A Copper Chaperone for Superoxide Dismutase That Confers Three Types of Copper/Zinc Superoxide Dismutase Activity in Arabidopsis

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The copper chaperone for superoxide dismutase (CCS) has been identified as a key factor integrating copper into copper/zinc superoxide dismutase (CuZnSOD) in yeast (Saccharomyces cerevisiae) and mammals. In Arabidopsis (Arabidopsis thaliana), only one putative CCS gene (AtCCS, At1g12520) has been identified. The predicted AtCCS polypeptide contains three distinct domains: a central domain, flanked by an ATX1-like domain, and a C-terminal domain. The ATX1-like and C-terminal domains contain putative copper-binding motifs. We have investigated the function of this putative AtCCS gene and shown that a cDNA encoding the open reading frame predicted by The Arabidopsis Information Resource complemented only the cytosolic and peroxisomal CuZnSOD activities, which has lost all CuZnSOD activities. However, a longer AtCCS cDNA, as predicted by the Munich Information Centre for Protein Sequences and encoding an extra 66 amino acids at the N terminus, could restore all three, including the chloroplastic CuZnSOD activities in the Atccs knockout mutant. The extra 66 amino acids were shown to direct the import of AtCCS into chloroplasts. Our results indicated that one AtCCS gene was responsible for the activation of all three types of CuZnSOD activity. In addition, a truncated AtCCS, containing only the central and C-terminal domains without the ATX1-like domain failed to restore any CuZnSOD activity in the Atccs mutant. This result indicates that the ATX1-like domain is essential for the copper chaperone function of AtCCS in planta.

Reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, are generated as a by-product in cells from physiological reactions such as electron flow in chloroplasts and mitochondria and some redox reactions (Fridovich, 1978). Accumulation of ROS might cause damages to living organisms (Imlay and Linn, 1988; Bowler et al., 1992; Mehdy, 1994). Superoxide dismutase (SOD), a group of metalloenzymes, belongs to the enzymatic defense system against ROS and catalyzes the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide (Beyer et al., 1991). Copper/zinc SOD (CuZnSOD), manganese SOD (MnSOD), and iron SOD (FeSOD) are three types of SODs reported in plants.

MnSOD is localized in mitochondria, whereas FeSOD is localized in chloroplasts (Jackson et al., 1978). CuZnSOD is present in three isoforms, which are found in the cytosol, chloroplast, and peroxisome (Kanematsu and Asada, 1989; Bowler et al., 1992; Bueno et al., 1995). In Arabidopsis (Arabidopsis thaliana), three CuZnSOD genes, CSD1, CSD2, and CSD3, have been identified. The CSD1 and CSD2 activities are detected in roots, leaves, stems, and siliques, and their proteins are localized in the cytosol and chloroplast, respectively. The CSD3 is proposed to be a peroxisomal protein because its carboxyl terminus contains the Ala-Lys-Leu tripeptide, a typical peroxisomal targeting signal (Kliebenstein et al., 1998). CuZnSOD is a homodimeric copper- and zinc-containing enzyme (McCord and Fridovich, 1969). Zinc is required for the structural integrity of the protein and influences enzyme activity, whereas copper plays a catalytic role in the disproportion of superoxide (Forman and Fridovich, 1973; Beem et al., 1974). The acquisition of both metal ions was assumed to be by passive diffusion; however, Rae et al. (1999) found that the intracellular concentration of free copper is undetectable under normal physiological conditions. Therefore, cells require copper chaperones to facilitate copper transfer to specific partners (Valentine and Gralla, 1997).

Copper chaperones are a novel class of proteins involved in intracellular trafficking and delivery of copper to copper-containing proteins (Harrison et al., 1999). The proteins involved in copper trafficking in yeast (Saccharomyces cerevisiae) are Lys7 (Horecka et al., 1995; Culotta et al., 1997), ATX1 (Lin and Culotta, 1995; Lin et al., 1997), and COX17 (Glerum et al., 1996). Lys7 is the largest by molecular mass among the three; it inserts copper into CuZnSOD and was designated as copper chaperone for SOD (CCS; Culotta et al., 1997). CCS possesses three functionally distinct protein domains. The N-terminal ATX1-like domain, bearing striking homology to ATX1 and containing the
MXCXXC copper-binding site, is required for CCS function under strict copper limitation conditions in vivo (Schmidt et al., 1999). The central domain displays sequence homology to its target protein, CuZnSOD, and physically interacts with SOD1 in human (*Homo sapiens*) and yeast (Casareno et al., 1998; Lamb et al., 2000, 2001; Schmidt et al., 2000). The C-terminal domain carries a conserved Cys (CXC) motif and may play a crucial role in copper transfer to CuZnSOD (Schmidt et al., 1999). Besides, the interaction with SOD1 also requires the C-terminal domain (Schmidt et al., 2000). Although the C-terminal domain is relatively short (30–40 residues), it is unique to CCS proteins from diverse species among identified copper

**Figure 1.** Sequence and expression of the *AtCCS* gene. A, The *AtCCS* gene structure. Black boxes represent the predicted exons and the gray box flanked by two ATGs represents part of the first exon not predicted in TAIR database. Lines between the boxes represent introns. ACCS-15 and ACCS-12 are primers designed for cloning the *AtCCS* cDNA (accession no. DQ003058). ACCS-8 is the primer used for the 5′-RACE experiment. ACCS-1 is the primer used for RT-PCR analysis. The same primers (ACCS-15 and ACCS-12) were also used for genomic PCR (G-PCR). *Actin2* (*ACT2*), shown in the inset section, was used as an internal control. The size of DNA markers (M) in kilobases (kb) was shown at the left. C, The 5′-end sequence of *AtCCS*. The positions of the two in-frame ATGs are boxed and the sequences corresponding to the primer ACCS-8 are underlined. Arrows indicate the 5′-end of the different clones obtained by 5′-RACE and the accession numbers for the four clones are DQ003054, DQ003055, DQ003056, and DQ003057, respectively. D, The deduced amino acid sequence and domain structure of *AtCCS* gene. Amino acid numbers are indicated at the right. Residues conserved in all CCS homologs are shown in bold. The putative chloroplast-targeting peptide and peroxisomal-targeting signal are boxed and underlined, respectively. The two conserved copper-binding motifs are shown with gray boxes.
chaperone proteins (Schmidt et al., 1999). Genes that complement yeast *lys7*, *atx1*, and *cox17* mutants have been identified in tomato (*Lycopersicon esculentum*) and Arabidopsis. Two of the cDNA clones, LeCCS (AF117707, tomato) and AtCCS (AF179371, Arabidopsis), are predicted to encode chloroplast-localized proteins but with an incomplete chloroplast-targeting transit peptide (Zhu et al., 2000). Wintz and Vulpe (2002) searched the Arabidopsis genome and concluded that AtCCS (AF179371.1, At1g12520) is the only yeast CCS homolog in Arabidopsis. The open reading frame of AtCCS contains two in-frame ATGs at the 5'-end of the predicted transcript. Because no other gene encodes for a potential cytosolic AtCCS-like chaperone in Arabidopsis, Wintz and Vulpe (2002) hypothesized that two transcription initiation sites may be involved in the production of different forms of AtCCS from the single AtCCS gene. However, The Arabidopsis Information Resource (TAIR) database predicts the initiation codon of AtCCS gene to be the second ATG, resulting in a polypeptide of 254 amino acids, while the Munich Information Centre for Protein Sequences (MIPS) predicts the initiation codon of AtCCS gene to be the first ATG, resulting in a polypeptide of 320 amino acids. Trindade et al. (2003) cloned a StCCS gene from potato (*Solanum tuberosum*) that also carried a plastidic transit peptide and was expressed only in stem-like tissues; unlike Arabidopsis, potato has another copy of the StCCS homolog that is expressed in leaves.

In yeast, a truncated yCCS without the ATX1-like domain complemented the *lys7* null mutant weakly, compared with the intact yCCS. This result indicated that the ATX1-like domain is important for the yCCS activity (Schmidt et al., 1999). Furthermore, in vitro studies of LeCCS suggested that the ATX1-like domain is indispensable for copper and/or zinc ion binding during the metal delivery process (Zhu et al., 2000).

To further study the function of AtCCS, we have obtained an AtCCS knockout mutant. We show here that the mutant has lost all three forms of CuZnSOD activity. Complementation of the mutant with the cDNA encoding the 320-amino acid polypeptide, as annotated by MIPS, restored all three activities. The results suggest that one AtCCS gene encodes both the cytosolic and the chloroplastic forms of AtCCS and activates CuZnSOD activities at different subcellular locations. In addition, we show that the ATX1-like domain of AtCCS is essential for the copper chaperone function in planta.

**RESULTS**

**AtCCS Gene in Arabidopsis**

In Arabidopsis, the AtCCS gene (At1g12520) is composed of six exons and five introns. Analysis of the genomic sequence relevant to this gene revealed the existence of an additional ATG located upstream of the annotated initiation codon, and this additional ATG is in frame with the open reading frame annotated in TAIR database (Fig. 1A). This additional ATG is the start codon predicted in the MIPS Arabidopsis database (http://mips.gsf.de/proj/thal/db/index.html). In TAIR database, the AtCCS gene is predicted to encode a 254-amino acid polypeptide containing no signal peptide for organelle targeting (designated hereinafter as AtCCScytosolic [AtCCScyt]). Whereas, the AtCCS gene in the MIPS database is predicted to contain an additional 66 amino acids carrying a putative chloroplastic targeting signal (designated hereinafter as AtCCS chloroplastic [AtCCScp]). However, the upstream ATG was not found in four cDNA clones encoding AtCCS (AF179371, AF061517, Ay050357, and GSLTSIL10ZC12) identified in the MIPS database. In
our results, the upstream ATG is present in a 979-bp reverse transcription (RT)-PCR product amplified from rosette leaves and flowers using a specific primer set, ACCS-15 and ACCS-12 (Fig. 1, A and B). This confirmed the existence of the upstream ATG in the AtCCS transcript. Sequence comparison between the genomic PCR and RT-PCR products amplified by the same primer pair positioned the exons and introns as shown in Figure 1A. Thirteen cDNA clones obtained by 5′-RACE were randomly selected for sequencing. The results indicated that the first ATG existed in the 5′-end of all 13 AtCCS transcripts. Moreover, 10 clones were mapped to one nucleotide upstream, while the other clones were mapped to four, 14, and 26 nucleotides upstream of the first ATG, respectively (Fig. 1C, arrows).

In Figure 1D, bioinformatic searches (on Web sites of http://www.cbs.dtu.dk/services/ChloroP and http://www.cbs.dtu.dk/services/TargetP) revealed that the additional 66 amino acids encoded by the sequence between the first and second ATG comprise a potential chloroplast-targeting transit peptide. The following 67th to 161st amino acids contain a highly conserved domain, MXXCXXC, which is a homologous domain of the ATX1 copper chaperone. The central domain (162nd to 290th amino acids) shares 19.1% sequence similarity to CSD1, and 22.7% similarity to both CSD2 and CSD3 of Arabidopsis. The last 30 residues of the C-terminal domain are unique to CCS and contain another highly conserved CXC motif.

CuZnSOD Activity and AtCCS Expression Pattern

The pattern for SOD activity in Arabidopsis is shown in Figure 2A. In addition to the two CuZnSOD activities as reported in Kliebenstein et al. (1998), an extra SOD activity migrating ahead the two CuZnSODs was also identified in reproductive tissues (CSD3, Fig. 2A). This activity band was characterized as a CuZnSOD due to the inhibition of the activity by KCN (data not shown). Based on the analysis of SOD activity in the csd1 knockout mutant (SALK_109389) and transgenic Arabidopsis plants containing an RNA interference CSD2 construct (C.-C. Chu, S.-M. Pan, and T.-L. Jinn, unpublished data), the three CuZnSODs activity bands were identified. The band with slowest mobility is CSD2, the second band is CSD1, and the fastest band is CSD3. CSD1 and CSD2 activities were detected in all tissues tested with the highest CSD1 activity detected in reproductive tissues. CSD3 activity was observed only in flowers and siliques, not in vegetative tissues (Fig. 2A).

We then analyzed the expression of the AtCCS gene. In northern-blot analyses, the AtCCS cDNA probe detected only one hybridization signal in flowers but not in rosette leaves (Fig. 2B). However, low levels of expression could be detected in rosette and cauline leaves by RT-PCR (Fig. 2C). In immunoblot analyses for protein expression, for some unknown reasons, our anti-AtCCS serum could detect AtCCS only when used in nondenaturing, but not on denaturing, PAGE (data not shown). As shown in Figure 2D, the anti-AtCCS antiserum detected several signals in stems and flowers when used in nondenaturing PAGE. Very weak signals were sometimes also observed in leaves (Fig. 2, D and E). The AtCCS signals did not result from antiserum cross-hybridization with
SOD since they migrated at different positions in the native gels (Fig. 2A, western). Furthermore, overexpression of a cDNA encoding AtCCS<sub>cyt</sub> (AtCCS<sub>cyt</sub>/wild type) increased the AtCCS signals detected (Fig. 2E), supporting that the bands detected on the native gels were indeed AtCCS.

Characterization of the Atccs Knockout Mutant

An Atccs mutant (SALK_025986), caused by a T-DNA insertion in the second exon (Fig. 3A), was obtained. Lines homozygous for the T-DNA insertion were identified by PCR and confirmed by Southern-blot analyses (Fig. 3B). Incomplete digestion might be the cause of higher M<sub>r</sub> bands seen in the Atccs sample digested with XbaI. In this Atccs mutant, neither the AtCCS transcript nor the AtCCS protein was detected (Fig. 3, C and D).

Almost no CuZnSODs activity was detected in the Atccs mutant (Fig. 4A) even in the presence of CuZnSOD transcripts (Fig. 4B) and proteins (Fig. 4C), which indicated that AtCCS was necessary for the activation of all types of CuZnSOD activity. The protein levels of CSD1 and CSD2 in the Atccs mutant were decreased in all tissues tested compared to the levels in the wild type (Fig. 4C). However, the steady-state level of CSD1

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**Figure 3.** The Atccs knockout mutant. A, Schematic representation of the positions of T-DNA insertion and related restriction enzyme sites in the AtCCS gene. B, Southern-blot analyses of Atccs homozygous T-DNA-insertion mutant. The genomic DNA of the wild type (WT) and Atccs mutant was digested with ClaI and XbaI and the AtCCS genomic clone was used as the probe. Size markers of DNA in kilobases (kb) are shown at the left. C, Northern-blot analyses of AtCCS gene expression in flowers from the wild type (WT) and Atccs mutant. The labeled AtCCS and ACT2 cDNA probes were used for cohybridization. ACT2 was used as a loading control of RNA. D, Immuno-blot analyses of AtCCS in flowers of the wild type (WT) and Atccs mutant by non-denaturing PAGE. Arrowheads indicate the AtCCS signals.
and CSD2 transcripts were not reduced in the Atccs mutant (Fig. 4B).

**Different Recovery of CuZnSOD Activities in the Atccs Mutant Complemented with AtCCScyt, AtCCScp, and AtCCScp-mutant**

Because CSD1, CSD2, and CSD3 activities could be simultaneously detected in flowers, in subsequent studies only results from flower tissue were presented. The Atccs mutant was transformed with AtCCScyt, the TAIR-predicted 254-amino acid open reading frame of the AtCCS gene (the transformants were designated as AtCCScyt/Atccs), or with a 1.64-kb genomic fragment (Fig. 1, A and B) that was sufficient to encode the MIPS-predicted 320-amino acid open reading frame (designated as AtCCScp/Atccs), to test for the recovery of the CuZnSOD activities. Both constructs were driven by the cauliflower mosaic virus 35S promoter. Immunoblot analyses revealed that AtCCS proteins were indeed expressed in AtCCScyt/Atccs and AtCCScp/Atccs transgenic plants (Fig. 5B). The CuZnSOD activity profile in different AtCCScyt/Atccs transgenic lines was the same as that of the wild type except that the chloroplastic CSD2 activity was never recovered (Fig. 5A, AtCCScyt/Atccs 1 to 3). However, T1 transgenic individuals transformed with AtCCScp were able to recover all three types of CuZnSOD activity (Fig. 5A, AtCCScp/Atccs 1 to 3). Recovery of all three types of CuZnSOD activity was also observed when the Atccs mutant was transformed with the AtCCScp cDNA driven by the 35S promoter (data not shown). These results indicated that AtCCScp, but not AtCCScyt, was sufficient to recover all three CuZnSOD activities in the Atccs mutant. To further test the importance of the 66 amino acids at the N terminus of AtCCScp, a mutant construct (AtCCScp-mut, Fig. 6A) was generated by introducing a guanine nucleotide before the second ATG through site-directed mutagenesis. CuZnSOD activity profile in the AtCCScp-mut/Atccs T1 transgenic plants was again missing the CSD2 band (Fig. 6B). This result suggested that the additional 66 amino acids in AtCCScp comprised a plastidic transit peptide necessary to restore the chloroplastic CSD2 activity.

**Chloroplastic Localization of AtCCScp**

To investigate if the first 66 amino acids of AtCCScp function as a chloroplast-targeting transit peptide, 35S-labeled AtCCScyt and AtCCScp were synthesized by in vitro transcription/translation and incubated with isolated pea (Pisum sativum) chloroplasts under...
import conditions. The AtCCScp cDNA directed the synthesis of two proteins, one 34 kD and one 29 kD (Fig. 7, lane 5). The AtCCScyt cDNA directed the synthesis of only the 29-kD protein (Fig. 7, lane 1), suggesting that the 29-kD product from AtCCScp was a result of internal initiation of translation from the second ATG. The 29-kD protein synthesized from AtCCScyt could not be imported into chloroplasts (Fig. 7, lane 3). In contrast, when the protein products from AtCCScp were incubated with chloroplasts, a 29-kD protein was produced (Fig. 7, lane 7), and this 29-kD protein was fully resistant to thermolysin (Fig. 7, lane 8), indicating its localization within the chloroplasts. Since the 29-kD protein synthesized from AtCCScy could not be imported into chloroplasts (Fig. 7, lane 3). In contrast, when the protein products from AtCCScp were incubated with chloroplasts, a 29-kD protein was produced (Fig. 7, lane 7), and this 29-kD protein was fully resistant to thermolysin (Fig. 7, lane 8), indicating its localization within the chloroplasts. Since the 29-kD protein synthesized from AtCCScy could not be imported into chloroplasts (Fig. 7, lane 3), the 29-kD chloroplast-localized protein produced after import of AtCCScp (Fig. 7, lane 7) most likely resulted from import of the 34-kD protein directed by AtCCScp. These results indicated that the first 66 amino acids of AtCCScp were necessary for the chloroplast import of AtCCScp.

Interaction between AtCCScyt and CSD1

We further investigated the possibility of direct interaction between AtCCScyt and CSD1 using the yeast two-hybrid assay. The AtCCScyt cDNA and CSD1 cDNA were fused with GAL4 DNA binding domain (BD-AtCCScyt) and activation domain (AD-CSD1), respectively. Cells containing both the BD and AD constructs were produced by mating of haploid cells containing individual plasmids. Diploid cells were then streaked on selection media to test for protein interaction. AD-CSD1 and BD-AtCCScyt together resulted in growth of the yeast transformants while cells transformed with individual binding or activation construct or with control plasmids did not grow (Fig. 8). This result suggested that AtCCScyt physically interacted with CSD1.

Importance of the ATX1-Like Domain in Conferring CuZnSOD Activity

A truncated AtCCS gene containing only the central and C-terminal domains (hereinafter referred as AtCCS)/Atccs, encoding the 162th–320th amino acids, Fig. 1D) was transformed into the Atccs mutant (designated as AtCCSD2D3/Atccs lines) to test for the importance of the ATX1-like region. The expression of the AtCCS/Atccs transcript in AtCCSD2D3/Atccs T1 transgenic lines was confirmed by northern-blot analyses (data not shown). Among 70 AtCCSD2D3/Atccs T1 transgenic lines, no CuZnSOD activity was observed in any of the plants even when protein extracts were prepared from detached leaf stalks that had been first incubated with a 1 mM CuSO4 solution at 25°C for 4 h (Fig. 9A). However, a weak CuZnSOD activity did appear if the leaf protein extracts were prepared first and the extracts were incubated directly in a 1 mM CuSO4 solution at 25°C for 4 h (Fig. 9B). This partial restoration of CuZnSOD activity was due to AtCCSD2D3 since no activity was observed in the Atccs mutant without the transgene (Fig. 9B, Atccs). Furthermore, the
CSD3 activity, never observed in vegetative tissues, was detected in the leaf extracts of wild type plants under such copper-supplemented conditions (Fig. 9, A and B, wild type, compare control and + CuSO₄).

**DISCUSSION**

One AtCCS gene was demonstrated to activate three forms of CuZnSODs localized in different subcellular locations in this study. The results obtained from the AtCCS<sub>cyt</sub>/Atccs, AtCCS<sub>cp</sub>/Atccs, and AtCCS<sub>cp-mut</sub>/Atccs transgenic plants and the in vitro protein import analysis indicated that the open reading frame from the first ATG encoded a plastidic AtCCS containing a chloroplastic transit peptide and was responsible for CSD2 activation (Figs. 5–7). Import of cytosolically synthesized precursor proteins into chloroplasts is usually very efficient and no precursor proteins are normally detected in the cytosol (Sun et al., 2001). Furthermore, the AtCCS precursor with the chloroplast-targeting transit peptide may not possess the chaperone function before the removal of the transit peptide. Therefore, a cytosolic form of AtCCS without the transit peptide is most likely also produced from AtCCS<sub>cp</sub> from the second ATG for the activation of CSD1 and CSD3. Results from AtCCS<sub>cyt</sub>/Atccs and AtCCS<sub>cp-mut</sub>/Atccs transgenic plants also support that the second ATG has the potential to be used as a translation initiation site. However, detection of the two proteins synthesized from AtCCS<sub>cp</sub> will be difficult since the cytosolic form and the processed mature form in plastids would be almost the same size (Fig. 7).

Several mechanisms have been reported for translation of two proteins from a single gene (Danpure, 1995; Small et al., 1998; Silva-Filho, 2003). With respect to AtCCS, it is not clear whether the two forms of AtCCS proteins resulted from two different transcripts or from alternative translation initiation from the same transcript. Sequences obtained from the 5'-RACE suggested that there might be at least two different types of AtCCS transcripts (Fig. 1C). Due to the possibility of mRNA degradation and abortion of the reverse transcriptase before reaching the 5'-end of mRNA, one would expect that the clones obtained from 5'-RACE should contain different lengths of 5'-untranslated region. However, 10 out of 13 of the clones from 5'-RACE extended only one nucleotide beyond the first ATG, and this high ratio suggested that this type of transcript existed in vivo. The size of the 5'-untranslated region in this transcript is too short, and thus the 40S subunit of ribosome may escape the first ATG (Kozak, 1991) and use the downstream ATG as a translation initiation site, resulting in the production of the cytosolic AtCCS. Meanwhile, the three clones with four, 14, and 26 nucleotides upstream of the first ATG suggested that there should be a longer

**Figure 7.** Import of AtCCS into isolated chloroplasts. In vitro-translated and [³⁵S]Met-labeled AtCCS<sub>cyt</sub> (lane 1) and AtCCS<sub>cp</sub> (lane 5) proteins were treated with thermolysin directly (lanes 2 and 6) or incubated with isolated pea chloroplasts under import conditions. The reisolated chloroplasts were further incubated in import buffer (lanes 3 and 7) or in import buffer containing thermolysin (lanes 4 and 8). Chloroplasts were reisolated, solubilized in sample buffer, and analyzed by SDS-PAGE and fluorography. Arrow indicates the precursor form of AtCCS<sub>cp</sub> and arrowhead indicates the imported mature form of AtCCS<sub>cp</sub> which is the same size as AtCCS<sub>cyt</sub>. Asterisk indicates the thermolysin-resistant fragment from both AtCCS<sub>cp</sub> and AtCCS<sub>cyt</sub>. The protein molecular mass markers in kD are shown at the left.

**Figure 8.** Analyses of AtCCS<sub>cp</sub> and CSD1 interaction by yeast two-hybrid assays. Chimeric proteins were constructed in which the open reading frame of AtCCS<sub>cp</sub> was fused to the GAL4 DNA-binding domain (BD-AtCCS<sub>cp</sub>) and that of CSD1 fused to the GAL4 activation domain (AD-CSD1). Haploid strains transformed with individual plasmids were mated and selected on the medium without Leu, Trp, His, and Ade. + for positive control and − for negative control as described in "Materials and Methods."
of proteins were applied to the SOD activity assay (Fig. 4A). A very small amount of CuZnSOD activity was also observed in rosette leaves, cauline leaves, and stems when the protein loading was increased to 120 μg (data not shown). These results revealed the existence of a CCS-independent pathway in addition to the CCS-dependent pathway for CuZnSOD activity. The CCS-independent pathway might activate a small amount of CSD2 that is sufficient to overcome the oxidative stress under normal growth condition. In yeast, ySOD1 activation is completely dependent on CCS (Culotta et al., 1997) due to the presence of the specific dual Pros at the 142nd and 144th amino acid in the ySOD1 protein sequence (Carroll et al., 2004). Whereas, the mammalian SOD1 can obtain some copper independent of CCS (Wong et al., 2000) and this CCS-independent pathway involves the reduced form of glutathione (Carroll et al., 2004). The specific dual Pros are largely restricted to Ascomycota fungi, (Carroll et al., 2004) and Arabidopsis CuZnSOD sequences do not contain these two specific Pro residues.

In the copper-deficient mice and the CCS knockout mice, the level of SOD1 protein was significantly reduced to different levels in different tissues, even though the level of SOD1 transcript was not altered (Prohaska et al., 2003). The authors suggested a post-transcriptional mechanism for the reduction of SOD1 protein when copper is limited. In Arabidopsis, the Atccs mutant showed a reduced protein level of the two forms of CuZnSOD, but the amount of CuZnSODs transcripts was not reduced (Fig. 4, B and C). This result is consistent with that from mice. However, a transcriptional regulation of CuZnSODs expression cannot be ruled out in Arabidopsis.

Protein translocation into chloroplasts and peroxisomes occur posttranslationally (Schnell and Hebert, 2003; Soll and Schleiff, 2004). Therefore, there is the possibility that activation of different CuZnSODs could take place in the cytosol before CuZnSOD proteins are delivered to different subcellular locations. However, the CSD2 activity was detected only in AtCCScp/Atccs but not in AtCCScp_mutation/Atccs transgenic plants (Figs. 5 and 6). These results indicated that CSD2 was activated in chloroplasts by chloroplast-localized AtCCS. On the other hand, although AtCCS contains a Ser-Lys-Val tripeptide at its C terminus, this tripeptide has been reported to function as a peroxisomal-targeting signal only in yeast, but not yet in higher plants (Elgersma et al., 1996; Hayashi et al., 1996; Mullen et al., 1997a, 1997b; Reumann, 2004). In addition, proteins can be fully folded in the cytosol before they are transported into the peroxisome (Walton et al., 1995), and therefore CSD3 could be fully assembled with copper and zinc before it is transported into peroxisomes. Therefore, whether the activation of CSD3 occurred in the cytosol or peroxisomes still remains to be demonstrated.

The importance of the ATX1-like domain in copper binding has been studied in yeast (Schmidt et al., 1999). In yeast, the free copper-ion concentration in an

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**Figure 9.** The importance of the AtCCS ATX1-like domain for CuZnSOD activation. The activity of CuZnSODs was analyzed in the wild type (WT), different T1 transgenic lines of AtCCSD2D3/Atccs (1 to 3) and Atccs mutant. A and B, Detached rosette leaves (A) and leaf protein extracts (B) were treated with a 1 mM CuSO4 solution at 25°C for 4 h (water treatment for controls). All samples were then analyzed for CuZnSOD activity. Positions of the CSD1, CSD2, and CSD3 activities are indicated at the left.

AtCCS transcript that would encode a chloroplastic form of AtCCS. Our proposal is consistent with the hypothesis of Wintz and Vulpe (2002). The authors suggested that the expression of the cytosolic and plastidic forms of AtCCS involves two transcription initiation sites in the AtCCS gene.

Rizhsky et al. (2003) reported that CSD2 knockdown plants had reduced CSD2 expression at the RNA and activity levels. Growth of the CSD2 knockdown plants was suppressed and the flowering time was delayed under a constant light of 55 to 100 μmol m⁻² s⁻¹. However, under a 16-h light/8-h dark photoperiod cycle at 100 μmol m⁻² s⁻¹, we did not observe any obvious difference in growth among the wild type, the Atccs mutant, the AtCCScyt/Atccs, and the AtCCS cp-mut / Atccs transgenic plants. In the Atccs mutant, approximately 6% of CuZnSOD activity compared with that of the wild type was observed in flowers when 30 μg...
unstressed yeast cell is less than 10^{-18} \text{ M} (Rae et al., 1999) and the ATX1-like domain is necessary only under such strict copper-limited conditions (Schmidt et al., 1999). If the growth media is supplemented with CuSO_4, yCCS without the ATX1-like domain could fully restore SOD1 activity (Schmidt et al., 1999). The authors have proposed that a copper-binding CXC motif in the C-terminal domain is sufficient for copper binding. However, cooperation with the ATX1-like domain results in maximal yCCS activity. In this study, the CuZnSOD activity was not restored when the rosette leaves from AtCCSD2D3/Atccs transgenic plants were immersed in 1 mM CuSO_4 solution (Fig. 9A). However, when protein extracts from the AtCCSD2D3/Atccs transgenic plants were directly mixed with 1 mM CuSO_4, a weak CuZnSOD activity was restored (Fig. 9B). Because direct incubation of the extracts with CuSO_4 would provide a higher concentration of copper to the AtCCSD2D3 mutant, our results agree with the results from yeast that AtCCSD2D3 can still function if copper is readily available and the ATX1-like domain is required when the availability of copper is limited. Copper content in Arabidopsis leaves has been reported to be 15.7 mg/kg dry weight (Abdel-Ghany et al., 2005), but the free copper-ion concentration is still unclear. The result that the AtCCS2D3 mutant failed to restore SOD activities suggests that the concentration of available copper is very low under normal growth conditions.

MATERIALS AND METHODS

Plants, Growth Condition, and CuSO_4 Treatment

The Atccs mutant (SALK_025986) of Arabidopsis (Arabidopsis thaliana) Columbia ecotype was obtained from the Arabidopsis Biological Resource Center (The Ohio State University). Seeds were sown in BIO-MIXTTING SUBSTRATUM (Agricultural Materials Company), incubated in the dark for 2 to 4 d at 4°C, and transferred to a growth chamber for germination. The seedlings were grown under 16-h light/8-h dark at 23°C/21°C at a light intensity of 60 to 100 \text{ μmol} \text{ m}^{-2} \text{ s}^{-1}.

For experiments shown in Figure 9, the detached rosette leaf stalks were immersed in 1 mM CuSO_4 solution, or crude protein extracts prepared from rosette leaves were incubated with 1 mM CuSO_4. Both treatments were performed at 25°C for 4 h. Protein extracts were then prepared from the detached leaves and samples from both treatments were assayed for CuZnSOD activity as described by Pan et al. (2001).

Analysis of the CCS Gene Sequence

Sequence information of genes, proteins, and cDNAs were retrieved through searching public databases with the BLAST algorithm (Altschul et al., 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), TAIR (http://www.arabidopsis.org), and MIPS Arabidopsis database (http://mips.gsf.de/proj/thal/db/index.html). The chloroplast-targeting signal was predicted with CHLOROP version 1.1 (Emanuelsson et al., 1999; http://www.cbs.dtu.dk/services/ChloroP) and TargetP version 1.01 (Emanuelsson et al., 2000; http://www.cbs.dtu.dk/services/TargetP).

Southern-Blot Analysis

Genomic DNA was isolated from rosette leaves according to Dellaporta (1993). DNA was digested with restriction enzymes, separated on a 1% (w/v) agarose gel, transferred to a nylon membrane (Roche), and hybridized with the AtCCS-genomic fragment labeled with Digoxigenin-11-dUTP by PCR with the primers ACCS-15, 5'-GCTTAGACTGGAAGACGAATAAGT-3' (the Xbal site is underlined and the first ATG was boxed) and ACCS-12, 5'-GGCGAGCTCTTAAACCTTACTGGCCACG-3' (theSac site is underlined). The membrane was washed twice at 65°C for 10 min in 2× sodium chloride/sodium phosphate/EDTA buffer supplemented with 0.1% (w/v) SDS. Hybridization signals were detected by the chemiluminescent reaction with disodium 3-[4-methoxyxyspiro[1,2-dioxetane-3,2'-benzoxazol]-2'-yl]-1,2,3-triazolo[3,1-l][1,2,4]triazole-3-carboxylate (Roche) according to the manufacturer's instructions.

Northern-Blot Analysis

Total RNA was isolated with TRIzol reagent (Invitrogen) and 10 μg total RNA was separated on a 1.2%-formaldehyde agarose gel and hybridized with AtCCS, CSD1, CSD2, and ACT2 cDNA fragments labeled with Digoxigenin-11-dUTP by PCR. Hybridization signals were detected as described for Southern-blot analyses. The cDNA probes were amplified by PCR with the following gene-specific primers: AtCCS, ACCS-15 and ACCS-12 (as described in Southern-blot analyses); CSD1, 5'-GCCCATGCGAGGAGGATGGT-3' (the Ncol site is underlined) and 5'-ATCCCGGCCCCTGGAGACCAATGAT-3' (the Smal site is underlined); CSD2, 5'-ACGGATCATCTCTCCGAGTCTCTCCCT-3' (the BamH site is underlined) and 5'-ATCTCTAACAGCCTACATCAGT-3' (the Xbal site is underlined); ACT2, 5'-AGCTCCTGGCAGTCCGAGGGTGG-3' (the Sacl site is underlined); ACT2, 5'-ATCTCTAACAGCCTACATCAGT-3' (the SacI site is underlined); ACT2, 5'-AGCTCCTGGCAGTCCGAGGGTGG-3' (the Sacl site is underlined); ACT2, 5'-AGCTCCTGGCAGTCCGAGGGTGG-3' (the Sacl site is underlined); ACT2, 5'-AGCTCCTGGCAGTCCGAGGGTGG-3' (the Sacl site is underlined).

The hybridization signals were detected by the chemiluminescent reaction with disodium 3-[4-methoxyxyspiro[1,2-dioxetane-3,2'-benzoxazol]-2'-yl]-1,2,3-triazolo[3,1-l][1,2,4]triazole-3-carboxylate (Roche) according to the manufacturer's instructions.

RT-PCR and 5'-RACE of the AtCCS Gene

For RT-PCR, Superscript II (Invitrogen) kit was used and primed with the oligo (dT) primer for cDNA synthesis. The RT reaction mixture (1 μL) was used for subsequent PCR with the following gene-specific primers: AtCCS, ACCS-15 and ACCS-12, or ACCS-1, 5'-ATCTCTAACAGCCTACATCAGT-3' (the SacI site is underlined); ACCS-11, 5'-GCTGCTGGTGCTCTCCGGTTG-3' (Fig. 1A), and internal control ACT2, ACT2-376s and ACT2-376 sc, or UbQ10, 5'-GATCTTGGCGGAAAACATGGAGGAGGTG-3' (UBQ1) and 5'-CCAATTTCAATAGAAAGAGGATCGAAGGTG-3' (UBQ2).

The 5'-terminal sequence of the AtCCS transcript was obtained using the BD SMART RACE cDNA Amplification kit (CLONTECH) according to the manufacturer’s instructions (5'-RACE). Total RNA (1 μg) from the flowers in the wild type was used for cDNA synthesis. The subsequent PCR was amplified with the universal primers mix provided in the kit and the gene-specific reverse primer, ACCS-8. The PCR products of the 5'-RACE were cloned into the yT&A vector (Yeastem Biotech) for DNA sequencing.

Protein Extraction and Quantification

Tissues were ground (tissue:medium ratio 1:3, w/v) with 150 mM Tris-HCl pH 7.2, and the homogenate was centrifuged at 13,000 g for 15 min. The protein concentration was determined by the method of Bradford (1976) with the Bio-Rad protein assay reagent (Bio-Rad) and bovine serum albumin was used as the protein standard.

Electrophoresis and SOD Activity Analysis

Total protein (30 μg) was separated on a 10% nondenaturing polyacrylamide gel in Tri-Gly buffer (pH 8.3). A photochemical method modified from Beauchamp and Fridovich (1971) was used to visualize SOD activity. The gel was soaked in 0.1% (w/v) nitroblue tetrazolium solution for 15 min, rinsed with distilled water, and transferred to 100 mM potassium phosphate buffer (pH 7.0) containing 0.028 mM riboflavin and 28 mM TEMED (N,N,N’,N’-tetramethyl-ethylenediamine) for another 15 min. After being washed with distilled water, gel was illuminated on a light box, with a light intensity of 30 μmol m^{-2} s^{-1} for 15 min to initiate the photochemical reaction. The SOD activity was verified by KCN and H_2O_2 as described by Pan et al. (1999). KCN is an inhibitor of CuZnSOD, whereas H_2O_2 inhibits both CuZnSOD and FeSOD. The MnSOD activity is not inhibited by either chemical.
Anti-AtCCS Serum Preparation

The coding region of AtCCS gene predicted in TAIR database was amplified by PCR with the gene-specific primers 5’-TCTGAGTCCATGCGG- 
ACTGCTCTCATT-3’ (the BamHI site is underlined) and 5’-TCTGGGAGTG- 
TATTTAACCTTCTTG-3’ (the XhoI site is underlined) that introduced the 
two restriction sites on the two ends. The PCR fragment was cloned into 
the pENTR1-A (Amersham Biosciences), blocked with 5% nonfat-milk in 
1X PBST (phosphate-buffered saline containing 0.05% Tween 20) for 1 h, 
and then incubated with anti-AtCCS antiserum (1:1,000 dilution) for another 
hour. The washed membrane was incubated with 1:3,000 diluted goat anti-rabbit 
IgG conjugated with alkaline phosphate (Perkin-Elmer); the signals in the 
membrane were detected by colorimetric reaction with 5-bromo-4-chloro-3-
indolyl phosphate/nitroblue tetrazolium (Perkin Elmer phosphor for CZeSD2 im-
munoblot assay, 30 μg of total protein was separated on a 10% SDS-PAGE and 
transferred to a polyvinylidene difluoride membrane with the anti-CDs1 
(1:1,000 dilution) and -CS2D2 (1:2,000 dilution) anti-sera as described in 
Kliebenstein et al. (1998).

Immunoblot Analyses

For the detection of AtCCS, 60 μg of total protein was separated on a 10% 
nondenaturing polyacrylamide gel and transferred to a polyvinylidene dif-
fluoride membrane (Amersham Biosciences), blocked with 5% nonfat-milk in 
1X PBST (phosphate-buffered saline containing 0.05% Tween 20) for 1 h, and 
then incubated with anti-AtCCS antiserum (1:1,000 dilution) for another 
hour. The washed membrane was incubated with 1:3,000 diluted goat anti-rabbit 
IgG conjugated with alkaline phosphate (Perkin-Elmer); the signals in the 
membrane were detected by colorimetric reaction with 5-bromo-4-chloro-3-
indolyl phosphate/nitroblue tetrazolium (Perkin Elmer phosphor for CZeSD2 im-
munoblot assay, 30 μg of total protein was separated on a 10% SDS-PAGE and 
transferred to a polyvinylidene difluoride membrane with the anti-CDs1 
(1:1,000 dilution) and -CS2D2 (1:2,000 dilution) anti-sera as described in 
Kliebenstein et al. (1998).

In Vitro Transcription/Translation of AtCCS cyt 
and AtCCS cp Proteins, and Protein Import into 
Isolated Chloroplasts

The AtCCS cp and AtCCS cyt cDNA fragments were amplified by RT-PCR 
with the following specific primers: AtCCS cyt ACCs-1 and ACCs-12; and 
AtCCS cp ACCs-15 and ACCs-12 (Fig. 1A). The RT-PCR products were cloned into 
the 3′ TA vector for DNA sequencing. Then the AtCCS cyt and AtCCS cp 
cDNA fragments were subcloned into the KpnI and BamHI sites of pBlueScript 
SK+ (Stratagene). These constructs were used as templates for in vitro 
transcription/translation using the TNT T7 coupled wheat germ extract 
kit (Promega) in the presence of [35S]Met according to the manufacturer’s 
transcription/translation using the TNT T7 coupled wheat germ extract 
kit (Promega) in the presence of [35S]Met according to the manufacturer’s 
protocol.

Yeast Two-Hybrid Analysis

The AtCCS cyt cDNA and the Arabidopsis CSD1 cDNA were amplified by 
PCR and subcloned into the pGBK7 and pACT2 yeast (Saccharomyces 
cerevisiae) expression vectors (Matchmaker 3, CLONTECH, in-framed with 
the GAL4 DNA BD and the GAL4 AD, respectively. The resulting BD-AtCCS cyt 
and AD-CSD1 vectors were then transformed into the yeast strain AH109 
(MAT α and Y187 (MAT α) using a modified lithium acetate method. For 
positive and negative controls, yeast cells were transformed with the plasmids 
pACT2-T α (AD-T α) and pGBK7-T3 (BD-p53), or with the plasmids pACT2-T α (AD-T α) and pGBK7-Lam (DB-Lamin C), respectively. Yeast cells were incubated into yeast peptone dextrose medium 
and cultured overnight. Cells were then streaked on synthetic dextrose 
medium lacking Leu, Trp, His, and Ade.

Gene Construction and Plant Transformation

The pPZP200GB with β-glucuronidase and BAR (BASTA resistance gene) 
cassettes was derived from pBI221 (CLONTECH) and pSK-35S-BAR (John 
Walker, University of Missouri) in this vector. This binary vector was used 
to clone AtCCS cp and AtCCS cpD2D3. The cDNA fragment corresponding to 
AtCCS cyt and AtCCS cpD2D3 was amplified by PCR with the following gene-
specific primers: AtCCS cyt 5’-ATTCTGAGTACCGACTGTCCTCACT-3’ (the 
XhoI site is underlined) and 5’-GCCAAGTCCTTAAAACCTTCTGGCAGC-3’ 
(the SacI site is underlined); and AtCCS cpD2D3, 5’-TCTGAGTACCGAT-
TATTAAACCTTACTGGCCACG-3’ (the SacI site is underlined). The PCR 
products were cloned into the Xhol/SacI sites of pZPP200GB by replacing 
the β-glucuronidase fragment. The obtained plasmids were named 
pPZP200GB-AtCCS cyt and pPZP200GB-AtCCS cpD2D3, respectively.

The AtCCS cyt and AtCCS cpD2D3 genomic fragments were amplified by 
PCR with the following specific primers: AtCCS cyt ACCS-15 and ACCS-12; 
AtCCS cp ACCS-15, 5’-AAATGCGCATCGCTTCAGGTGCAGT-3’ (the 
first ATG is boxed and the introduced extra G nucleotide is underlined), and 
ACCs-12. The PCR products were cloned into the 3′ TA vector for DNA se-
quencing. Then the KpnI/SacI fragments corresponding to AtCCS cyt and 
AtCCS cpD2D3 were cloned into pBB-HYG (Becker et al., 1992) for plant trans-
formation. The obtained plasmids were named pBB-HYG-AtCCS cyt and pBB-
HYG-AtCCS cpD2D3, respectively.

Yeast Two-Hybrid Analysis

The plasmids for plant transformation were transformed into Agrobacterium 
tumefaciens C58 by electroporation. Agrobacterium cells containing each 
plasmid were transformed into the Atccs mutant by the floral-dipping method 
(Clough and Bent, 1998). Transgenic plants of AtCCS cyt/Atcs and ACCS cpD2D3/ 
Atcs were selected by spraying seedlings at 7, 9, and 11 d after germination 
with a solution of 0.4% BASTA herbicide (McDowell et al., 1998). The AtCCS cyt/ 
Atcs and AtCCS cpD2D3/Atcs transgenic plants were selected by half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) containing 
25 μg mL−1 hygromycin.

Sequence data from this article can be found in the GenBank/EMBL data 
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