PC is essential for photosynthetic electron transport in plants (4).

Another major copper enzyme in plants is copper/zinc superoxide dismutase (Cu,Zn-SOD). Superoxide dismutase (SOD) aids in the scavenging of reactive oxygen species (ROS) by converting O$_2^-$ to H$_2$O$_2$ (5). SOD enzymes are classified based on the metal cofactors (5). In green tissues of Arabidopsis the major isoforms of Cu,Zn-SOD localize to the cytoplasm (CSD1) and to the chloroplast stroma (CSD2), whereas the peroxisomal isoform (CSD3) constitutes a minor activity (6). In chloroplasts, another SOD called FSD1, which uses an iron cofactor, can be found. A manganese requiring MnSOD localizes to mitochondria (6). Next to its roles in photosynthesis and superoxide scavenging in chloroplasts, copper is also involved in respiratory electron transport in mitochondria as a cofactor in the cytochrome c oxidase (COX) complex (7). Therefore the allocation of available copper over these two organelles may also be crucial for maintaining an optimum energy budget in a cell.

The copper delivery system to PC and Cu,Zn-SOD in plant cells has been studied in some detail (1). Copper enters the cell by means of the CopT family of transporters (8, 9). A cytosolic form of the copper chaperone for SOD (CCS) delivers copper to CSD1 (10). Two P-type ATPases, PAA1 and PAA2, are required for efficient copper delivery across the plastid envelope and the thylakoid membrane, respectively, in Arabidopsis (11, 12). Once copper is transported into the stroma by PAA1, it can be transferred to the stromal form of CCS, which can target it to stromal CSD2 (10, 13). On the other hand, stromal copper can also be transferred to PC via PAA2.

In illuminated chloroplasts, the possible one electron reduction of oxygen at PSI results in the generation of superoxide (14). The potential damage caused by superoxide or by superoxide-derived ROS is minimized by the combined action of stromal Fe-SOD or Cu,Zn-SOD enzymes, which convert superoxide to oxygen and peroxide, and by stromal ascorbate peroxidases, which convert the formed peroxide into water. Despite the presence of these ROS scavenging systems, the chloroplast is a cell organelle, where plants encounter a high risk of generating damaging ROS. Whereas copper delivery is important for energy metabolism and superoxide scavenging, excess copper is toxic and growth of plants on tissue culture media containing copper above 20 μM causes visible damage to plants (15). Free copper ions may participate in the Fenton reaction, resulting in the production of highly reactive and damaging hydroxyl radicals. However, the risk of free copper ions may be minimized by a strict regulation of copper delivery in a cell. For example, the free copper concentration was estimated to be less than 10$^{-18}$ M.
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in a yeast cell, whereas the total copper concentration is \(~70 \mu M\) (16).

In plants, the chloroplast is particularly sensitive to copper toxicity, which is illustrated by the phenotypes of mutants in genes that encode chloroplast copper transporters. The sensitivity to excess copper is reduced in the Arabidopsis paa1 mutant, in which copper delivery to the stroma is severely impaired (11, 12). In contrast, paa2, in which copper is not delivered to the thylakoid lumen, is hypersensitive to high copper concentrations (12).

Major copper proteins such as PC and Cu,Zn-SOD may act as copper sinks under conditions where copper supply is in excess. Therefore, copper homeostasis may involve the regulation of the abundance of these copper sinks. Previously, it was shown that the levels of three copper proteins, CSD1, CSD2, and to a lesser extent PC increase in response to elevated copper concentrations (12). The abundance of CSD1 and CSD2 was found to be regulated in response to copper availability at the mRNA abundance level. Whereas CSD1 and CSD2 expression was shut off when copper is limiting, the iron-requiring FSD1 was up-regulated. In contrast, the PC level is likely to be a consequence of copper delivery to the thylakoid lumen because apo-PC without copper is unstable (17, 18). The regulation of SOD expression would allow efficient delivery of limited copper to PC, which is essential, yet also maintain plastid SOD activity. Based on these observations, we proposed a model, in which the copper available to chloroplasts is sensed and the resulting information is used to direct the expression of CSD1 and CSD2.

Micro-RNAs are small 21–22-long RNA molecules that, in plants, contribute to the regulating the expression of specific genes by directing an endo-ribonuclease machinery to degrade target mRNAs (19). Micro-RNAs have been shown to direct a transfection analysis and predicted to target CSD1 and CSD2. The micro-RNA family, miR398, was identified by computational analysis and predicted to target CSD1, CSD2, and COX5b-I mRNAs (21, 23, 27). In a recent report, the miR398 family was shown to control CSD1 and CSD2 expression in response to environmental stress (28). Here we present evidence that transcripts of CSD1, CSD2, and also COX5b-I, which encodes a plant homolog of the zinc-binding subunit of the mitochondrial copper enzyme cytochrome c oxidase, are regulated by miR398. Regulation of miR398 is predominantly in response to copper. Consequently, CSD1 and CSD2 transcripts and proteins do not accumulate when copper is below a critical threshold even under stress conditions. However, when copper is sufficient a slight additive effect of copper and stress can be observed on miR398 expression. We propose a regulatory mechanism in which the expression of chloroplast SOD isozymes is determined by metal cofactor availability, a mechanism that involves miR398, the expression of which is regulated by copper availability.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Arabidopsis thaliana ecotype Columbia gl1 was used as the wild type, and the background of all transgenic plants was also Columbia gl1. Plants were grown on agar-solidified MS (Murashige-Skoog) medium including 1% sucrose (Sigma) with CuSO4 as indicated, and were grown under controlled conditions (light intensity of 40 \(\mu mol m^{-2} s^{-1}\), 16-h light/8-h dark cycle at 23 °C).

Stress Treatments—For high-light stress, 2-week-old seedlings grown on MS medium or MS plus copper at a light intensity of 120 \(\mu mol m^{-2} s^{-1}\), in a 12-h light/12-h dark cycle were transferred in the middle of the light cycle to continuous high light (950 \(\mu mol m^{-2} s^{-1}\)). Control plants were maintained at low light intensity throughout the experiment. For methyl viologen (MV) treatment, MS and MS plus copper-grown seedlings were sprayed with 100 \(\mu M\) MV (Sigma). After 24 h, unstressed and stress-treated seedlings were harvested and used for RNA and protein extraction.

Plasmid Construction—To construct CSD2::LUC, the CSD2 promoter region was amplified from genomic DNA by PCR using the primers CSD2pro-F (5'-AGTGAGATCTTGT-CAAAATGGAAAGAGCG-3') and CSD2pro-R (5'-GATTTGCTCATCCTCTCTCTCTGT-3'). The underlined sequence is the restriction site used for the cloning. When restriction sites are not indicated in primers, the site present in the genome sequence was used for the cloning. The amplified product was digested with XbaI and Ncol, then cloned into the pBI211-LUC+ vector (29), and confirmed by sequencing. The primers used for PCR amplification were CSD2ox-F (5'-TTCTCTCTAGC-CTAACAATGAGCGCTCAGAAA-3') and CSD2ox-R (5'-TCAAAACATGAGCGCATGGAAGAA-3') for CSD2::LUC, CSD2ox-F and CSD2ox-R (5'-CTAGTGAGAGCTGACGGAAGAA-3') for CSD2::LUC, and CSD2ox-F and CSD2ox-R (5'-CTTCTCATCCTCTCTCTCTGT-3') for CSD2::LUC. The amplified products were inserted into Ncol-digested CSD2::LUC, then confirmed by restriction digestion and sequencing. For construction of 3SS::CSD2, the primers used for PCR amplification were CSD2ox-F and CSD2ox-R (5'-AACGAATTCAACAGCATGATTCCAG-3'). The PCR product was inserted into XbaI-EcoRI-digested pBI121 vector (GenBank accession number AF485783). To create 3SS::miR398, the primers used for PCR amplification were miR398box-F (5'-TTGTGATCGTTGATTTGATTACG-3'), and miR398box-R (5'-GAGACGAGGAGACGACGAGCTGTTTCC-3'). The PCR product was inserted into XbaI-SacI-digested pBI121 vector. To construct 3SS::CSD2ina, the primers were CSD2ox-F and CSD2ina-R (5'-TCCATGATTGATGATTCCAG-3') for template 1, and CSD2ina-F (5'-ATTTGACGTGATTGATGATTAC-3') and CSD2ox-R for template 2. The two templates were fused by recombinant PCR (30) using primers CSDox-F and CSDox-R. The PCR product was inserted into pBI121 in the same way as 3SS::CSD2.

Luciferase (LUC) and SOD Assays—LUC activity was detected in 2-week-old seedlings. 1 mM Luciferine (Molecular Probes, Eugene, OR) was sprayed onto plants. Images of emitted light were captured by a CCD (charge-coupled device) camera, and light intensities were calculated using AQUACOSMOS software (Hamamatsu Hotonics, Japan). SOD isozymes were extracted and assayed on native polyacrylamide gels as described (11).
RNA Extraction and RNA Gel Blot Analysis—Total RNA was isolated from 3-week-old seedlings grown on MS nutrient-agar plates using the TRIzol reagent (Invitrogen). For transcript analysis, 10 μg of total RNA was subjected to electrophoresis on a 1% agarose gel containing 4% formaldehyde, transferred to a nylon membrane, and probed with 32P-labeled probes. The probes for CSD1, CSD2 (12), and APX1 (31) were obtained by PCR amplification. Radioactive probes were synthesized with an oligo-labeling kit (Amersham Biosciences) using random primers. Hybridization and washing was performed as described (12).

For small RNA analysis, 20 μg of total RNA (40 μg total RNA for Fig. 3B) was loaded per lane and resolved on a denaturing 17% polyacrylamide gel containing 8 m urea in TBE buffer (89 mM Tris base, 89 mM boric acid, 1 mM EDTA) and transferred electrophoretically to Hybond N+ (Amersham Biosciences). DNA oligonucleotide probes specific for miR398a (5'-AACGGG-TGACCGAAGACA-3') and miR398b or miR398c (5'-CAGGGGTGACCTGAGAACACA-3') were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Fermentas, Hanover, MD). The blots were also analyzed with an oligo-labeling kit (Amersham Biosciences). The blots were prehybridized for 1 h and hybridized overnight using ULTRAhybrid-oligo-labeling kit (Amersham Biosciences) according to instructions. Primers used were as follows: 5’-TCTTACACCTGTGAAATGTTGC-3’ and 5’-CTTA-GTCTGACACGTGAAAC-3’. The blots were washed four times, 20 min each (two times with 2× SSC, 0.2% SDS, one time with 1× SSC, 0.1% SDS, and one time with 0.5× SSC, 0.1% SDS) at 50°C. Membranes were briefly dried and bands were visualized using a phosphorimager.

RT-PCR Analysis—10 μg of total RNA was treated with DNase I (Fermentas) and cDNA was synthesized using Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI) according to instructions. Primers used were as follows: 5’-TCCTACACCTGTGAAATGTTGC-3’ and 5’-CTTACACCTGTGAAATGTTGC-3’. The PCRs were performed in a final volume of 50 μl containing 2 units of Taq polymerase, 10, 15, 20, 25, 30, 35, and 40 cycles of amplification were performed in an Eppendorf Mastercycler, each consisting of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C. The amplified products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining.

Immuno blot Analysis—Rosette leaves from Arabidopsis plants grown on MS-agar plates or MS supplemented with CuSO4 were harvested and frozen immediately in liquid nitrogen. Total protein was extracted as described (12) and the protein concentration was determined according to Bradford (32). 20 μg of total proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Antibodies for immunoblot detection of PC (12) and SOD isoforms (6) have been described. Antibody for Hsp70 was obtained from Invitrogen. Antibodies for cytochrome f and COX2 were gifts from Dr. A. Makino (Tohoku University, Japan) and Dr. K. Noguchi (University of Tokyo, Japan). Antibodies for alternative oxidase (AOX) were obtained from Agrisera (Vannas, Sweden). Ferritin-specific antibodies were a kind gift of Dr. J. F. Briat (INRA Montpellier, France). For the detection of COX2 and AOX crude mitochondrial fractions were prepared as described (33).

RESULTS
CSD2 mRNA Abundance Is Post-transcriptionally Regulated by Copper Availability—The protein levels of two Cu,Zn-SODs were analyzed in Arabidopsis seedlings cultured at three different copper concentrations (Fig. 1A). Standard MS plant growth medium (34) contains 0.1 μM CuSO4 and is widely used for plant tissue culture, however, this concentration may be suboptimal for Arabidopsis seedlings, because the photosynthetic electron transport rate is close to but not at full capacity (11, 12). Furthermore, Cu,Zn-SOD expression is very low and FSD1 is expressed perhaps to compensate (Fig. 1A) (12). The expression pattern was drastically changed, and CSD1 and CSD2 were predominantly accumulated in seedlings cultured at 5 and 10 μM CuSO4. Previously, it was shown that protein levels of SODs were correlated to the transcript levels (12). The protein level of PC was only mildly elevated in response to increases in copper in the media in this range (Fig. 1A); more severe reduction of PC levels and photosynthetic electron transport occurs at much lower copper levels (12).

To study the molecular mechanism that regulates the CSD2 mRNA level in response to copper availability, we constructed transgenic Arabidopsis plants expressing LUC under the control of the CSD2 promoter (CSD2pro::LUC). For this construct the intergenic region with the upstream gene and the 5’-untranslated region including the translational initiation codon of CSD2 was used. The in vivo LUC activity was monitored with a CCD camera in seedlings cultured on low copper (0.1 μM CuSO4) and sufficient copper (5 and 10 μM CuSO4) media. Unexpectedly, the same level of luminescence was detected at all copper conditions (Fig. 1B). This result suggests that the level of CSD2 mRNA is not regulated in response to copper by transcriptional activity via the promoter.

The CSD2 mRNA level may therefore be regulated in response to copper availability post-transcriptionally. To investigate this possibility, we generated transgenic Arabidopsis plants that constitutively transcribe CSD2 mRNA (CSD2ox lines). Despite the control by the constitutive CaMV35S promoter, the accumulation of CSD2 mRNA strictly depended on copper concentrations (Fig. 1C) as was observed for the endogenous gene transcribed from the CSD2 promoter. Although the RNA level was higher in the CSD2ox lines than in the wild type even under low copper conditions, it was drastically elevated under sufficient copper conditions. To test the possibility that the activity of the CaMV35S promoter could perhaps somehow respond to copper availability, we tested the accumulation of mRNA derived from a different gene, PGR5 as a control. PGR5 is involved in photosystem I cyclic electron transport (35) and
its expression is unlikely to be affected by copper availability. In CaMV35S::PGR5 lines the PGR5 mRNA level was not influenced by copper concentrations (data not shown), indicating that copper availability does not affect the transcriptional activity of the CaMV35S promoter. Consistent with the results of RNA gel blot analysis, the CSD2 protein level responded to copper availability in CSD2ox lines (Fig. 1D). The activity of CSD2 also responded to the copper concentration in CSD2ox lines, as in the wild type (Fig. 1E).

To test if copper regulation of CSD2 requires activity of the protein, we also constructed transgenic Arabidopsis plants that constitutively express inactive CSD2 (CSD2ina lines). In Cu/Zn-SODs, five well conserved histidines are involved in the binding of a copper ion. The bound copper ion can cycle between the Cu(I) and Cu(II) redox states, which is necessary for catalytic activity (36, 37). A change of the conserved His, which corresponds to His-48 in yeast and human Cu,Zn-SOD, should result in altered copper binding with loss of activity in CSD2ina lines. As expected, in lines that overaccumulate the CSD2ina protein we did not observe increased activity of SODs (Fig. 1E). In fact, compared with the wild type, the CSD2 activity in CSD2ina lines was even slightly reduced, probably as a result of competition for copper binding with overaccumulating CSD2ina (Fig. 1E). Despite the lack of activity of CSD2ina, the protein (Fig. 1F) and mRNA (Fig. 1G) levels were precisely regulated by copper, as in the lines overexpressing active CSD2 (CSD2ox-1). The level of CSD2 mRNA accumulation was lower in the two CSD2ina lines, even though both constructs share
miR398 Targets CSD2 mRNA and Its Expression Is Regulated in Response to Copper Availability—A bioinformatic analysis of the Arabidopsis genome has suggested that a micro-RNA family, miR398, targets the mRNA of CSD1, CSD2, and COX5b-1 (21). The targets of miR398 (Fig. 2A) are the coding region of CSD2 mRNA and the 5′-untranslated region of CSD1 mRNA (28) as well as the 5′-untranslated region of COX5b-1 mRNA (21). The miR398 family consists of three genes, miR398a (localized to chromosome II), miR398b and miR398c (both localized to chromosome V). The sequences of mature miR398b and miR398c are identical, whereas the 3′ end nucleotide is different in miR398a (Fig. 2A). Recently, miR398 was shown to be involved in the regulation of CSD1 and CSD2 expression under oxidative stress conditions (28). We were interested to see if CSD2 expression in response to copper is regulated via miR398. To test the possibility that copper availability regulates miR398 expression, the accumulation of miR398 RNA was analyzed under different copper conditions. The RNA gel blot analysis revealed that miR398 RNA accumulated at low copper concentration (0.1 μM) but not at sufficient copper concentrations (5 and 10 μM) (Fig. 2B). We used probes designed to hybridize to miR398a or miR398b/c, however, because these probes differ by only one base they most likely cross-react. Expression of the precursors for miR398a and miR398b/c can be distinguished by RT-PCR, using gene-specific primers. RT-PCR analysis indicated that both miR398a and miR398b/c are regulated by copper and that the expression of miR398b/c is much higher than that of miR398a (data not shown). We conclude that miR398 expression is regulated by copper.

miR398 Is Involved in the Down-regulation of CSD2 mRNA under Low Copper Conditions—The expression of CSD2 is post-transcriptionally suppressed under low copper conditions (Fig. 1). At the same time the expression of miR398 is induced (Fig. 2). These results suggest that the CSD2 mRNA level may be regulated via miR398 depending on copper availability. We compared protein levels at different copper concentrations in the wild type and two mutant plant lines that are defective in the processing machinery; for example, Arabidopsis contains four DICER-like genes (41) and 10 members of the ARGONAUTE family (42). To directly test miR398 RNA accumulation we performed RNA gel blot analysis. miR398 RNA accumulation was only marginally affected in dcl1, ago1 under low copper, whereas no accumulation was observed at 5 μM copper, as expected. In contrast, miR398 RNA was below the detection limit at 0.05 μM copper in hen1 even after loading 40 μg of total RNA (Fig. 3B). However, by RT-PCR a weak signal was detected in hen1 grown at low copper (Fig. 3B). The reduced miR398 accumulation in hen1 is consistent with an increase in CSD1 and CSD2 protein levels in this mutant on low copper (Fig. 3A). Taken together with the result of the miR398 RNA gel blot (Fig. 2B), the partial loss of copper regulation observed in micro-RNA processing mutants suggests that miR398 is involved in the down-regulation of CSD2 and CSD1 mRNA at low copper concentrations.

To test if miR398 is directly involved in the degradation of CSD2 mRNA under low copper conditions, we constructed transgenic Arabidopsis plants that express either a truncated or a full-length version of CSD2 fused with the LUC gene. The CSD2Δ1::LUC construct encodes the 132 amino acids from the N terminus of CSD2 and its mRNA does not include the putative miR398 target site. The CSD2Δ2::LUC construct encodes for 151 amino acids of CSD2 but its mRNA still contains the miR398 target site (Fig. 3C). All transgenic plants were grown on low- and sufficient copper media, and then used to analyze LUC activity by luminescence. As expected, the LUC activity in CSD2full::LUC plants was regulated by copper availability (Fig.
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3D, lower panel). In contrast, in CSD2Δ1::LUC, which lacks the miR398 binding sequence, the same level of luminescence was detected under both low and sufficient copper conditions (Fig. 3D, upper panel). In CSD2Δ2::LUC, however, the LUC activity again strictly depended on the copper concentration (Fig. 3D, middle panel). We observed a similar level of luminescence under sufficient copper conditions, when miRNA398 is not expressed, in all lines. The results based on LUC activity were also confirmed by the direct analysis of the chimeric RNA levels by RT-PCR (Fig. 3E). These results indicate that a region within the 57 ribonucleotides, which are absent in CSD2Δ1::LUC but present in CSD2Δ2::LUC and which includes the miR398 target site, is essential for the regulation by copper availability. All the results strongly suggest that miR398 is directly involved in the degradation of the CSD2 transcript on low copper.

Overexpression of miR398 Destabilizes Both CSD1 and CSD2 mRNAs—The miR398 target site is essential for regulating the CSD2 mRNA stability in response to copper availability (Fig. 3). To test if miR398 is directly involved in the degradation of the CSD2 mRNA, we constructed transgenic Arabidopsis plants, which constitutively express miR398b (Fig. 4). In four independent T1 lines cultured on soil, the level of miR398b RNA was found to be significantly higher than that in wild-type seedlings (Fig. 4A). We also observed that some other lines with the same construct exhibited a reduction in the miR398b expression possibly due to co-suppression (data not shown). The RNA level of miR398a was not affected in transgenic lines. In wild-type seedlings grown on soil containing copper, the level of miR398 RNA was low and we observed a high expression for CSD1 and CSD2 both at the mRNA and protein levels (Fig. 4, A and B). As a consequence of overexpression of miR398, however, the expression of CSD1 and CSD2 was signifi-
The overexpression of miR398b represses CSD1 and CSD2 expression. A, transcript analysis by RT-PCR for the indicated genes in the 35SmiR398 lines (T1 generation) and wild-type seedlings (WT). Seedlings were first cultured for 14 days on MS medium containing sufficient copper (1 μM) for kanamycin selection and then transferred to soil. Total RNA was extracted from plants 7 days after transfer to soil. The number of PCR cycles was 25 for miR398b, CSD2, CSD1, PC, and Actin, which was optimum to detect these transcripts semi-quantitatively in the wild type. The number of PCR cycles was 40 for miR398a due to its lower expression level. B, immunoblot analysis of the same plants used in A. C, elevated miR398 RNA levels and suppression of CSD2 mRNA at 5 μM copper co-segregate in the T2 generation. RT-PCR analysis was performed for the indicated transcripts in individual T2 plants grown on MS medium supplemented with 5 μM CuSO4. Two or three T2 plants originated from four independent T1 lines (11, 15, 21, and 23) were analyzed. The wild-type plants grown at 5 μM and 0.1 μM CuSO4 were used as controls.

The expression of miR398 is regulated in response to differences in copper level—Sunkar et al. (28) have shown that miR398 is down-regulated by oxidative stress. Excess copper may also cause oxidative stress. Indeed the down-regulation of miR398 was also observed after spraying seedlings with 100 μM copper (28). However, our analysis indicated that expression of
due to gene silencing.
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**A**

![Graph showing copper levels and target expression](image1)

**B**

![Graph showing miR398 expression levels](image2)

**C**

![Graph showing CSD2 and EtBr levels](image3)

**D**

![Graph showing miR398 and copper levels](image4)

**E**

![Graph showing CSD and APX1 levels](image5)

**F**

![Graph showing CSD and Hsp70 levels](image6)

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**FIGURE 6. Copper availability is the predominant factor that controls miR398-mediated Cu,Zn-SOD expression.** A, immunoblot detection of CSD2, CSD1, FSD1, PC and Hsp70 indicates that plants switch from the use of FeSOD to Cu,Zn-SOD at a threshold low copper concentration. Northern blot detection of B, miR398 levels, and C, CSD2 mRNA levels in response to copper. U6 small nuclear RNA was detected as a loading control. D, the combined effects of copper availability and oxidative stresses on miR398 expression measured by RNA blot. E, the combined effects of copper availability and oxidative stresses on CSD2, CSD1, and APX1 (ascorbate peroxidase 1) expression measured by RNA blots. F, the combined effects of copper availability and oxidative stresses on protein levels for CSD1, CSD2, feritin, and Hsp70. Plants were grown on MS media with the indicated copper concentrations at 100 μM copper (highest concentration) for 2 weeks before harvest. For the oxidative stress treatments, plants grown under the same condition were transferred to high light intensity (950 μmol m⁻² s⁻¹) for 24 h or exposure to 100 μM MV in the light (100 μmol m⁻² s⁻¹) for 24 h.

miR398 and the targets CSD1, CSD2, and COX5b-1 are regulated by copper concentrations in a range of 0.1–5 μM, which are probably too low to cause oxidative stress. To further investigate the exact levels of copper that are required to regulate miR398 expression, we investigated the regulation of gene expression in response to changes in a low range of copper concentrations. We investigated the effects on the levels of protein (Fig. 6A), miR398 (Fig. 6B), and CSD2 mRNA (Fig. 6C). The results indicate that the shift from FSD1 to CSD2 and CSD1 takes place at less than 0.5 μM copper, where copper stress is likely to be very minimal. miR398 levels were found to vary between 0.1 μM copper (highest miR398 levels) and 5 μM copper (absence of miR398). At 2 μM copper miR398 was almost undetectable already and at this concentration CSD2 protein and mRNA was at its highest expression level. Direct measurements of copper contents in seedlings by ICP-AES indicated that healthy soil-grown plants of the same age have a shoot copper content comparable with plants grown on MS medium supplemented with 0.7 μM copper (data not shown). These observations strongly suggest that it is the availability of copper, which regulates CSD protein levels via miR398 and that this regulation is a part of the essential copper homeostasis network in a plant cell.

Given that miR398 is involved in the regulation by copper availability, how does oxidative stress as reported by Sunkar et al. (28) relate to this process? To assess the possibility that oxidative stress independently regulates the expression of miR398 and thus that copper and oxidative stress have additive effects, we investigated miR398 expression as well as protein and mRNA levels for CSD1 and CSD2 in plants grown in a range of copper conditions, without or with oxidative stress (Fig. 6, D–F). High-light treatment (950 μmol m⁻² s⁻¹) of plants that were adapted to lower light (120 μmol m⁻² s⁻¹) results in excess excitation of reaction centers and possible over-reduction of the electron transfer chain, leading to oxidative stress. A high concentration (100 μM) of MV, which was used also by Sunkar et al. (28), was applied as another means to induce oxidative stress. MV causes superoxide formation most likely at photosystem I. The treatments with high light and MV caused visible photodamage to plants (data not shown). The level of
miR398 (Fig. 6D) and CSD1 and CSD2 mRNA (Fig. 6E) and protein levels (Fig. 6F) were regulated by copper availability even under the oxidative stresses. Although high-light treatment slightly down-regulated the expression of miR398 especially at intermediate copper levels of 0.5–1 μM, it caused only a subtle effect on the RNA or protein levels for CSD1 and CSD2. Compared with high light, MV affected the miR398 expression more significantly, but its effect on the protein levels of CSDs was also very mild. These results support the notion that the expression of miR398 is regulated predominantly by copper availability, although extreme oxidative stress slightly effects additively.

The expression of ascorbate peroxidase 1 (APX1) is considered to be one of the most sensitive markers of oxidative stress (45, 46). APX1 expression is not elevated by our copper treatments under control conditions (Fig. 6E), suggesting that oxidative stress is minimal in this range of copper concentration. Nevertheless, under the same conditions expression of miR398 and CSDs is clearly regulated by copper in a reciprocal fashion with miR398, suggesting that it is the availability of copper itself and not oxidative stress that regulates CSD1 and CSD2 expression. Another marker of oxidative stress is ferritin. Ferritin expression is known to be regulated by enhanced levels of superoxide or peroxide (47, 48). Indeed ferritin protein levels were up-regulated by MV treatment. However, copper treatment did not increase ferritin levels, in contrast, if anything ferritin expression was reduced by higher copper availability. Taken together our data strongly suggest that the regulation of CSDs expression via miR398 predominantly responds to copper availability although oxidative stress has a slight additive effect.

**DISCUSSION**

Consistent with a bioinformatic prediction (21), miR398 targets mRNAs that encode the copper proteins, CSD1, CSD2, as well as COX5b-1. As a regulator of abundant copper proteins, miR398 is a key factor in the control of copper homeostasis in the plant cell. The most abundant copper protein, PC, is not subject to this regulation by copper availability (Figs. 1 and 4). This regulation allows limited copper to be preferentially transferred to PC, which is essential for photosynthesis in higher plants. In contrast to PC, CSD2 function is not essential and the enzyme is replaced by FSD1, which uses iron as a cofactor under low copper conditions. The down-regulation of CSD2 expression seems to be coordinated with CSD1. We think that the miR398-mediated response to copper limitation is part of a homeostatic control mechanism allowing plants to thrive in a range of copper regimes. Indeed, Arabidopsis seedlings grew healthy at all concentrations tested and only minor effects were seen on photosynthesis by varying copper levels between 0.1 and 10 μM (11, 12).

Whereas the physiological significance of CSD1 and CSD2 regulation can be understood in the context of copper homeostasis, the effect of miR398 on COX5b-1 expression is less obvious. COX5b-1 is one of two COX5b genes in Arabidopsis and encodes for the zinc-binding subunit 5b of cytochrome c oxidase. COX5b-1 mRNA is targeted by miR398 but the available data indicate that COX5b-2 mRNA is not. Therefore, because microarray data (www.genevestigator.ethz.ch/at) indicate that COX5b-2 is expressed in most tissues, we expect that at least some COX5b function is maintained even under low copper conditions. The function of plant and mammalian subunit 5b (COX4 in yeast) is not fully clear at this point. The copper in cytochrome c oxidase is bound by the three core subunits (COX1, -2, and -3) (49), which are encoded by the mitochondrial genome in most eukaryotes. It is well established that reduced cytochrome c oxidase activity is an effect of severe copper deficiency in plants (50). However, the stable accumulation of the core subunit COX2 under the copper concentrations that we tested and the lack of up-regulation of the AOX level may be taken to indicate that the copper conditions applied were not adverse for function of cytochrome c oxidase. Perhaps miR398 regulates the respiratory electron transport chain in a very subtle manner in response to copper availability. Such regulation may be more significant in tissues in which mitochondria become a larger copper sink, such as in non-photosynthetic cells.

Whereas we favor a role of miR398 primarily in the context of copper homeostasis, the same micro-RNA was reported to regulate CSD1 and CSD2 expression in response to oxidative stress (28) and our data indicate that copper and stress can have a slight additive effect on miR398 expression under certain conditions (Fig. 6). However, it is unclear whether plants utilize up-regulation of CSD1 and CSD2 in a stress response in nature. The notion that CSD1 and CSD2 have a limited role under oxidative stress is suggested by the fact that CSD1 and CSD2 mRNA were elevated by a shift from very low light intensity (60 μmol photons m⁻² s⁻¹) to intermediate light intensity (125 μmol photons m⁻² s⁻¹), but no increased transcription was observed by higher light intensity (6). For Arabidopsis, a light intensity of 125 μmol photons m⁻² s⁻¹ is not stressful, whereas 60 μmol photons m⁻² s⁻¹ limits photosynthesis.

We have shown that copper availability is sensed by plants to regulate the expression of miR398. Oxidative stress may influence this copper signal. However, at this point the exact nature of the signal or signals that direct miR398 expression are unclear. First, it is possible that the copper concentration is directly sensed by machinery in the stroma or cytosol, and the resulting signal is used to affect miR398 expression. This type of mechanism may be used in Chlamydomonas reinhardtii where copper homeostasis is regulated via the CRR1 transcription factor (51). On the other hand copper may, even at low concentrations, promote ROS formation and such ROS could be sensed to direct miR398 expression. The latter type of signal seems, however, less specific.

In addition to SOD function in the context of oxidative stress prevention, our data suggest also a role in metal homeostasis. The role of individual SOD enzymes in response to oxidative stress is perhaps overstated in the literature because cells have redundancy in their ROS scavenging machineries (52). Plants with a T-DNA insertion in the CCS gene encoding a copper chaperone for both Cu,Zn-SODs have virtually no active CSD1 and CSD2, yet these plants are phenotypically similar to the wild type (10) even on media or soil with sufficient copper, a
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condition where FSD1 is also absent. Furthermore, we could observe overexpression of miR398 and suppression of CSD1 and CSD2 expression in T1 and T2 seedlings (Fig. 4). Consistent with the phenotype of the CCS knock-out lines, the miR398 overexpressors did not exhibit any visible phenotypes despite their severe reduction in both Cu,Zn-SOD activities. This observation is also consistent with the observed normal growth of seedlings cultured on MS medium containing 0.1 μM copper, in which miR398 induces degradation of both CSDs (Fig. 1A). We propose that miR398 is involved in the regulation of copper homeostasis rather than the response to oxidative stress.

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