REGULATION OF COPPER HOMEOSTASIS BY microRNA 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 microRNA, 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 (Cu) must be delivered to a number of important enzymes that are active in various sub-cellular locations (1). One of the most abundant Cu proteins in photosynthetic tissues is plastocyanin (PC), which localizes to the thylakoid lumen of chloroplasts and mediates electron transport between the cytochrome *bf* complex and photosystem I. Although a heme-containing cytochrome *c* can serve as an alternative to PC under low-Cu 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 Cu enzyme in plants is copper/zinc superoxide dismutase (Cu/ZnSOD). 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/ZnSOD localize to the cytoplasm (CSD1) and to the chloroplast stroma (CSD2), while 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, Cu 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 Cu over these two organelles may also be crucial for maintaining an optimum energy budget in a cell.

The Cu delivery system to PC and
Cu/ZnSOD in plant cells has been studied in some detail now (1). Cu enters the cell by means of the CopT family of transporters (8,9). A cytosolic form of the Cu chaperone for SOD (CCS) delivers Cu to CSD1 (10). Two P-type ATPases, PAA1 and PAA2, are required for efficient Cu delivery across the plastid envelope and the thylakoid membrane, respectively in Arabidopsis (11,12). Once Cu 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 Cu 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 FeSOD or Cu/ZnSOD 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. While Cu delivery is important for energy metabolism and superoxide scavenging, excess Cu is toxic and growth of plants on tissue culture media containing Cu above 20 µM causes visible damage to plants (15). Free Cu ions may participate in the Fenton reaction, resulting in the production of highly reactive and damaging hydroxyl radicals. However, the risk of free Cu ions may be minimized by a strict regulation of Cu delivery in a cell. For example, the free Cu concentration was estimated to be less than 10^{-18} M in a yeast cell, whereas the total Cu concentration is ~70 µM (16).

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

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

MicroRNAs 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). MicroRNAs have been shown to direct a variety of developmental processes (20) but also have been implied in responses to stress (21,22) and in controlling plant nutritional status including sulfur (21,23) and phosphate (24-26). The microRNA family, miR398, was identified by computational analysis and predicted to target CSD1, CSD2 and COX5b-1 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-1, which encodes a plant homolog of the Zn-binding subunit of the mitochondrial Cu enzyme cytochrome c oxidase, are regulated by miR398. Regulation...
of miR398 is predominantly in response to Cu. Consequently, CSD1 and CSD2 transcripts and proteins do not accumulate when Cu is below a critical threshold even under stress conditions. However, when Cu is sufficient a slight additive effect of Cu 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 which involves miR398, the expression of which is regulated by Cu 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-Aldrich, St. Louis, MO) with CuSO4 as indicated, and were grown under controlled conditions (light intensity of 40 µmol m⁻² s⁻¹, 16h-light/8h-dark cycle at 23ºC).

Stress treatments - For high-light stress, two week-old seedlings grown on MS medium or MS plus Cu at a light intensity of 120 µmol m⁻² s⁻¹, in a 12h light /12h dark cycle were transferred in the middle of the light cycle to continuous high light (950 µmol m⁻² s⁻¹). Control plants were maintained at low light intensity throughout the experiment. For methyl viologen (MV) treatment, MS and MS plus Cu-grown seedlings were sprayed with 100 µM MV (Sigma-Aldrich, St Louis, MO). After 24 h, unstressed and stress-treated seedlings were harvested and used for RNA and protein extraction.

Plasmid construction - To construct CSD2pro::LUC, the CSD2 promoter region was amplified from genomic DNA by PCR using the primers CSD2pro-F (5'-AGTGAGATCTTTGCAAAATGGGACAGC-3') and CSD2pro-R (5'-GCATTCTCATCTCCTTCTGG-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 NcoI, then cloned into the pBl221-LUC+ vector (29), and confirmed by sequencing. The primers used for PCR amplification were CSD2ox-F (5'-TTCTCTAGACGTCAAAATAGAAGC-3') and CSD2cut3-R (5'-CTAACAACATGACCATGGAGCTCACAGAA-3') for CSD2Δ1::LUC, CSD2ox-F and CSD2cut2-R (5'-CATAAATGCCATGGCCATGCTTGA-3') for CSD2Δ2::LUC, and CSD2ox-F and CSD2full-R2 (5'-CTTTTCGAGGTGTGATGGCATGGGCG-3') for CSD2full::LUC. The amplified products were inserted into NcoI-digested CSD2pro::LUC, then confirmed by restriction digestion and sequencing. For construction of 35S::CSD2, the primers used for PCR amplification were CSD2ox-F and CSD2full-R (5'-AACGAAATTCATAAGCATTG-3').

The PCR product was inserted into XbaI-EcoRI digested pBl121 vector (GenBank accession number AF485783). To create 35S::miR398b, the primers used for PCR amplification were miR398box-F (5'-TTTGATCTAGATTTAGAATA-TGGGGAAC-3'), and miR398box-R (5'-GAAGAGGAAGCACGGAGGAC-3'). The PCR product was inserted into XbaI-SacI digested pBl121 vector. To construct 35S::CSD2ina, the primers were CSD2ox-F and CSD2ina-R (5'-CCTCTGTGGATTTGAGTGGATTTGG-3') for template 1, and CSD2ina-F (5'-ATTTTGAGTGGATTTGG-3') and CSD2ox-R (5'-GAAGAGGAAGCACGGAGGAC-3') for template 2. The two templates were fused by recombinant PCR (30) using primers CSD2ox-F and CSD2ox-R. The PCR product was inserted into pb121 in the same way as 35S::CSD2.

Luciferase (LUC) and SOD assays - LUC activity was detected in two-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, 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 three-week-old seedlings grown on MS nutrient-agar plates using the TRlzol reagent (Invitrogen, Carlsbad, CA). 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-labelled 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, Piscataway, NJ) 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, Piscataway, NJ). DNA oligonucleotide probes specific for miR398a (5'-AAGGGGTGACCTGAGAACACA-3') and miR398b or miR398c (5'-CAGGGGTGACCTGAGAACACA-3') were end-labeled with γ32P-ATP using T4 polynucleotide kinase (Fermentas, Hanover, MD) and cDNA was synthesized using Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI) according to instructions. Primers used were as follows: 5'-TCCTACAACCTGTGAATGTTCG-3' and 5'-CTTATCGACACTGGAACAC-3' for CSD2, 5'-GAGTTGCAGTTTTGAACACA-3' and 5'-TTCTTTGGAAACGTAAGTGCAG-3' for CSD1, 5'-CGATCGAAACCAAGCTAACC-3' and 5'-GGACTCAAATTAAGAGGCTTTC-3' for PC, 5'-GAGAGATTCAGGTGCCCAG-3' and 5'-AGAGCCAGCGGGTTCATTA-3' for Actin, 5'-ATTTCAGAGGATGCATGTA-3' and 5'-CCTCACCCCTTTGAATCTCCC-3' for miR398a, 5'-GATCTTGACAGGGTTGATGTA-3' and 5'-GGTCACCCCTGCTGAGCTCTT-3' for miR398b, 5'-CAGCTATTCTGATTACACCC-3' and 5'-GGAATCCATTTCTGATCACA-3' for LUC. 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 by ethidium bromide staining.

**Immunoblot 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 (Carlsbad, CA). Antibodies for cytochrome f and COX2 were gifts from Dr. A Makino (Tohoku University, Japan) and Dr. K
Nogichi (University of Tokyo, Japan). Antibodies for alternative oxidase (AOX) were obtained from Agrisera (Vannas, Sweden). Ferritin specific antibodies were a kind gift of Dr. JF 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 Cu availability - The protein levels of two Cu/ZnSODs were analyzed in Arabidopsis seedlings cultured at three different Cu 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 sub-optimal for Arabidopsis seedlings, since the photosynthetic electron transport rate is close to but not at full capacity (11, 12). Furthermore, Cu/ZnSOD 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 predominately 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 Cu in the media in this range (Fig. 1A); more severe reduction of PC levels and photosynthetic electron transport occurs at much lower Cu levels (12).

To study the molecular mechanism that regulates the CSD2 mRNA level in response to Cu 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 (UTR) 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-Cu (0.1 µM CuSO4) and sufficient-Cu (5 and 10 µM CuSO4) media. Unexpectedly, the same level of luminescence was detected at all Cu conditions (Fig. 1B). This result suggests that the level of CSD2 mRNA is not regulated in response to Cu by transcriptional activity via the promoter.

The CSD2 mRNA level may therefore be regulated in response to Cu 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 the Cu 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-Cu conditions, it was drastically elevated under sufficient-Cu conditions. To test the possibility that the activity of the CaMV35S promoter could perhaps somehow respond to Cu 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 Cu availability. In CaMV35S::PGR5 lines the PGR5 mRNA level was not influenced by Cu concentrations (data not shown), indicating that Cu 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 Cu availability in CSD2ox lines (Fig. 1D). The activity of CSD2 also responded to the Cu concentration in CSD2ox lines, as in the wild type (Fig. 1E).

To test if Cu regulation of CSD2 requires activity of the protein, we also constructed transgenic Arabidopsis plants which constitutively express inactive CSD2 (CSD2ina lines). In Cu/ZnSODs, five well-conserved histidines are involved in the binding of a Cu ion. The bound Cu 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 His48 in yeast and human Cu/ZnSOD, should result in altered Cu binding with loss of activity in CSD2ina lines. As expected, in lines that over-accumulate the CSD2ina protein we did not observe increased activity of SODs (Fig. 1E). In fact, compared to the wild type, the CSD2 activity in CSD2ina lines was even slightly reduced, probably as a result of competition for Cu binding with over-accumulating CSD2ina (Fig. 1E). Despite the lack of activity of CSD2ina, the protein (Fig. 1F) and mRNA (Fig. 1G) levels were precisely regulated by Cu, as in the lines over-expressing active CSD2 (CSD2ox-1). The level of CSD2 mRNA accumulation was lower in the two CSD2ina lines, even though both constructs share the same promoter. This difference is most likely explained by the position effect and a consequence of the random insertion of T-DNA during Agrobacterium-mediated transformation. We conclude that CSD2 expression is regulated by Cu independently of the activity of the product.

miR398 targets CSD2 mRNA and its expression is regulated in response to Cu availability - A bioinformatic analysis of the Arabidopsis genome has suggested that a microRNA 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’ UTR of CSD1 mRNA (28) as well as the 5’UTR 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 Cu is regulated via miR398. To test the possibility that Cu availability regulates miR398 expression, the accumulation of miR398 RNA was analyzed under different Cu conditions. The RNA gel blot analysis revealed that miR398 RNA accumulated at low-Cu concentration (0.1 µM) but not at sufficient-Cu concentrations (5 and 10 µM) (Fig. 2B). We used probes designed to hybridize to miR398a or miR398b/c, however since 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 Cu 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 Cu.

miR398 is involved in the down-regulation of CSD2 mRNA under low-Cu conditions - The expression of CSD2 is post-transcriptionally suppressed under low-Cu 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 Cu availability. We compared protein levels at different Cu concentrations in the wild type and two mutant plant lines that are defective in the maturation of microRNAs: hua enhancer 1 (hen1) (38), dicer-like 1 (dcl1) (39). We also investigated a mutant defective in microRNA directed mRNA processing: argonaute 1 (ago1) (40). In wild-type seedlings, the levels of CSD1 and CSD2 proteins were at the detection limit at 0.1 µM Cu (Fig. 3A). In contrast, CSD1 and CSD2 protein accumulated even at the low-Cu concentration in the mutant backgrounds, particularly in hen1. The defect in the response to Cu is only minor which is probably due to the redundancy in the microRNA processing machinery; for example Arabidopsis contains four DICER-like genes (41) and ten members of the ARGONAUTE family (42). To directly test miR398 RNA accumulation we performed RNA blot analysis. miR398 RNA accumulation was only marginally affected in dcl1, ago1 under low Cu while no
accumulation was observed at 5 μM Cu, as expected. In contrast, miR398 RNA was below the detection limit at 0.05 μM Cu in hen1 even after loading 40 μg total RNA (Fig. 3B). However, by RT-PCR a weak signal was detected in hen1 grown at low Cu (Fig. 3B). The reduced miR398 accumulation in hen1 is consistent with an increase in CSD1 and CSD2 protein levels in this mutant on low Cu (Fig. 3A). Taken together with the result of the miR398 RNA gel blot (Fig. 2B), the partial loss of Cu regulation observed in microRNA processing mutants suggests that miR398 is involved in the down-regulation of CSD2 and CSD1 mRNA at low-Cu concentrations.

To test if miR398 is directly involved in the degradation of CSD2 mRNA under low-Cu 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-Cu media, and then used to analyze LUC activity by luminescence. As expected, the LUC activity in CSD2full::LUC plants was regulated by Cu availability (Fig. 3D, lower panel). In contrast, in CSD2Δ1::LUC, however, the LUC activity again strictly depended on the Cu concentration (Fig. 3D, middle panel). We observed a similar level of luminescence under sufficient-Cu 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 Cu availability. All the results strongly suggest that miR398 is directly involved in the degradation of the CSD2 transcript on low Cu.

Over-expression of miR398 destabilizes both CSD1 and CSD2 mRNAs - The miR398 target site is essential for regulating the CSD2 mRNA stability in response to Cu 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 Cu, the level of miR398b RNA was low and we observed a high expression for CSD1 and CSD2 both at the mRNA and protein levels (Fig. 4A and B). As a consequence of over-expression of miR398, however, the expression of CSD1 and CSD2 was significantly reduced both at the mRNA and protein levels in the four lines. In contrast to the effect on CSD1 and CSD2 expression, the over-expression of miR398b did not affect the levels of PC either at the mRNA or protein level (Fig. 4A and B).

Next we tested if we could observe repression of CDS2 expression by miR398b overproduction in the presence of 5 μM Cu, a condition where CSD2 is highly expressed in wild-type plants because endogenous miR398 levels are very low. Although we selected for and confirmed the presence of the transgene in the T2 generation, we noted that the over-expression of miR398 was unstable in the T2 generation, possibly due to gene silencing. We exploited this phenomenon to investigate if miR398 overproduction and CSD2 silencing segregated together. To test this, we cultured individual T2 seedlings originated from four T1 lines on medium containing 5


µM Cu and extracted RNA from each seedling. RT-PCR analysis showed that the level of miR398b RNA remained higher than in the wild type in seven out of nine T2 seedlings, but was reduced to the wild-type level in two seedlings (#21-3 and #23-3) possibly due to gene silencing (Fig. 4C). RT-PCR indicated that the higher accumulation of miR398b RNA co-segregates with the repression of CSD2 expression even in the presence of 5 µM Cu. Taken together all the results (Fig. 1-4), we conclude that Cu availability regulates the stability of mRNA both for CSD1 and CSD2 via miR398.

COX5b mRNA is also a target of miR398 - Next to CSD1 and CSD2 mRNAs, COX5b-1 mRNA was also predicted to contain the target site of miR398 (21). To test the possibility that COX5b mRNA stability is also regulated by Cu via miR398, COX5b mRNA level was determined under various Cu concentrations (Fig. 5A). Consistent with the target prediction, the COX5b mRNA level was lower at 0.1 µM Cu compared to 5-10 µM Cu. Interestingly, the instability of COX5b mRNA at low Cu concentrations was somewhat enhanced in plants that overexpress CSD2 (Fig. 5A). Compared to the strict regulation of CSD1 and CSD2 mRNA levels via miR398 (Fig. 1), the COX5b mRNA level was only mildly influenced by Cu availability. Consistent with this observation, the accumulation of the cytochrome c oxidase complex, which was evaluated by the level of COX2 protein, was not affected by Cu availability (Fig. 5B). AOX is involved in respiratory electron transport to oxygen, which bypasses cytochrome c oxidase (43). The protein level of AOX was not influenced by Cu availability (Fig. 5B). The Arabidopsis genome includes another gene encoding COX5b (COX5b-2, At1g80230) and both genes are expressed (44). We did not find a target site for miR398 in COX5b-2 mRNA. The two genes are very similar in their coding sequence (72% identity at the amino acid level). It is probable that the expression of two genes is differentially regulated by Cu availability, and this regulation may be physiologically significant in certain tissues or under special conditions.

The expression of miR398 is regulated in response to differences in Cu level - Sunkar et al (28) have shown that miR398 is down-regulated by oxidative stress. Excess Cu may also cause oxidative stress. Indeed the down-regulation of miR398 was also observed after spraying seedlings with 100 µM Cu (28). However, our analysis indicated that expression of miR398 and the targets CSD1, CSD2 and COX5b-1 are regulated by Cu 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 Cu which are required to regulate miR398 expression, we investigated the regulation of gene expression in response to changes in a low range of Cu 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 Cu, where Cu stress is likely to be very minimal. miR398 levels were found to vary between 0.1 µM Cu (highest miR398 levels) and 5 µM Cu (absence of miR398). At 2 µM Cu miR398 was almost undetectable already and at this concentration CSD2 protein and mRNA was at its highest expression level. Direct measurements of Cu contents in seedlings by ICP-AES indicated that healthy soil-grown plants of the same age have a shoot Cu content comparable to plants grown on MS medium supplemented with 0.7 µM Cu (data not shown). These observations strongly suggest that it is the availability of Cu, which regulates CSD protein levels via miR398 and that this regulation is a part of the essential Cu homeostasis network in a plant cell.

Given that miR398 is involved in the regulation by Cu 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 Cu 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 Cu conditions, without or with oxidative stress (Fig. 6D, E and F). High-light treatment (950 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) of plants that were adapted to lower light (120 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) results in excess excitation of reaction centers and possible over-reduction of the electron transfer chain, leading to oxidative stress. A high concentration (100\( \mu \text{M} \)) of methyl viologen (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 Cu availability even under the oxidative stresses. Although high-light treatment slightly down-regulated the expression of miR398 especially at intermediate Cu levels of 0.5-1 \( \mu \text{M} \), it caused only a subtle effect on the RNA or protein levels for CSD1 and CSD2. Compared to 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 Cu 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 Cu treatments under control conditions (Fig. 6E), suggesting that oxidative stress is minimal in this range of Cu concentration. Nevertheless, under the same conditions expression of miR398 and CSDs is clearly regulated by Cu in a reciprocal fashion with miR398, suggesting that it is the availability of Cu itself and not oxidative stress which 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, Cu treatment did not increase ferritin levels, in contrast if anything ferritin expression was reduced by higher Cu availability. Taken together our data strongly suggest that the regulation of CSDs expression via miR398 predominantly responds to Cu availability although oxidative stress has a slight additive effect.

**DISCUSSION**

Consistent with a bioinformatic prediction (21), miR398 targets mRNAs that encode the Cu proteins, CSD1, CSD2 as well as COX5b-1. As a regulator of abundant Cu proteins, miR398 is a key factor in the control of Cu homeostasis in the plant cell. The most abundant Cu protein, PC, is not subject to this regulation by Cu availability (Fig. 1 and 4). This regulation allows limited Cu 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-Cu conditions. The down-regulation of CSD2 expression seems to be coordinated with CSD1. We think that the miR398-mediated response to Cu limitation is part of a homeostatic control mechanism allowing plants to thrive in a range of Cu regimes. Indeed, Arabidopsis seedlings grew healthy at all concentrations tested and only minor effects were seen on photosynthesis by varying Cu levels between 0.1 and 10 \( \mu \text{M} \) (11,12).

Whereas the physiological significance of CSD1 and CSD2 regulation can be understood in the context of Cu 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 Zn-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, since microarray...
indicate that COX5b-2 is expressed in most tissues, we expect that at least some COX5b function is maintained even under low Cu conditions. The function of plant and mammalian subunit 5b (COX4 in yeast) is not fully clear at this point. The Cu 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 Cu deficiency in plants (50). However, the stable accumulation of the core subunit COX2 under the Cu concentrations that we tested and the lack of up-regulation of AOX level may be taken to indicate that the Cu 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 Cu availability. Such regulation may be more significant in tissues in which mitochondria become a larger Cu sink, such as in non-photosynthetic cells.

While we favor a role of miR398 primarily in the context of Cu homeostasis, the same microRNA was reported to regulate CSD1 and CSD2 expression in response to oxidative stress (28) and our data indicate that Cu 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 was 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, while 60 µmol photons m⁻² s⁻¹ limits photosynthesis.

We have shown that Cu availability is sensed by plants to regulate the expression of miR398. Oxidative stress may influence this Cu 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 Cu concentration is directly sensed by a 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 Cu homeostasis is regulated via the CRR1 transcription factor (51). On the other hand Cu 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 Cu chaperone for both Cu/ZnSODs 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 Cu, a condition where FSD1 is also absent (Abdel-Ghany et al., unpublished). Furthermore, we could observe over-expression of miR398 and suppression of CSD1 and CSD2 expression in T₁ and T₂ seedlings (Fig. 4). Consistent with the phenotype of the CCS knockout lines, the miR398 over-expressors did not exhibit any visible phenotypes despite their severe reduction in both Cu/ZnSOD activities. This observation is also consistent with the observed normal growth of seedlings cultured on MS medium containing 0.1 µM Cu, in which miR398 induces degradation of both CSDs (Fig. 1A). We propose that miR398 is involved in the regulation of Cu homeostasis rather than the response to oxidative stress.
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FOOTNOTES

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1The abbreviations used are: Cu, copper; PC, plastocyanin; SOD, superoxide dismutase; ROS, reactive oxygen; COX, cytochrome c oxidase; MS, Murashige-Skoog, MV, methyl viologen; CCD, (charge-coupled device); LUC, luciferase, AOX, alternative oxidase;

FIGURE LEGENDS

Fig. 1. Cu/ZnSOD expression is regulated by Cu availability at a post-transcriptional step. A, Proteins extracted from rosette leaves of seedling cultured at different Cu concentrations were fractionated by SDS-PAGE, and detected by using specific antibodies. Hsp70 was detected as a loading control. B, Cu does not affect the LUC activity in CSD2pro::LUC lines. Upper panel: luminescence recorded by a CCD camera of the wild type (WT) and CSD2pro::LUC line (#123)
grown at the indicated Cu concentrations. Lower panel: luminescence intensities were quantified in three independent transgenic lines (#17, #28 and #123). Data are averages of four independent replicates. C, RNA blot analysis of CSD2 mRNA in the wild type (WT) and CSD2 over expressing lines (CSD2ox-1 and CSD2ox-2). D, Protein blot analysis of CSD2 in the same plant lines used in RNA analysis. E, Analysis of SOD activities in the wild type (WT), CSD2ox-1 and two lines over-expressing inactive CSD2 (CSD2ina-1 and CSD2ina-2). F, Immunoblot analysis of CSD2 expression in the same plants used in E. Hsp70 was detected as a loading control. G, RNA blot analysis of CSD2 in the same plants used in E.

Fig. 2. miR398 is predicted to target three Cu enzymes and is itself regulated by Cu. A, Sequence alignments of the miR398 family members, and their putative target sequences in CSD1, CSD2, and COX5b-1 mRNAs. B, Small RNA blot analysis of miR398 using the indicated probes. Lower panel, ethidium bromide staining of the upper portion of the same gel.

Fig. 3. Regulation of Cu/ZnSOD expression by Cu involves microRNA A, Comparison of CSD1, CSD2, FSD1, PC and Hsp70 proteins accumulation, as measured by immunoblotting, in response to Cu availability in the wild type (WT) and mutant plants defective in microRNA processing. B, Expression of miR398b in plants analyzed by RNA gel blot analysis (upper panel) and RT-PCR (lower panel). C, Schematic presentation of deletion derivatives of CSD2 fused with the LUC gene. Vertical arrows indicate the miR398 binding site. D, Luminescence images of two independent transgenic lines (#41 and #42). Two-week-old seedlings of CSD2∆1::LUC, CSD2∆2::LUC, and CSD2full::LUC cultured on the media containing 0.1 and 5 µM Cu were used for analysis. E, RT-PCR analysis of chimeric RNA. The number of PCR cycles was 25 for miR398b and Actin, and 20 for chimeric RNA (LUC).

Fig. 4. Overexpression of miR398b represses CSD1 and CSD2 expression. A, Transcript analysis by RT-PCR for the indicated genes in the 35S::miR398 lines (T1 generation) and wild-type seedlings (WT). Seedlings were first cultured for 14 days on MS medium containing sufficient Cu (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 Cu 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.

Fig. 5. Accumulation of COX5b-1 mRNA is affected by Cu availability. A, RNA blot analysis of COX5b-1. Total RNA was extracted from the wild type (WT) and a CSD2 over-expressor (CSD2ox-1) cultured on the medium containing Cu indicated. B, Immunoblot blot analysis of COX2 and AOX protein levels in wild type seedlings cultured on the medium containing Cu indicated.

Fig. 6. Cu availability is the predominant factor that controls miR398-mediated Cu/ZnSOD expression. A, Immunoblot detection of CSD2, CSD1, FSD1, PC and Hsp70 indicates that plants switch from the use of FeSOD to Cu/ZnSOD at a threshold low Cu concentration. Northern blot detection of B, miR398 levels and C, CSD2 mRNA levels in response to Cu. U6 snRNA was detected as a loading control. D, The combined effects of Cu availability and
oxidative stresses on miR398 expression measured by RNA blot. E, The combined effects of Cu availability and oxidative stresses on CSD2, CSD1 and APX1 (ascorbate peroxidase 1) expression measured by RNA blots. F, The combined effects of Cu availability and oxidative stresses on CSD2, ferritin and Hsp70. Plants were grown on MS media with the indicated Cu concentrations at 100 µmol m⁻² s⁻¹ for two 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.
Figure 1
A

\[
\text{miR398a} \quad 3'\text{-}\text{UUCGCCACUGGACUCUUGUGU} \quad 5' \\
\text{miR398b&c} \quad 3'\text{-}G\text{UUCGCCACUGGACUCUUGUGU} \quad 5'
\]

\[
\text{CSD1} \quad 5'\text{-}AAGGGGUUUCCUGAGAUCACA \quad 3' \\
\text{miR398a3} \quad \text{UUCGCCACUGGACUCUUGUGU} \quad 5'
\]

\[
\text{CSD2} \quad 5'\text{-}UUCGCCACUGGACCCAGAACA \quad 3' \\
\text{miR398a3} \quad \text{UUCGCCACUGGACUCUUGUGU} \quad 5'
\]

\[
\text{COX2b-1} \quad 5'\text{-}AAGGGUUGACCCUGAGAUCACA \quad 3' \\
\text{miR398a3} \quad \text{UUCGCCACUGGACUCUUGUGU} \quad 5'
\]

B

![Figure 2]

Figure 2
Figure 3
Figure 4
Figure 5

A

|          | WT  | CSD2ox-1 |
|----------|-----|----------|
| COX5b    |     |          |
| 18S      |     |          |
| 0.1      | 5   | 10       |
| 0.1      | 5   | 10       |

B

|          |       |          |
|----------|-------|----------|
| COX 2    |       |          |
| AOX      |       |          |
| 0.1      | 5     | 10       |

(µM Cu)
Figure 6
