Regulation of superoxide dismutase expression by copper availability
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The most abundant copper proteins in green tissues are plastocyanin (PC) in thylakoids and copper/zinc superoxide dismutase (Cu/ZnSOD) of which the major isoforms are found in the cytosol and in the chloroplast stroma. An iron superoxide dismutase (FeSOD) can also be found in the stroma. The expression of superoxide dismutases (SODs) has been studied mainly in the context of abiotic stress. However, the availability of metal cofactors may also determine SOD expression patterns. Indeed, in Arabidopsis thaliana, Cu/ZnSOD enzymes were only expressed when copper was sufficient. This observation was made for plants grown on sucrose-containing tissue culture media and regulation of SOD expression by copper has not been tested for other species. To investigate the effect of copper on SOD expression, we used a hydroponic set-up in which plants grew without any evident stress symptoms. We observed that A. thaliana, Brassica juncea, Lycopersicum lycopersicum, Zea mays and Oryza sativa, downregulated Cu/ZnSOD in response to copper limitation. Under this condition, FeSOD expression was upregulated to replace Cu/ZnSOD in the stroma in all plants except Z. mays, in which FeSOD was not detectable. Copper limitation did not affect PC accumulation in any of the plants except Z. mays. Comparisons of leaf copper contents and SOD expression suggest that Cu/ZnSOD and FeSOD expression levels are good indicators of impending copper deficiency. Plants that downregulate Cu/ZnSOD and upregulate FeSOD under copper limitation can maintain superoxide scavenging and save copper for use in PC, which is essential for photosynthesis.

Introduction
The trace element copper (Cu) is required as a cofactor for several processes including photosynthesis, respiration, ethylene perception, oxidative stress reduction, cell expansion and cell-wall lignification (Marschner 1995).

In plant chloroplasts, two major Cu-containing proteins are found, plastocyanin (PC) and copper/zinc superoxide dismutase (Cu/ZnSOD). PC is one of the most abundant proteins in the thylakoid lumen (Kieselbach et al. 1998, Schubert et al. 2002) and is essential for electron transfer between the cytochrome b6f complex and photosystem I (Weigel et al. 2003). Polyphenol oxidase is another Cu protein found in the thylakoids of some plants, such as spinach (Kieselbach et al. 1998), but not in other species such as Arabidopsis thaliana (Schubert et al. 2002). In the chloroplast stroma, Cu/ZnSOD requires Cu, along with Zn, as cofactors to catalyze the dismutation of superoxide radicals (O2−) into H2O2 (Asada 1999). In

Abbreviations – Cu/ZnSOD, copper/zinc superoxide dismutase; ddH2O, double-distilled H2O; DW, dry weight; FeSOD, iron superoxide dismutase; ICP-AES, inductively coupled plasma-atomic emission spectrometry; MnSOD, Mn superoxide dismutase; PC, plastocyanin; PSII, photosystem II.
A. thaliana, seven SOD genes have been identified, with the major activities coming from CSD1, CSD2, FSD1 and MSD1 (Kliebenstein et al. 1998). CSD1 and CSD2 both encode a Cu/ZnSOD with CSD1 activity in the cytosol and CSD2 activity in the stroma. FSD1 encodes a chloroplast localized SOD that utilizes Fe as the cofactor (FeSOD), while MSD1 encodes a mitochondrial SOD (MnSOD) that utilizes Mn as the cofactor (Kliebenstein et al. 1998). Other important Cu proteins in plant cells include cytochrome-c oxidase in the mitochondria, the ethylene receptors in the endomembrane system and various apoplastic oxidases (for a review see Pilon et al. 2006).

Because of its redox potential, Cu can exist in both the Cu$^{2+}$ and Cu$^{1+}$ form in living organisms. The reversible oxidation–reduction of Cu makes it very useful as a cofactor in electron transfer reactions. However, the redox activity of Cu could also result in oxidative stress if Cu would be present as a free ion. In yeast, the estimated free Cu ion concentration in the cell was at least 12 orders of magnitude below the total Cu concentration (Rae et al. 1999). In order to allow efficient delivery of essential metal ions and at the same time avoid toxic excess, all organisms have evolved transport and sequestration systems (Nelson 1999). Copper can be limiting to plant productivity in crop species when below 5 mg kg$^{-1}$ dry weight (DW), whereas toxicity is reported above 30 µg g$^{-1}$ DW (Marschner 1995). The transport and delivery of Cu into and within plant cells has been reviewed recently (Pilon et al. 2006). Plant cells acquire Cu through CopT family transporters (Sancenon et al. 2003), while Cu is removed from the cytosol by the HMA5 transporter (Andrés-Colás et al. 2006). Within the cell, transport in chloroplasts is the best understood. Two Cu-transporting P-type ATPases, PAA1 and PAA2, are required for efficient delivery of Cu to PC in chloroplasts. A detailed biochemical and phenotypic analysis of mutations for PAA1 and PAA2 indicated that the transporters function in the chloroplast envelope and thylakoid membrane, respectively. A third component of the Cu-delivery system in plant plastids is the Cu-chaperone CCS, a functional homologue of a yeast metallochaperone for Cu (Abdel-Ghany et al. 2005a). Interestingly, CCS in plants has dual localization, in both cytosol and chloroplasts, and is required for activity of both CSD1 and CSD2 (Chu et al. 2005). Cu homeostasis not only requires mechanisms by which Cu is delivered to subcellular targets, but also regulated expression of targets for Cu delivery. Availability of Cu was found to be a major determinant of Cu/ZnSOD and FeSOD expression in A. thaliana (Abdel-Ghany et al. 2005a, 2005b). When Cu supply was sufficient, CSD1 and CSD2 were expressed and became active, yet their expression and activity diminished when Cu supply was limited. It was proposed that when Cu supply is limited, Cu is shuttled to PC rather than to CSD1 and CSD2, because PC is essential (Abdel-Ghany et al. 2005b). However, during low Cu conditions, SOD may still be important for the scavenging of superoxide radicals produced at photosystem I, so an alternative SOD isoform, FSD1, is used. In the photosynthetic alga Chlamydomonas reinhardtii, PC is not essential because a heme-containing cytochrome-c$_{6}$ can functionally replace it under Cu limitation (Quinn and Merchant 1995). Thus, the hierarchy of Cu use and the mechanism to reduce the effects of Cu limitation is different in Arabidopsis and Chlamydomonas.

The reciprocal expression of Cu/ZnSOD and FeSOD in response to Cu availability during non-stress conditions was only shown for A. thaliana plants grown on tissue culture medium. Thus far, regulation has not been verified in other growth conditions, and it has not been examined in other plant species. Information about micronutrient use at the cellular level could benefit agricultural crop production through a better understanding of the genetic programs by which plants optimize photosynthetic activity in their green tissues during sub-optimal Cu growth conditions. In this study, we present evidence that supports the model in which Cu is allocated preferentially to PC over cytosolic and stromal Cu/ZnSOD during Cu-limited growth for a variety of crop species.

**Materials and methods**

**Plant species and growth conditions**

The following species were used: A. thaliana (col-0), Brassica juncea (accession no. 173874), Zea mays (var. sugos), Oryza sativa (subsp. indica IR64), and Lycopersicon lycopersicum (Pole Brandywine). Plants were germinated on Whatman® filter paper soaked in double-distilled H$_{2}$O (ddH$_{2}$O). Seedlings (4–7 days old) were placed on 1/5X Hoagland’s hydroponic solution (Hoagland and Arnon 1938). For growth under Cu limitation, Cu was omitted from the medium, while 0.05 µM Cu was added for Cu-sufficient conditions. Hydroponic growth medium was contained in black plastic tubs 25 cm × 40 cm (6.8 l volume) and used in conjunction with a non-transparent acrylic cover having 15 evenly spaced holes where plantlets were placed and supported by the top half of a 1.5 ml microcentrifuge tube. The growth medium was replaced every 7 days and maintained daily with ddH$_{2}$O to compensate for evaporation. The solution was aerated continuously using aquarium pumps at 200–300 ml/min. To minimize Cu contamination of the hydroponic set-up, all equipment was washed with 25 mM EDTA and rinsed
repeatedly with ddH2O. Chemicals used for the Hoagland's solution were certified ACS (American Chemical Society) grade and purchased from Fisher Scientific (Fair Lawn, NJ). Pilot studies showed that even with Cu omitted from the medium, all plants seemed to grow without noticeable stress and plants indeed accumulated measurable Cu, probably as a result of minor Cu contamination in the chemicals used to prepare the medium. Each treatment consisted of three replicates and each replicate contained five plants. Plants were grown for a total of 35 days in 10 h/14 h light/dark cycles at a light intensity of 120 μmol photons m⁻² s⁻¹ and 23°C.

For growth in agar plates, tissue culture media was made using certified ACS grade chemicals from Fisher Scientific as described by Murashige and Skoog (1962) minus Cu. Copper was added for treatments at 0.1 μM and 5.0 μM, or omitted. Agar media contained 1% sucrose and 0.5% agarose. All media was made using distilled de-ionized H2O. Plants were grown in Magenta boxes (Sigma, St Louis, MO) for a total of 21 days in 12 h/12 h light/dark cycles at a light intensity of 120 μmol photons m⁻² s⁻¹ and 23°C.

**Protein extraction, SOD activity and immunoblotting**

Leaf samples were taken after 6 h into the light period and used for all measurements except where noted. In order to sample young, photosynthetically active leaves, the third, fourth and fifth leaf of each plant (whole leaf for dicots, middle third for monocots, whole shoot for A. thaliana) were harvested and frozen in liquid nitrogen directly after measurement of shoot fresh weight. Soluble leaf proteins for non-denaturing and SDS polyacrylamide gel analysis were extracted as described (Abdel-Ghany et al. 2005a). Protein concentration of extracts was determined according to Bradford (1976) using BSA as a standard. For SOD isozyme separation and activity analysis, 20 μg of protein was fractionated on non-denaturing gels then stained for activity as described (Abdel-Ghany et al. 2005a, Beauchamp and Fridovich 1971). Identification of SOD isozymes utilized 2 mM KCN to inhibit Cu/ZnSOD (Van Camp et al. 1996) and 3 mM H2O2 to inhibit Cu/ZnSOD and FeSOD (H2O2 results not shown). Antibodies used for immunodetection of SOD isoforms (Kliebenstein et al. 1998) and PC (Abdel-Ghany et al. 2005a) have been described. All SOD antibodies were raised against A. thaliana proteins. Rubisco large subunit antibody was purchased from AgriSera (Stockholm, Sweden), and PC (Abdel-Ghany et al. 2005a) was raised against spinach proteins. Each experiment was done in triplicate with identical results and representative gels are shown.

**Fluorescence, CO2 assimilation and starch accumulation**

Chlorophyll fluorescence was measured with a programmable, pulse-modulated, Hansatech Fluorometer FMS2 (Hansatech Instruments, Norfolk, UK) on overnight dark-adapted plants essentially as described by Maxwell and Johnson (2000). The following program was used to estimate chlorophyll fluorescence parameters: plants were exposed to a saturating light pulse (2050 μmol photons m⁻² s⁻¹) to estimate Fₚ/Fₚ₀ (photosystem II antennae efficiency). Light-adapted parameters were determined at actinic light intensities of 43, 350 and 770 μmol photons m⁻² s⁻¹ using a saturating pulse to determine photosystem II quantum efficiency (ΦPSII), calculated as (Fₚ₉₋Fₒ)/Fₚ₀; as well as photochemical quenching, calculated as (Fₚ₉₋Fₒ)/(Fₚ₋Fₒ), and non-photochemical quenching, calculated as (Fₚ₋Fₒ)/Fₚ. A far-red illuminating pulse was initiated after each saturating pulse to establish initial fluorescence (Fₒ) (Maxwell and Johnson 2000).

CO₂ assimilation was measured using a Licor 6400 (LI-COR Biosciences, Lincoln, NB). All measurements were made with leaf samples after 6 h into the light period. Ambient CO₂ of 360 μmol mol⁻¹ at an airflow of 200 μmol s⁻¹ and actinic light of 400 μmol photons m⁻² s⁻¹ were used to measure total change in CO₂ in μmol m⁻² s⁻¹. Starch staining as a qualitative indicator of starch accumulation was as follows: a leaf from each replicate was boiled in water for 1 min then boiled in 95% ethanol until white. Leaves were added to Lugol’s iodine solution for 4 min to stain for starch followed by rinsing in H₂O.

**Biomass and elemental analysis**

Shoot biomass for each plant was determined and grouped into a replicate of five plants. One gram of fresh leaves (stems not used), representative of the entire replicate, were dried for 5 days at 45°C. The dried leaves were reweighed, and percent of total leaf mass as dry mass was determined. For elemental analysis, 100 mg of dried leaf samples were digested with 1 ml nitric acid and heated for 2 h at 60°C then 6 h at 130°C. Digests were diluted to 10 ml with ddH₂O and analyzed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) for Cu, Fe, Mg, Mn, Zn, P, and S as described by Pilon-Smits et al. (1999).

**Statistical analysis**

Statistical analysis was performed using the Jump-in software package (SAS Institute, Cary, NC).
Results

To investigate if plants change their pattern of SOD expression in response to Cu availability, we grew several crop species in hydroponics. Our aim was to compare responses in photosynthetic tissues under conditions of mild Cu limitation and Cu sufficiency while avoiding stresses as a result of either critical deficiency or toxicity. Pilot experiments indicated that all plants showed a normal growth and appearance even when Cu was omitted. Under this condition, we measured Cu concentrations of at least 5 μg g⁻¹ DW, which is above reported critical deficiency levels for most plant species (Marschner 1995). For all experiments conducted, within each species and across all replicates, none of the plants showed any visible signs of chlorosis, anthocyanin production or variation of size in response to the Cu treatments as illustrated by the appearance of 35-day-old plants shown in Fig. 1.

With a reproducible hydroponic set-up established, we focused first on A. thaliana and the related crop plant B. juncea (Indian mustard) to examine if these plants alter expression of the major Cu proteins, PC and Cu/ZnSOD, in response to Cu supply. Arabidopsis thaliana plants grown in hydroponics had abundant Cu/ZnSOD activity with no noticeable FeSOD activity on Cu-sufficient medium, whereas FeSOD was the most evident activity on Cu-limited medium (Fig. 2A). The reciprocal changes in Cu/ZnSOD and FeSOD activity in response to Cu could be fully ascribed to a difference in the abundance of the corresponding polypeptides (Fig. 2B). Note that the CSD2 antibody recognizes a single protein in A. thaliana extracts, whereas the CSD1 antibody detects CSD1 but also cross-reacts with CSD2, which is similar in amino acid sequence (Kliebenstein et al. 1998). Importantly, we did not observe a change in the expression of the two PC isoforms in response to Cu feeding in hydroponics (Fig. 2B). Thus, in hydroponic conditions where A. thaliana growth depends fully on photosynthesis, we see very similar regulation of Cu protein expression compared with what was observed for tissue culture supplied with sucrose (Abdel-Ghany et al. 2005a). In B. juncea, we observed, along with MnSOD and FeSOD, three possible isoforms of Cu/ZnSOD based on their activity (Fig. 2A) and cross-reactivity to CSD1 and CSD2 antibodies (Fig 2B). Similar to what was seen for A. thaliana, we observed that both the activity and protein abundance of Cu/ZnSOD and FeSOD were regulated in response to Cu and that the expression was reciprocal. At the same time, PC expression was also not affected by Cu treatment (Fig. 2B). As expected, MnSOD was not affected by Cu feeding in B. juncea or any of the other species tested (Figs 2A, B and 3A). To ensure that the

Fig. 1. Hydroponically grown plants. Images of 35-day-old plants prior to sampling.
treatments indeed affected leaf Cu concentration, an elemental analysis (ICP-AES) was conducted on leaf samples. In this first study, leaf Cu concentrations decreased in Cu-limited plants, with *Arabidopsis thaliana* decreasing significantly (*P* < 0.05) (Fig. 2C). Copper content for all species and treatments remained within 5 and 30 μg g⁻¹ DW, a range considered to be between critical deficiency and toxicity for most plants (Marschner 1995). Iron content increased significantly (*P* < 0.05) in *B. juncea* when Cu was limited, while *Arabidopsis thaliana* showed no detectable change in Fe content in this experiment. Thus, under conditions of photosynthetic growth, both *A. thaliana* and *B. juncea* downregulated expression of their Cu/ZnSODs and upregulated FeSOD in response to mild Cu limitation, whereas PC levels are unaffected by this treatment. Therefore, these plants seem to prioritize Cu delivery to PC over SOD proteins during Cu limitation.

**Fig. 2.** Effects of Cu treatment on hydroponically grown plants. Hydroponically grown wild-type plants in 1/5 Hoagland’s with 0.05 μM added Cu (+) and omitted Cu (−) for 35 days (A) SOD isozyme activities from leaf homogenates. Total soluble proteins (25 μg per lane) were fractionated on non-denaturing 15% acrylamide gels and stained for total SOD activity. (B) Immunodetection of plastocyanin (PC) and SOD proteins. Proteins extracted from leaf homogenate (25 μg per lane) were fractionated by 15% SDS–PAGE. Each protein was detected by immunoblot analysis using specific antibodies. (C) Analysis of leaf Fe and Cu. Values are given as averages for three replicates in μg g⁻¹ DW ± s.e. of the mean. Significant differences between pairs are indicated with an asterisk (Student’s *t* test; *P* < 0.05).

**Fig. 3.** Effects of Cu treatment on hydroponically grown plants. All experimental procedures are the same as Fig. 2 except in (A) where gels were stained without an inhibitor (untreated) or with KCN (KCN), to inhibit Cu/ZnSOD, to differentiate Cu/ZnSOD from FeSOD.
To investigate if other unrelated plants show a similar response to Cu limitation, we compared the response to Cu in *B. juncea* to a number of crop species. To assess the effects of Cu feeding, we also analyzed how limited Cu growth conditions affected parameters indicative of productivity. In a second series of hydroponics, we grew the dicots, *B. juncea* and *L. lycopersicum* (tomato), next to the monocots, *Z. mays* (corn) and *O. sativa* (rice). *Arabidopsis thaliana* was omitted from this experiment because of its small size, which made it difficult to accurately measure all the parameters reported here. We analyzed the activity and abundance of SOD isoymes and PC as well as micronutrient levels for Cu and Fe (Fig. 3). Reciprocal regulation of Cu/ZnSOD and FeSOD in response to Cu treatments was observed for *B. juncea* and *L. lycopersicum* (Fig. 3A, B). However, for *B. juncea*, perhaps Cu was not as limiting in the second experiment compared with the first (Fig. 3) as evidenced by a higher Cu content and the observation of both FeSOD and Cu/ZnSOD on low Cu. We observed that *L. lycopersicum* had a high Cu content compared with the other species tested, even under Cu limitation (Fig. 3C). Even though Cu concentrations were relatively high in both *B. juncea* and *L. lycopersicum*, a significant (\(P < 0.05\)) Cu reduction in the limited Cu grown plants was measured, which apparently affected the regulation of FeSOD and Cu/ZnSOD abundance. The expression of PC did not change significantly for both dicot species, *B. juncea* and *L. lycopersicum* (Fig. 3B).

In the monocot species *Z. mays*, we did not detect FeSOD activity or a protein band with cross-reactivity to *A. thaliana* FSD1, indicating that FeSOD is below the detection limit in this species. MnSOD and several Cu/ZnSOD isoforms were readily detected in *Z. mays*. In *Z. mays* we did observe reduced activity and protein abundance of the Cu/ZnSODs during limited Cu growth conditions, although the shut-off was not complete. Interestingly, in *Z. mays*, we also observed a reduced expression of PC during Cu-limited growth (Fig. 3B). As the presence of FeSOD in *Z. mays* was not observed here, it is possible that FeSOD does not exist in this *Z. mays* species, or it is induced only during growth conditions not imposed here. For *O. sativa*, expression and activity of Cu/ZnSOD and FeSOD occurred for both Cu treatments with only a slight decrease in Cu/ZnSOD for the Cu-limited treatment, while FeSOD and PC appear unchanged. As FeSOD activity in *O. sativa* is low and unchanged between Cu treatments when compared with its Cu/ZnSOD counterpart, it is possible that Cu-limited conditions are relatively minor and not low enough to increase FeSOD expression.

Both monocot species showed reduced leaf Cu concentrations when Cu limited, with a significant \(P < 0.05\) reduction for *Z. mays* (Fig. 3C). Consistent with the first study, leaf Fe concentrations also showed a trend of increasing Fe during Cu-limited growth. Additional elemental analyses included phosphorous (P) and sulfur (S). Most plant species tested showed a trend of decreasing P and S when Cu limited (Table 1). However, instead of decreased, we found increased contents of P and S in Cu-limited *B. juncea* \(P < 0.05\). Other elements tested (Zn, Mn and Mg) were not significantly different between treatments in any of the plants tested (not shown).

We tested a number of parameters that are useful indicators of plant health and productivity (Table 1). Only those parameters that showed the greatest variation between treatments are shown and they indicated only a few significant differences between + and − Cu treatments for all of the plant species tested (Table 1).

![Table 1. Physiological measurements of hydroponic grown plants. Measurements were made on 35-day-old plants grown with 0.05 \(\mu\)M added Cu (+) and omitted Cu (−). CO₂ assimilation in \(\mu\)mol m⁻² s⁻¹ at 360 \(\mu\)mol CO₂ and 400 \(\mu\)mol photons m⁻² s⁻¹. Fv/Fm (PSII antenna efficiency) was measured on dark-adapted plants and PSIi (PSII quantum efficiency) was measured at 770 \(\mu\)mol photons m⁻² s⁻¹. Elemental analysis of phosphorous and sulfur are given in \(\mu\)g g⁻¹ DW. Values are given as averages for three replicates ± SE of the mean. Significant differences between pairs are indicated with an asterisk (Student’s t test; \(P < 0.05\)).](image-url)
Even though there were no major changes between Cu treatments, there were trends apparent in the data that correlate 
with SOD and PC expression levels. Noticeably, on low Cu compared to high Cu for \textit{Z. mays}, the decreasing trend in CO$_2$ assimilation, dry mass, and electron transport activity from PSII activity coincides with reduced PC expression. A reduced flux PSII was also observed for \textit{L. lycopersicum} under Cu limitation, albeit not significant. The accumulation of starch was also unchanged among treatments for all species, and is not shown here. Overall, we observed that all plant species tested reduced Cu/ZnSOD expression in response to Cu limitation. At the same time, we observed upregulation of FeSOD for dicots but not for the monocots. In all plants except for \textit{Z. mays}, the expression of PC was not significantly affected by Cu limitation.

The apparent lack of a response to Cu for FeSOD in \textit{O. sativa} could be the result of insufficient depletion of Cu in hydroponics for the species. We examined the effect of Cu on SOD expression for plants grown in agar media, where Cu can be depleted more easily. We compared \textit{Z. mays}, \textit{O. sativa} and \textit{B. juncea}. We analyzed 21-day-old seedlings grown without Cu or 0.1 \( \mu \text{M} \) Cu and 5.0 \( \mu \text{M} \) Cu added (Fig. 4). All of the proteins detected in \textit{B. juncea} and \textit{Z. mays} responded similarly to those seen using hydroponic growth conditions (Figs 2B and 3B). For \textit{O. sativa} grown in agar, protein expression levels of Cu/ZnSOD decrease on low Cu. For FeSOD, by loading 40 \( \mu \text{g} \) instead of 25 \( \mu \text{g} \) of protein, we clearly detected two FeSOD bands and observed a slight increase on low Cu (Fig. 4). Interestingly, the 0.1 \( \mu \text{M} \) Cu in agar growth media, which is the Cu concentration found in standard MS media (Murashige and Skoog 1962), does not support full Cu/ZnSOD expression in \textit{B. juncea} or \textit{Z. mays}, and appears to be insufficient for PC expression in \textit{Z. mays}.

**Discussion**

Preferential allocation of Cu to PC during limited Cu growth conditions was observed in this study for four out of five plant species. This observation underscores the essential role of PC in photosynthetic electron transport. Only in \textit{Z. mays} did we see a simultaneous reduction of the PC level together with Cu/ZnSOD under Cu limitation. Additionally, overall primary productivity effects were minor for all plant species, which indicates that plants have a certain plasticity that allows them to thrive on a range of Cu concentrations.

Copper-deficient plants exhibit chlorosis, stunted growth and apical meristem death with general critical Cu deficiencies ranging between 1 \( \mu \text{g} \) g$^{-1}$ and 5 \( \mu \text{g} \) g$^{-1}$ DW. The reported onset of critical Cu toxicity for many species is in the 20–30 \( \mu \text{g} \) g$^{-1}$ DW range (Marschner 1995). We observed that leaf Cu concentration varied, as expected, between sufficient and limited Cu treatments within each species. However, the differences between species were often larger. For instance, both \textit{B. juncea} and \textit{L. lycopersicum} contained more leaf Cu during limited Cu growth than \textit{Z. mays} grown on sufficient Cu. Therefore, the expression of Cu/ZnSOD, together with fluorescence measurements, may give a better indicator of Cu status.

As Cu limitation was correlated with a reduction in Cu/ZnSOD activity, it is possible that Cu availability is in part responsible for the expression of Cu/ZnSOD. During limited Cu growth conditions, preferential allocation of Cu to PC could occur by simply reducing Cu/ZnSOD. Studies analyzing the regulation of SODs, particularly Cu/ZnSOD, show increased mRNA and/or SOD activities during stress (Ehsani-Moghaddam et al. 2005, Kurepa et al. 1997a, 1997b, Pastori et al. 2000, Tewari et al. 2006), while the expression of MnSOD was constitutive under most conditions tested in \textit{A. thaliana} (Kliebenstein et al. 1998). However, these studies only supplied sufficient to toxic levels of Cu to the plants during growth.

In addition, over-expression studies for FeSOD in tobacco (Van Camp et al. 1996), poplar (Arisi et al. 1998), and \textit{Z. mays} (Van Breusegem et al. 1999) all lead to increased tolerance to oxidative stress. FeSOD has also been shown to increase naturally when plants are subjected to chloroplastic oxidative stress (Alscher et al. 2002, Tsang et al. 1991), high light (Kliebenstein et al. 1998) or Cu deficiency (Tewari et al. 2006, Yu and Rengel 1999), and decrease when exposed to excess Cu (Kurepa et al. 1997b, Tewari et al. 2006). However, those studies examining effects of deficient and excess Cu growth
conditions did not show clear reciprocal regulation of Cu/ZnSOD and FeSOD during non-stressed growth conditions; but rather linked the regulation of Cu/ZnSOD and FeSOD to Cu-related stress. Here we suggest that Cu availability is important in determining which SOD isoform plants express prior to any additional stress. However, it is important to note that regulation of SODs based on Cu status may not be uniform for all species. Zea mays, for example, may be less adapted to Cu limitation, or another important regulatory element not induced here is necessary for FeSOD expression during Cu limitation. A lack of FeSOD induction during low Cu growth while simultaneously downregulating Cu/ZnSOD for Z. mays was previously reported (Ruzsa and Scandalios 2003). Oryza sativa, however, appears to maintain both Cu/ZnSOD and FeSOD, at least in the range of Cu concentration tested here. It is possible that monocots have evolved with different mechanisms to reduce the effects of Cu limitation. With the O. sativa genome sequenced and the cloning of OsFeSOD complete (Kaminaka et al. 1999), a better understanding of SOD regulation in response to Cu in monocot species is possible.

In our study we also observed a reciprocal trend in leaf Cu and Fe concentration based on Cu availability, suggesting that Fe translocation to leaf tissue is increased during Cu limitation perhaps to compensate for Cu reduction. Several studies have indicated that Cu deficiency increases the expression of proteins likely involved in Fe uptake and shoot translocation (Mukherjee et al. 2006, Romera et al. 2003, Zheng et al. 2005).

We propose that plants that downregulate Cu/ZnSOD and upregulate FeSOD under Cu limitation can save Cu for use in PC, which is essential for photosynthesis. This plasticity in response to Cu nutrition allows plants to always have an SOD enzyme ready to scavenge superoxide radicals in the stroma and at the same time maintain PC activity for photosynthetic electron transport. We think it is reasonable that a Cu cofactor requiring protein is utilized to save Fe and perhaps buffer cellular Cu when Cu is sufficient. Downregulation of a Cu protein that can be functionally replaced by a Fe cofactor requiring protein makes sense when Cu is limited. These adaptations to varying Cu nutrient levels broaden the range of conditions under which plants can thrive.

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