Altered Cu Metabolism and Differential Transcription of Cu/Zn*Sod* Genes in a Cu/ZnSOD-Deficient Mutant of Maize: Evidence for a Cu-Responsive Transcription Factor†

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ABSTRACT: Maize inbred line A351 exhibits extremely low levels of Cu/Zn superoxide dismutase (SOD) isozymes, three cytosolic and one chloroplastic, which are increased by supplying copper to near-toxic concentrations. Activities of the copper enzymes cytochrome *c* oxidase and ascorbate oxidase are also reduced. The level of expression of the maize copper chaperone for SOD is normal to elevated. The gene transcript encoding chloroplastic SOD-1 is present at normal levels, whereas RNA levels of the cytosolic SODs are low and increase with added copper, suggesting a promoter element and copper-dependent transcription factor common to the three genes. Although a reduced level of high-affinity copper transport in A351 cannot be ruled out, high transcript levels of a constitutively expressed metallothionein, suggesting increased copper chelation capacity and creating a general copper-deprivation effect, seem to be a likely cause of the reduced levels of copper enzyme activity and Cu/Zn*Sod* gene transcripts. While exogenous copper does not affect the wild-type SOD activity or protein, it increases wild-type Cu/Zn*Sod* transcript levels in a response similar to that of several yeast genes involved in copper sequestration and antioxidant defense. A sequence that is highly homologous to those of the copper-responsive transcription factors ACE1 (*Saccharomyces cerevisiae*) and AMT1 (*Candida glabrata*) is present in the promoters of three maize Cu/Zn*Sod* genes.

Reactive oxygen species (ROS), resulting from either normal aerobic metabolism or various biotic and abiotic stresses, can lead to disease or cell death (*1*). To prevent or minimize the deleterious effects of ROS, aerobic organisms have developed complex, coordinated antioxidant defenses, both nonenzymatic and enzymatic. The latter include SODs,[1] ubiquitous metalloenzymes that catalyze the reduction of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (*2*). SODs are most often found as one of three forms: manganese (MnSODs), which are mitochondrial; iron (FeSODs), which are chloroplastic in plants and cytosolic in bacteria (and generally absent in animals); and copper/zinc (Cu/ZnSODs), which are either cytosolic or chloroplastic (*1*). The redox-active metal in each form is Mn, Fe, and Cu, respectively.

Cu is required as a cofactor for many critical enzymes in addition to Cu/ZnSODs; as such, it is indispensable for life. Paradoxically, at a concentration that is too high Cu is toxic, partly due to its ability in the presence of $O_2^{\bullet-}$ and H_2O_2 to produce •OH, the most damaging form of ROS, via $O_2^{\bullet-}$-dependent Fenton chemistry (*3*). Cu must therefore be tightly regulated as it is absorbed by organisms, transported into cells, targeted to cytosolic molecules and to subcellular compartments, and, ultimately, excreted. Cu research in mammals is drawing increasing interest, because aberrant Cu metabolism is implicated in various diseases. The earliest recognition of Cu-related pathologies came from studies on animals grazed on Cu-deficient soils and forage (*4*); thus, the uptake and metabolism of this trace metal in plants are also significant.

Antioxidant systems and copper metabolism intersect in diverse ways and at various times. Cu/ZnSODs, found in virtually all life forms, stand at three of these intersections. They require Cu for catalytic activity, reduce $O_2^{\bullet-}$ levels (thereby preventing the interaction of Cu and $O_2^{\bullet-}$ to produce •OH), and play a role in intracellular Cu buffering (*5*, *6*).

Unlike animals or single-celled organisms, in which generally only one Cu/ZnSOD and one MnSOD are found, the SOD gene−enzyme systems of plants comprise multiple forms of each. In maize, this system includes one chloroplastic and four cytosolic Cu/ZnSODs and a multigene family of mitochondrial MnSODs (*1*). The Cu/Zn isoforms, chloroplastic SOD-1 and cytosolic SOD-2, SOD-4, SOD-4A, and SOD-5, are encoded by the unlinked structural genes *Sod1*, *Sod2*, *Sod4*, *Sod4A*, and *Sod5*, respectively. The SOD gene−enzyme system of maize has been well-characterized both genetically and biochemically, and naturally occurring allelic and activity variants have been identified. This comprehensive body of knowledge in a higher eukaryote renders this a useful system in which to study the regulation and interaction of these enzymes and their genes under a variety of conditions. The low Cu/ZnSOD line A351 enables us to

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[1] Abbreviations: SOD, superoxide dismutase; *Sod*, gene or transcript encoding maize SOD; CCS, copper chaperone for SOD; MT, metallothionein; COX, cytochrome *c* oxidase; AAO, ascorbate oxidase.

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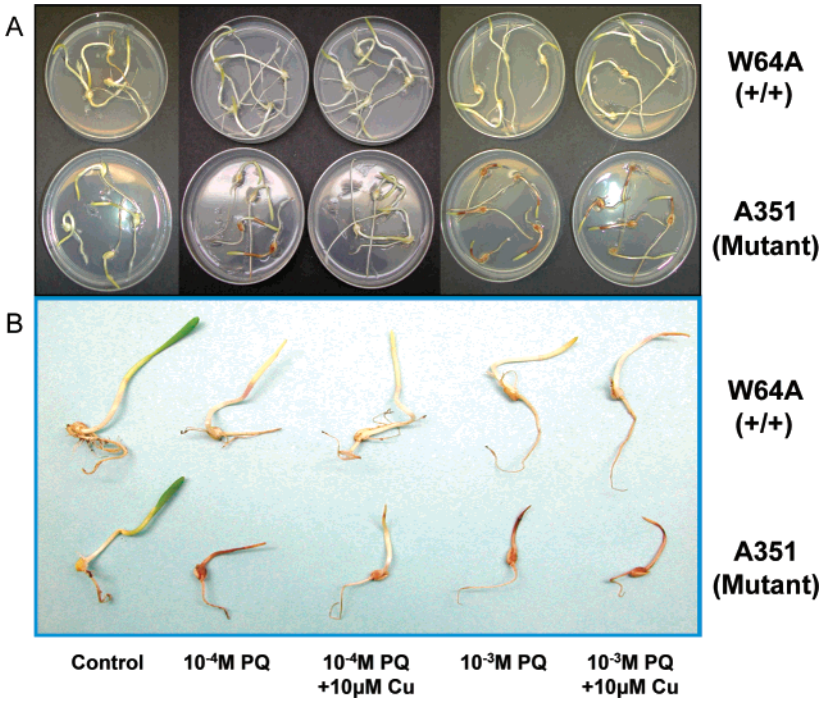

FIGURE 1: Effects of paraquat (PQ) with or without Cu on W64A vs A351: (A) 4 dpi etiolated seedlings treated in the dark for 24 h on agar plates with or without PQ, with or without Cu and (B) PQ-treated seedlings transferred to soil and a 12 h light/12 h dark cycle for 72 h.

examine more closely the roles of Cu/ZnSODs as they relate to copper metabolism.

This study was initiated to determine the basis for maize line A351's low Cu/ZnSOD phenotype, which is inherited as a quantitative trait (*7*). Because Cu is critical for SOD activity, studies were begun by adding Cu (with and without zinc) to the growth medium of A351 seedlings. From results in these initial experiments, aberrant Cu chemistry became the primary suspect responsible for the variant phenotype. In this paper, we present a model for Cu metabolism in maize with respect to the A351 SOD phenotype.

EXPERIMENTAL PROCEDURES

W64A and A351 maize stocks are maintained by this laboratory.

Paraquat Treatments. Seeds of W64A (standard SOD wild-type line) and A351 (low Cu/ZnSOD phenotype) were surface-sterilized for 10 min in 1% NaOClO, imbibed for 24 h in deionized water, and grown in constant darkness at 25 °C in sterilized trays, on Kimpack germination paper (Seedboro Equipment Co.) moistened with deionized water. At 4 days postimbibition (dpi), scutella and etiolated embryos were excised and plated on BMS medium with or without 10^{-3} or 10^{-4} M methyl viologen (paraquat, PQ) with or without 10 μM $CuSO_4$. Plates were incubated in constant darkness at 25 °C. After 24 h, representative seedlings were planted in a soil mix and grown for an additional 72 h, in a 12 h light/12 h dark cycle.

Copper Treatments. Seeds were surface-sterilized, imbibed in deionized water or in 1, 10, 100, 500, or 1000 μM $CuSO_4$, and planted as described above, the germination paper being moistened with solutions with the respective Cu concentrations. Trays were sealed with plastic film to retain moisture. Seedlings were grown to 7 dpi at 25 °C, in constant darkness, harvested, and separated into epicotyls, mesocotyls, roots, and scutella. One-half of each sample was immediately extracted for protein analysis; the other half was frozen in liquid nitrogen for subsequent RNA extraction. To minimize plant-to-plant variation, tissues from four to six seedlings, per treatment, were extracted together as one repetition for protein or RNA analysis. Two to three repetitions were carried out for each treatment. Treatments were repeated at least three times.

Protein Analysis. Samples were extracted in 25 mM glycylglycine (pH 7.4) and centrifuged for 5 min at 13 000 rpm and 4 °C. These crude extracts were used for all subsequent analyses. Activities of SOD (*8*), COX (modified from ref *9*), and AAO (*10*) were determined spectrophotometrically. The protein concentration was determined (*11*), and equal protein samples were separated on starch gels to produce SOD zymograms (*12*). Samples were also separated by 13% SDS−PAGE (*13*), transferred to nitrocellulose, and probed with polyclonal antibodies against maize SOD-1, SOD-3, or SOD-4 (*14*). Additional membranes were probed with antibodies against human and yeast CCS.

RNA Analysis. Total RNA was extracted (modified from ref *15*), and 10 or 20 μg of RNA from scutella or other tissues, respectively, was separated on 1.6% denaturing agarose gels and blotted to nylon membranes (*16*). The membranes were sequentially hybridized, in modified Church buffer (*17*), with ^{32}P-labeled (*18*) gene-specific DNA probes for *Sod1*, *Sod2*, *Sod4*, and *Sod4A*, and a full-length probe for the multigene *Sod3* family. Fresh membranes were probed with a nearly full-length cDNA for CCS from mouse brain and with four MT probes from *Arabidopsis* (*Arabidopsis thaliana*): MT1a, MT2a, MT2b, and MT3. As a final step, filters were probed with pHA2, containing an *18S* sequence, to confirm the equal loading of gels (*19*). RNA analyses were repeated three times, from three independent treatments.

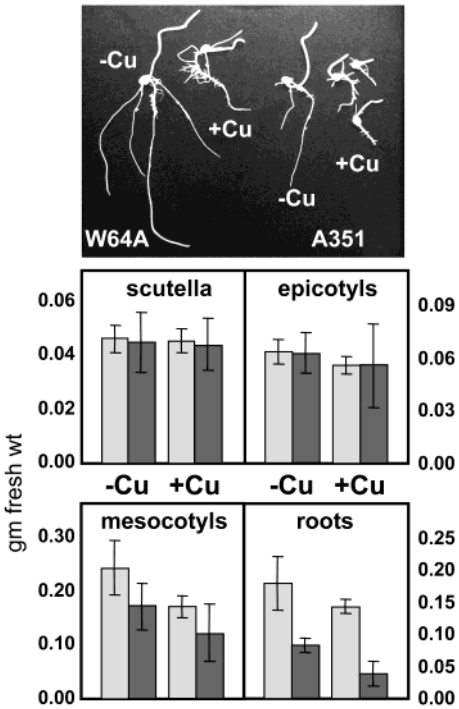

FIGURE 2: Effects of Cu on the growth of maize seedlings. The 7 dpi etiolated seedlings were grown with or without Cu, and for +Cu, [Cu] = 1 mM: (light gray) W64A and (dark gray) A351.

RESULTS

PQ Sensitivity in A351 and W64A with or without Cu. To confirm the PQ sensitivity of A351, seedlings were exposed to PQ in vitro with or without Cu. A351 seedlings exhibited browning of mesocotyls and inhibition of growth when exposed for 24 h to 10^{-3} or 10^{-4} M PQ. Addition of 10 μM Cu alleviated these symptoms, but only at 10^{-4} M PQ (Figure 1A). W64A seedlings were unaffected by a 24 h PQ exposure, with or without Cu. Control seedlings of both maize lines grew normally when transferred to soil, with shoots beginning to green after 3 days in a light/dark cycle (Figure 1B). PQ-treated W64A seedlings survived, though exhibiting chlorosis. A351 seedlings treated with 10^{-3} M PQ with or without Cu did not survive, nor did those treated with 10^{-4}M PQ alone. A351 seedlings exposed to 10^{-4} M PQ with Cu survived, though they were stunted, compared to W64A, and chlorotic.

Toxicity of Cu Treatments. Plants exhibit typical symptoms in response to increasing Cu levels. To determine whether the treatments that were used approached toxic levels, seedling growth and tissue weights were recorded. While the fresh weight of scutella and epicotyls changed little in both maize lines, the mesocotyl weight and length decreased with added Cu, and the root weight decreased significantly (Figure 2). Roots also decreased in length and exhibited increased growth of root hairs.

Effect of Exogenous Cu on Cu/ZnSOD Activity. A351 control tissues express significantly less SOD activity than the wild type, averaging 26−44% of that of W64A (Figure 3A). While SOD activity in W64A was unaffected by exogenous Cu, it rose significantly in A351, to near-wild-type levels at 1 mM Cu. This increase was observed in individual isozyme bands (Figure 3B) and in total activity (Figure 3A), the latter being due to increases in all Cu/ZnSODs (SOD-1, SOD-2, and SOD-4/4A). No change was noted in the SOD-3 band (Figure 3B) or in SOD activity measured with the addition of 1 mM KCN (data not shown), which inhibits Cu/ZnSOD activity.

Activities of Cu Enzymes COX and AAO. To determine whether A351's low enzyme activity is restricted to Cu/

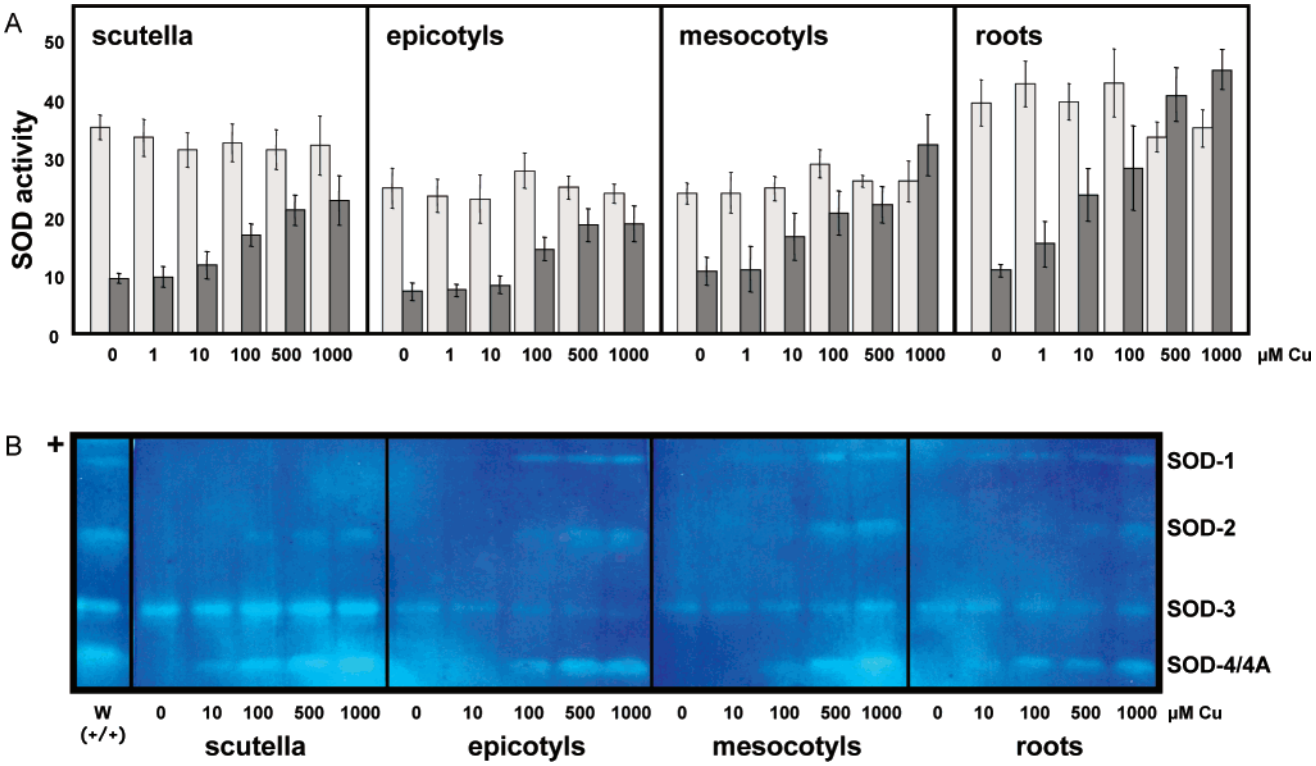

FIGURE 3: Effect of increasing copper concentration on SOD activity. (A) Total SOD activity, in units per milligram of total protein: (light gray) W64A and (dark gray) A351. (B) Zymograms stained for SOD (SOD-4 and SOD-4A isozymes comigrate).

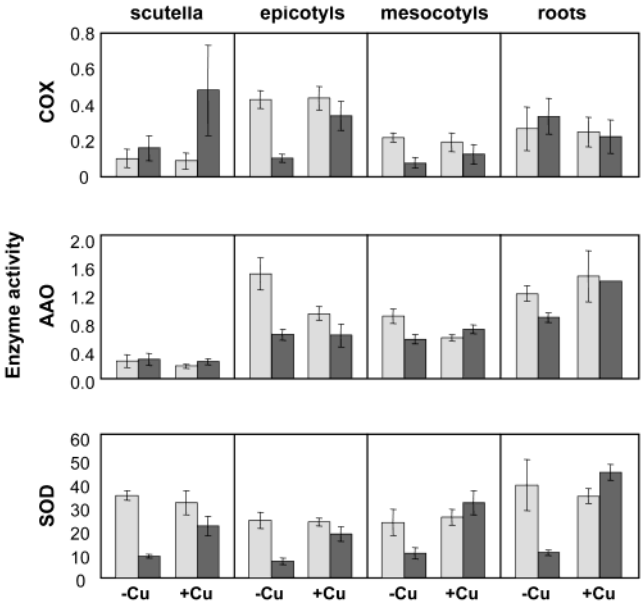

FIGURE 4: Activity of three Cu enzymes, with or without Cu. Activity is in units per milligram of total protein, and for +Cu, [Cu] = 1 mM: (light gray) W64A and (dark gray) A351.

ZnSOD, activities of two additional Cu enzymes were monitored (Figure 4). COX activity in A351 scutella and roots was no different than in W64A, whereas it was significantly reduced in A351 epicotyls and mesocotyls (24−36% of that of the wild type). In response to 1 mM Cu, COX levels were unchanged in W64A but increased in A351 scutella and epicotyls. AAO activity was significantly reduced in all A351 tissues except scutella, ranging from 42 to 71% of that of the wild type. With 1 mM Cu, AAO activity decreased in W64A epicotyls and mesocotyls while remaining unchanged in A351. (The apparent increase in AAO levels in A351 roots is based on only one measurement and may not be statistically valid.)

Protein Levels of Cu/ZnSODs with or without Cu. Although Cu/ZnSOD isozyme activity was known to be reduced in A351, the corresponding protein levels had not previously been determined. To measure the amount of SOD protein with or without Cu, Western blots were probed with antibodies recognizing the various maize SODs. In all A351 tissues, changes in the immunoreactive protein (Figure 5) corresponded closely to changes in isozyme activity. No changes were noted in W64A protein, corresponding to the lack of change in the observed enzyme activity.

Effect of Cu on Sod Transcripts. To determine the relative transcript levels of *Sod*s in A351 with or without Cu, RNA blots were probed with gene-specific or full-length DNA fragments for the maize *Sod* genes. While levels of the *Sod1* transcript were comparable in both maize lines, increasing somewhat and to the same extent with added Cu, levels of *Sod2*, *Sod4*, and *Sod4A* RNA were extremely low in A351 and increased with Cu, consistent with changes in the corresponding isozyme levels (Figure 6). *Sod3* remained unaffected. In W64A, levels of the Cu/Zn*Sod* gene transcripts also increased, but to a much lesser degree.

Expression of MTs in A351 and W64A. Probing with DNA sequences from *Arabidopsis* MTs was carried out to analyze relative MT levels in A351 and W64A. A transcript detected by MT1a was present in most A351 tissues but absent in

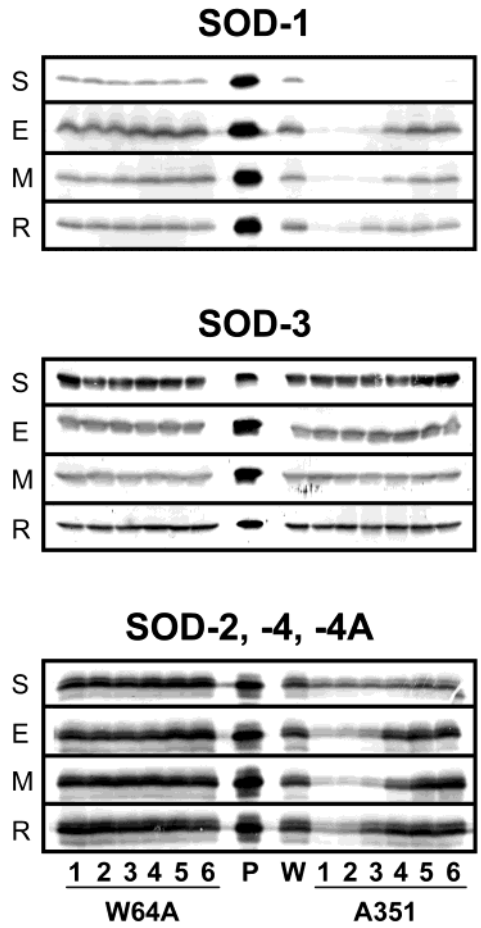

FIGURE 5: Western blots probed with SOD antibodies. S is scutella, E epicotyls, M mesocotyls, and R roots. Concentration of Cu: 0 μM (1), 1 μM (2), 10 μM (3), 100 μM (4), 500 μM (5), and 1 mM (6). P is pure protein (SOD-1, SOD-3, or SOD-4). W is a W64A 0 μM control (SOD-4 antibodies cross-react with SOD-2 and SOD-4A).

W64A. It exhibited no significant change in response to Cu (Figure 7A,B). Where detectable, levels of RNA recognized by MT2a, MT2b, and MT3 were not significantly different in A351 and W64A (Figure 7A). The level of MT2a RNA, as detected in scutella, showed a slight increase in both maize lines in response to an increasing Cu level (Figure 7B).

Maize Cu Chaperone for the SOD (ZmCCS) Protein and Transcript. Yeast and human antibodies to CCS (yCCS and hCCS) were used to probe Western blots of maize tissue extracts to determine whether this protein is expressed, and at what levels, in A351. An ~35000 kDa protein was recognized by the yCCS antibody in scutella and epicotyls, though the strength and size of the signal varied (Figure 8A). In scutella, bands with similar intensities, but slightly larger than yCCS, were recognized in both maize lines. In epicotyls, the signal was stronger in A351 than in W64A, and the latter band was smaller, corresponding more closely to pure yCCS. A mouse CCS DNA probe detected a band in maize epicotyl RNA (Figure 8B), which was equally strong in both lines.

A Maize Promoter Sequence that Is Highly Homologous to a Yeast Cu-Responsive Element. On the basis of the reactions of wild-type Cu/Zn*Sod* genes to exogenous Cu, we searched the promoters of *Sod1*, *Sod4*, and *Sod4A* for the Cu-responsive ACE1 sequence from genes encoding Cu/Zn*SOD1* and MTs *CUP1* and *CRS5* in *Saccharomyces cere-*

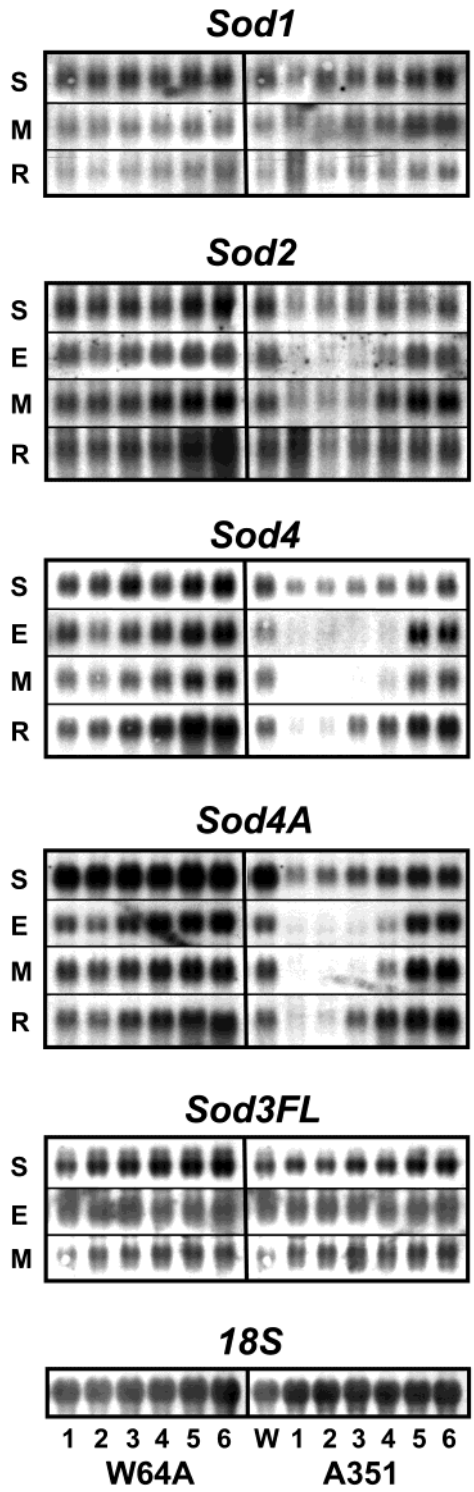

FIGURE 6: Responses of *Sod* RNAs to increasing Cu concentrations. *Sod1*, *Sod2*, *Sod4*, and *Sod4A* are Cu/Zn*Sod*s. *Sod3* is an Mn*Sod*. *18S* is a ribosomal loading control. DNA fragments are gene-specific except where noted as full length (FL). S is scutella, E epicotyls, M mesocotyls, and R roots. Concentration of Cu: 0 μM (1), 1 μM (2), 10 μM (3), 100 μM (4), 500 μM (5), and 1 mM (6). W is a W64A 0 μM control.

visiae and the homologous AMT1 sequence from genes encoding MTs and the *AMT1* gene itself in *Candida glabrata*. The consensus sequence was found intact in maize *Sod1* and *Sod4A*, and nearly intact in *Sod4* (Figure 9). The conserved T in the AT-rich region is absent in *Sod4*, being replaced with an A.

DISCUSSION

In maize line A351, activities of three Cu enzymes, Cu/ZnSOD, COX, and AAO, are significantly reduced compared with those of wild-type W64A (Figure 4). A decreased activity of multiple Cu-containing enzymes is typical of general Cu deprivation. In leaves of Cu-deficient clover, activities of several Cu enzymes are depressed, including AAO (*20*), and Cu-starved yeast and rats exhibit decreased Cu/ZnSOD and COX activities (*21*−*23*). Even when nutritional levels of Cu are optimal, aberrant Cu metabolism within an organism can mimic symptoms of external deprivation. In the nicotianamine-free mutant of tomato, which exhibits Cu deficiency in the shoot, cytosolic and chloroplastic Cu/ZnSOD levels are reduced, even when exogenous Cu is supplied (*24*). Various disruptions in the yeast Cu trafficking pathway also result in lower levels of Cu enzyme activities. A lack of high-affinity Cu transport leads to a general decrease in Cu enzyme activities (*25*), whereas the lack of a particular Cu chaperone results in a decreased activity or a lack of a particular enzyme, such as SOD or COX (*26*).

Altered Cu metabolism in A351 is also suggested by two other observations. A351, like other reported SOD-deficient mutants (*3*), is more sensitive to PQ than is W64A (Figure 1), and added Cu alleviates this sensitivity. An exogenous supply of near-toxic levels of Cu to A351 seedlings (Figure 2) results in near-normal levels of SOD activities, proteins, and transcripts (Figures 3, 5, and 6), ruling out aberrancies in the *Sod* structural genes themselves.

For Cu-requiring systems to function normally, a highly regulated Cu homeostasis is necessary, maintained in most organisms by a complex scheme of cellular uptake and efflux, intracellular distribution and sequestration, and the regulation of genes involved in these processes. While the greatest amount of information is available for yeast, mammals, and bacteria (*25*−*27*), homologous systems have been reported in plants (*28*−*30*). On the basis of our results and on the basis of systems in other organisms, we present a model of Cu uptake and distribution in maize relative to the A351 Cu/ZnSOD phenotype (Figure 10).

Cu uptake by cells occurs via Cu transport proteins (CTRs), which have been well-characterized in yeasts and mammals (*25*, *31*). Although less is known about the transport of Cu across plant cell membranes, a protein able to complement yeast null for high-affinity Cu transport has been reported in *Arabidopsis* (*32*). Disruption of Cu transport in yeast results in a Cu/ZnSOD null phenotype (*33*). The SOD activity can be partially restored by the addition of Cu (*31*, *33*). Although no change in AAO activity is observed in maize line A351 (Figure 5B), COX and SOD activities are both increased by exogenous Cu (Figures 3 and 4). This reduced activity, restored by increasing concentrations of extracellular Cu, of three distinct Cu enzymes suggests either a lack of Cu transport into A351 cells or some mechanism whereby Cu entering the cells is prevented from reaching these enzymes.

To ensure the appropriate distribution of Cu within cells while preventing indiscriminate binding or sequestration, target-specific Cu chaperones bind Cu as it enters the cell and deliver it to intracellular sites (*26*). Chaperones have been identified that deliver Cu to the secretory system (*34*),

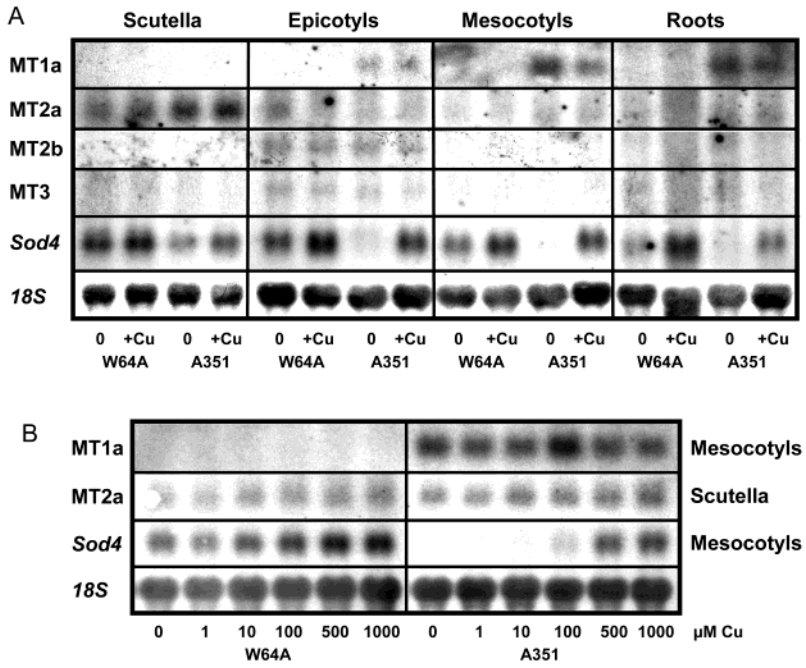

FIGURE 7: Presence of putative *ZmMt* transcripts and their responses to Cu. RNA blots probed with *Arabidopsis* MT DNA sequences. *Sod4* is a maize control, and *18S* is a ribosomal loading control. (A) For +Cu, [Cu] = 1 mM. (B) The concentration of Cu is in micromolar.

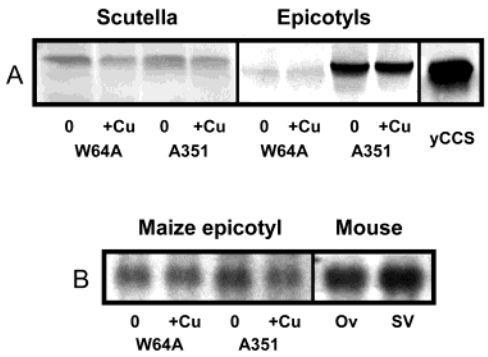

FIGURE 8: Expression of the maize copper chaperone for SOD (CCS) protein and RNA. For +Cu, [Cu] = 1 mM. (A) Western blot probed with yeast CCS antibodies. The yeast control is pure yCCS. (B) RNA blot probed with mouse CCS DNA. Mouse RNA controls are ovaries (Ov) and seminal vesicles (SV).

to mitochondria for incorporation into COX (*9*), and to cytosolic Cu/ZnSODs (*35*, *36*). No chaperones had been confirmed in maize, although several have been reported in other plants (*29*), including CCS in tomato and *Arabidopsis* (*37*).

Yeast mutants null for CCS exhibit a phenotype similar to Cu/ZnSOD null mutants, with their attendant PQ sensitivity (*36*). Addition of exogenous Cu overcomes the lack of CCS and restores SOD activity, although somewhat short of wild-type levels (*38*), similar to the results observed in A351 (Figure 3), suggesting that the maize CCS homologue might be absent. Since homologous Cu chaperones are highly conserved among species, we attempted to detect the ZmCCS protein and transcript using yeast and human CCS antibodies and a mouse CCS DNA clone. Blots hybridized with yCCS confirm the presence of a CCS homologue in both maize lines (Figure 8A). The Zm*Ccs* transcript is also detected, confirming normal expression of this gene in A351 (Figure 8B). These data indicate that the A351 SOD phenotype is not due to aberrant CCS.

Many key enzymes in higher plants, including mitochondrial COX and cytosolic AAO, require Cu. Although no Cu chaperone to mitochondria has yet been reported in plants, the presence of highly conserved, homologous systems for all other chaperones found to date leads us to postulate that this chaperone is present in maize as well. No chaperone has yet been identified for Cu transport to AAO (or to any cytosolic Cu enzyme other than SOD) or to chloroplasts, though a putative copper transporter, CutA, targeted to the chloroplast, has been identified in *Arabidopsis* on the basis of sequence comparison (*39*). How and where maize SOD-1 acquires Cu, whether in the cytosol, where it is translated, or in the chloroplast lumen, its ultimate destination, remain unclear, but metal acquisition by photosynthetic Cu proteins in the green algae *Chlamydomonas reinhardtii* has been well-characterized. Plastocyanin receives its cofactor in the lumen, requiring delivery of Cu to this compartment (*40*). In cyanobacteria, an ATX1 homologue has recently been reported that delivers copper to thylakoid membranes (*41*), where, in the absence of chloroplasts and mitochondria, are found the photosynthetic machinery and respiratory machinery of this organism, including plastocyanin and cytochrome oxidase. Since COX, AAO, and SOD-1 activities are all reduced in A351, we cannot unequivocally state that the respective putative Cu chaperones are normal. However, the pleiotropic Cu-deprivation effect in A351 is more likely due to a single global cause, affecting overall intracellular Cu levels, rather than to multiple, target-specific defects.

Chaperones are deemed necessary due to the normally high Cu-chelation capacity of most cells (*38*). Supranormal competition for intracellular Cu may be responsible for the A351 phenotype, possibly due to MTs. These low-molecular weight, cysteine-rich proteins with high metal binding capacity play important roles in metal sequestration and in defense under conditions of metal excess in animals and yeast (*27*, *42*) and may share these functions in plants (*30*, *43*). Plant MT genes and their Cu-binding products have been

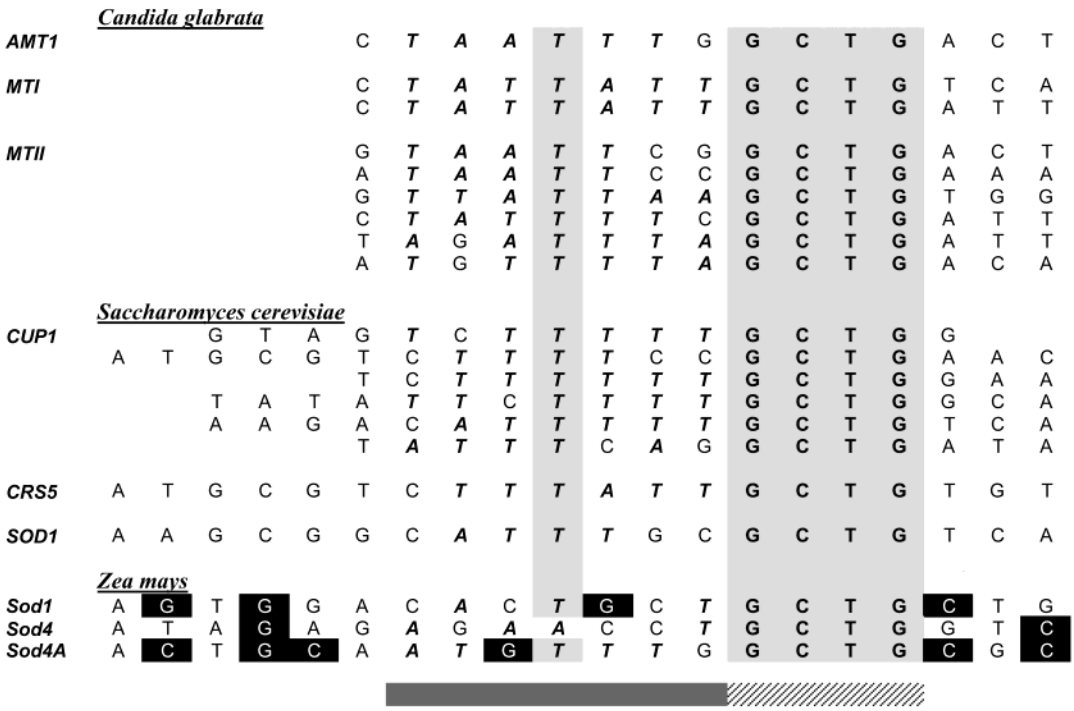

FIGURE 9: Cu-responsive elements in yeast genes compared with sequences from maize *Sod* gene promoters. The light gray blocks are consensus sequence (TnnnGCTG, where n is any nucleotide). The cross-hatched bar is the core sequence. The medium gray bar is an AT-rich area (bold and italicized As and Ts); white lettering on a black background denotes *Zea mays*-specific nucleotides. The *AMT1* gene itself contains the AMT1 sequence (yeast sequences adapted from refs *29*, *49*, and *54*).

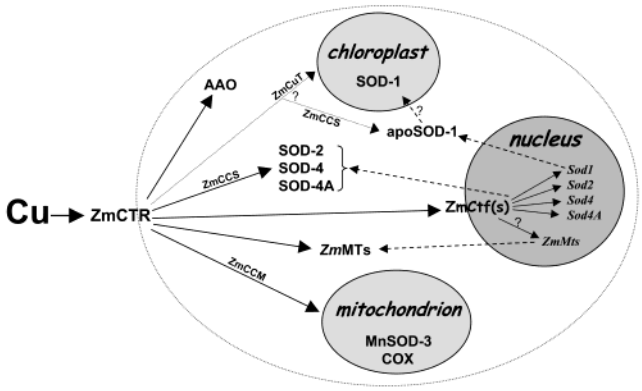

FIGURE 10: Model for Cu transport in maize cells relative to the A351 phenotype. Zmx is a *Z. mays* homologue of molecules reported in other species. Cu enters cells via a CTR (Cu transport protein). It is delivered to mitochondrial enzymes by CCM (Cu chaperone to mitochondria) and to cytosolic SODs by CCS (Cu chaperone for SOD). ApoSOD-1 may receive its cofactor in the cytosol, via CCS, or in the chloroplast, via a chaperone targeted to that organelle, designated CuT (Cu chaperone/transporter to chloroplasts). Cytosolic AAO also requires Cu, and MTs sequester Cu in the cytosol. Ctfs (Cu-responsive transcription factors) may receive Cu in the cytosol or in the nucleus and may be responsible for Cu-dependent basal transcription of *Sod2*, *Sod4*, and *Sod4A*, as seen in A351, and for Cu-responsive transcription of *Sod1* in A351 and of all Cu/Zn*Sod* genes in W64A.

best characterized in *Arabidopsis*. Maize line A351 exhibits elevated levels of a transcript homologous to MT1a (Figure 7), which is constitutively expressed in *Arabidopsis* seedlings. As in that species (*44*), maize MT1a does not respond significantly to exogenous Cu. The same transcript is undetectable in the maize wild-type line, regardless of Cu concentration. These transcript levels suggest a significant difference in Cu-chelation capacity between W64A and A351, leading to a lack of readily available Cu in A351 and leading, in turn, to the respective activity levels of the three Cu enzymes that have been examined.

It is interesting to note that the higher levels of MT1a do not render A351 Cu tolerant. In fact, A351 seems to succumb more readily to Cu toxicity, as seen in its more drastically retarded growth, typical of Cu toxicity (*30*), compared with W64A (Figure 2). This increased sensitivity to Cu supports overchelation capacity being the cause for the A351 phenotype, since near-toxic levels of Cu are needed to restore SOD and COX activity levels and Cu/Zn*Sod* RNA levels, indicating that Cu-chelation limits have been reached.

Although both Cu-dependent and Cu-independent transcription of various Cu proteins has been reported (*21*−*23*, *40*), differential transcription of genes encoding multiple Cu protein isoforms within one organism, as is observed in A351 (Figure 6), had not previously been reported. The defining factor in A351 is the ultimate destination of the holoenzyme: chloroplast versus cytosol. While the transcript levels of *Sod1*, encoding chloroplastic SOD-1, are not significantly different in both maize lines and increase only moderately with added Cu, in sharp contrast to SOD-1 protein and activity levels, transcript levels of *Sod2*, *Sod4*, and *Sod4A* (Figure 6) correlate closely with activity and protein levels of cytosolic SOD-2, SOD-4, and SOD-4A (Figures 3B and 5).

The low basal transcript levels of the cytosol-destined isoforms in A351 may be due to a promoter element and Cu-dependent transcription factor common to the three genes. Though significantly different, the promoters of *Sod4* and *Sod4A* are more similar to one another than to the *Sod1* promoter, making a differential effect with respect to intracellular destination plausible. Although no factor has been reported to play a role in basal *Sod* gene expression,

there are such examples among MTs. Basal and metal-induced expression of trout MTs may be due to separate transcription factors (*45*). In the yeast *S. cerevisiae*, ACE2 plays a role in basal levels of the MT *CUP1* gene transcript while ACE1 is involved in both basal and metal-induced *CUP1* expression (*27*, *46*). While certain mutations in ACE1 lead to low basal transcript levels that are not increased by Cu, a comparable mutation in a putative metal-induced transcription factor in maize line A351 is unlikely, since added Cu restores transcripts to wild-type levels (Figure 6).

Since normal levels of the *Sod1* transcript are detected in A351 (Figure 6), a translational or post-translational mechanism must be in place either to prevent the production of immunodetectable and active SOD-1 protein (Figures 3 and 5) or to allow for its degradation in the absence of Cu. In *C. reinhardtii*, the plastocyanin transcript is produced and translated under conditions of Cu deficiency and the apoprotein is imported into chloroplasts, but without incorporation of Cu, it is rapidly degraded by a protease that is active only under conditions of Cu deficiency (*40*). A similar mechanism may be at work in A351, allowing for normal levels of the *Sod1* transcript but little to no enzyme activity or protein.

Although SOD enzyme activity in W64A remains unaffected by added Cu (Figures 3A and 5), wild-type transcript levels for all Cu/Zn*Sod* genes increase steadily (Figure 6). In addition to acting as a critical enzyme cofactor, Cu also regulates several nuclear genes involved in yeast Cu metabolism, including CTRs and MTs, through the action of Cu-responsive transcription factors (*27*, *47*). One of these is the ACE1 trans-acting factor, which is brought into a conformational state capable of binding DNA only after activation by Cu (*48*). ACE1 activates the *S. cerevisiae* Cu/Zn*SOD1* and MT *CUP1* and *CRS5* genes (*29*). While there have been no reports of a similar effect on *C. glabrata* Cu/Zn*SOD*, a homologous factor, AMT1, activates the MT genes of *C. glabrata* (*49*). ACE1 and AMT1 binding sites share a consensus sequence, which is present multiple times in the yeast *CUP1* gene and in the *C. glabrata MTI* and *MTII* genes (*49*, *50*). In contrast, there is only a single ACE1 copy in the yeast *CRS5* and *SOD1* genes (*51*, *52*). The responses to Cu of each of the genes are proportional to the frequency of occurrence of the ACE1 or AMT1 binding sequence (*27*, *49*, *51*). In maize, the responses to Cu of the Cu/Zn*Sod* genes in W64A (Figure 6) most closely resemble those of the yeast *CRS5* and *SOD1* genes. Single copies of the ACE1/AMT1 sequence have been identified in the promoters of maize *Sod1*, *Sod4*, and *Sod4A* (Figure 9). These sequences are highly homologous to 15−20 bp sequences of ACE1/AMT1 binding sites and include the core sequence, GCTG, downstream of an AT-rich region. The response of the W64A Cu/Zn*Sod* genes to Cu agrees with the presence of a single copy of this sequence. This is the first report of Cu-activated transcriptional regulation of Cu/Zn*Sod* genes in a plant.

In conclusion, the low Cu/ZnSOD phenotype of maize line A351 is most likely due to a lack of readily available intracellular Cu, as supported by the reduced activity levels of two other Cu enzymes, COX and AAO. The restoration of enzyme activity through the addition of Cu points to a defect either in Cu transport or in intracellular Cu delivery. Although disruption of high-affinity Cu transport cannot be ruled out, high RNA levels of the constitutively expressed MT1a lead us to postulate that a chronic intracellular overchelation capacity for Cu in A351 results in a general Cu-deprivation effect. Included in this effect, basal transcript levels of the cytosol-destined isoforms of SOD are shown to be Cu-dependent, while transcript levels of the chloroplast-destined form are Cu-independent. The restoration of Cu enzyme activity levels and of *Sod* transcript levels depends on the chelation capacity of the cells being exceeded, as is apparent in the increasingly evident symptoms of Cu toxicity as wild-type levels of Cu enzymes and *Sod* transcripts are achieved.

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