Copper Chaperone-Dependent and -Independent Activation of Three Copper-Zinc Superoxide Dismutase Homologs Localized in Different Cellular Compartments in Arabidopsis[1][W][OA]

Chien-Hsun Huang[2], Wen-Yu Kuo[2], Celeste Weiss, and Tsung-Luo Jinn*

Institute of Plant Biology and Department of Life Science, National Taiwan University, Taipei 10617, Taiwan (C.-H.H., W.-Y.K., T.-L.J.); and Department of Biochemistry and Molecular Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel (C.W.)

Superoxide dismutases (SODs) are important antioxidant enzymes that catalyze the disproportionation of superoxide anion to oxygen and hydrogen peroxide to guard cells against superoxide toxicity. The major pathway for activation of copper/zinc SOD (CSD) involves a copper chaperone for SOD (CCS) and an additional minor CCS-independent pathway reported in mammals. We characterized the CCS-dependent and -independent activation pathways for three CSDs localized in different cellular compartments in Arabidopsis (*Arabidopsis thaliana*). The main activation pathway for CSD1 in the cytoplasm involved a CCS-dependent and -independent pathway, which was similar to that for human CSD. Activation of CSD2 in chloroplasts depended totally on CCS, similar to yeast (*Saccharomyces cerevisiae*) CSD. Peroxisome-localized CSD3 via a CCS-independent pathway was similar to nematode (*Caenorhabditis elegans*) CSD in retaining activity in the absence of CCS. In Arabidopsis, glutathione played a role in CCS-independent activation, as was reported in humans, but an additional factor was required. These findings reveal a highly specific and sophisticated regulation of CSD activation pathways in planta relative to other known CCS-independent activation.

Superoxide dismutases (SODs) are a group of metalloenzymes that defend against free radical species by disproportionating O_2^- into hydrogen peroxide and oxygen molecules (Beyer et al., 1991; Bowler et al., 1992). By a specific metal cofactor required for this superoxide-scavenging activity (McCord and Fridovich, 1969), they are classified as copper/zinc SOD (CuZnSOD), iron SOD (FeSOD), manganese SOD (MnSOD), or nickel SOD (Alscher et al., 2002; Zelko et al., 2002). Most eukaryotic cells contain two types of SODs, with CuZnSOD largely localized in the cytoplasm (Crapo et al., 1992) and MnSOD localized in mitochondria (Weisiger and Fridovich, 1973; Marres et al., 1985). Plants contain MnSOD, FeSOD, and many isoforms of CuZnSOD in different cellular compartments (Jackson et al., 1978; Kanematsu and Asada, 1989; Bowler et al., 1992; Bueno et al., 1995). Arabidopsis (*Arabidopsis thaliana*) has three CuZnSOD (CSD) isoforms: CSD1 in the cytoplasm, CSD2 in chloroplasts, and CSD3 in peroxisomes (Kliebenstein et al., 1998; Alscher et al., 2002).

The metal cofactors for SODs are transition metals, with the ability to readily accept or donate an electron for the superoxide dismutation process. However, these metal ions in free form via the Haber-Weiss reaction could produce harmful hydroxyl radicals (Halliwell and Gutteridge, 2007). Hence, free transition metals generally exist in a complexed form, with activated SOD required as a metallochaperone. Cu chaperones involved in Cu trafficking in yeast (*Saccharomyces cerevisiae*) are ATX1 (Lin and Culotta, 1995; Lin et al., 1997), COX17 (Glerum et al., 1996), and Lys-7 (Horecka et al., 1995). Lys-7 can fully restore the *lys7*Δ (i.e. *ccs*Δ) mutant function outside of Lys biosynthesis as a Cu chaperone for superoxide dismutase (CCS), which is a functional homolog of the human CCS (Horecka et al., 1995; Culotta et al., 1997). The CCS interacts with CSD, assisting in Cu incorporation and catalysis of disulfide bond formation, thereby resulting in an active CSD (Casareno et al., 1998; Lamb et al., 2001; Brown et al., 2004; Furukawa et al., 2004). In Arabidopsis, a single *AtCCS* gene product activated CSDs in the cytoplasm and chloroplast (Chu et al., 2005); however, the phenotype of the *AtCCS*-knockout mutant (*Atccs*) was found to be normal (Chu et al., 2005; Cohu et al., 2009). Similarly, a CCS-independent

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[2] These authors contributed equally to the article.

* Corresponding author; e-mail jinnt@ntu.edu.tw.

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activation pathway for CSD was found in mice (Wong et al., 2000). These data suggest the existence of additional CSD-activating factors.

To date, yeast SOD1 (ySOD1) activation has been found to fully depend on yeast CCS (yCCS; Carroll et al., 2004), whereas the nematode (*Caenorhabditis elegans*) CSD (wSod-1) is exclusively activated independently of CCS (Jensen and Culotta, 2005). Human CSD (hSOD1) is largely activated by CCS but retains about 25% to 50% of its activity in the absence of CCS (Carroll et al., 2004). However, CCS-independent pathways for SOD activation in plants are not well understood.

It has long been suggested that reduced glutathione (GSH) may participate in intracellular Cu homeostasis by forming Cu-GSH complexes when transferring Cu to CSD (Ciriolo et al., 1990) and metallothionein (Ferreira et al., 1993) in vitro. GSH was required for CCS-independent activation of hSOD1 in yCCS mutant strains with defective GSH metabolism, and wSod-1 was inactive in the presence of CCS when GSH was depleted in yeast (Carroll et al., 2004; Jensen and Culotta, 2005). However, a direct interaction between GSH and CSD has yet to be demonstrated. Results from mutagenesis studies showed that amino acid residues 142 and 144 near the C terminus of human and yeast CSDs were important in the CCS-independent pathway (Carroll et al., 2004; Jensen and Culotta, 2005). When these residues were replaced by dual Pro residues in ySOD1, CCS-independent activities for both hSOD1 and wSod-1 were inhibited. A recent study further confirmed that the Pro at residue 144 but not 142 restricted ySOD1 disulfide formation in the absence of CCS, which played a key role in blocking CCS-independent activation (Leitch et al., 2009a). The existence of additional essential factors in this pathway remains to be determined.

In this study, we investigated the dependence of Arabidopsis CSD activity on the CCS metallochaperone in Arabidopsis and yeast knockout strains. The three CSD proteins in Arabidopsis showed unique levels of activity by CCS-dependent and -independent activation pathways. Moreover, the CSD2 lost the ability of CCS-independent activation, possibly because of an inhibitory factor(s) in chloroplasts, which has not been reported in any other species. We also confirmed in Arabidopsis, as in hSOD1, that GSH was involved in the CCS-independent pathway, with an additional factor cooperatively assisting in CSD activation.

RESULTS

CCS-Independent Activation of Arabidopsis CSD1 and CSD3 in Yeast

In our previous study (Chu et al., 2005), we observed residual CSD activity in *Atccs* (6%–30% of the wild-type levels), which indicated the existence of a CCS-independent pathway for CSD activation in Arabidopsis (Supplemental Fig. S1). Because *AtCCS* is a functional homolog of *yCCS* (Abdel-Ghany et al., 2005), the Arabidopsis CSD might be activated in the yeast system, so we expressed each of the three Arabidopsis *CSD* genes in yeast *ySOD1*- and *yCCS*-knockout strains (*sod1*Δ and *ccs*Δ, respectively; Fig. 1). Yeast *sod1*Δ and *ccs*Δ are auxotrophic for Lys when grown in air (Culotta et al., 1997).

In-gel SOD activity assay revealed a strong CSD1 activity band in *sod1*Δ and a weaker but visible band in *ccs*Δ (Fig. 1A, top, lanes 1 and 2), which demonstrated

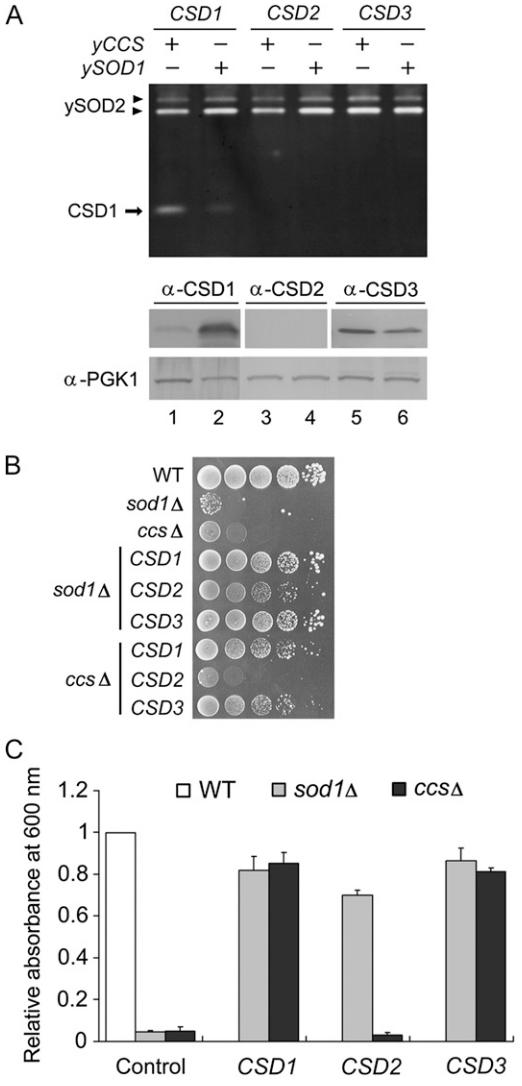

Figure 1. The activities of three Arabidopsis CSDs in yeast *sod1*Δ and *ccs*Δ. A, Lysates of yeast expressing Arabidopsis *CSD1*, *CSD2*, and *CSD3* were analyzed by in-gel SOD activity assay (top) and immunoblotting (bottom). Strains expressed on the *sod1*Δ or *ccs*Δ background are represented as *yCCS*+/*ySOD1*− or *yCCS*−/*ySOD1*+, respectively. B and C, Viability of yeast *sod1*Δ and *ccs*Δ expressing *CSD1*, *CSD2*, or *CSD3* under Lys-lacking conditions. Plate (B) and liquid (C) assays are described in "Materials and Methods." Cell density values are relative to the wild-type (WT) level. All data were from four independent tests (means ± SD).

CCS-independent activation of CSD1. *CSD2* and *CSD3* were overexpressed in yeast, but we detected no activity (Fig. 1A, top, lanes 3–6), and the CSD2 protein was not detectable (Fig. 1A, bottom, lanes 3 and 4).

Because of the limited sensitivity of the in-gel SOD activity assay, we used a more sensitive method, the Lys-independent aerobic growth assay, to detect the relatively weak CSD activity in yeast in vivo (Wallace et al., 2004). With this assay, Lys biosynthesis ability was maintained when ySOD1 activity was just higher than 2% of the wild-type level to rescue the Lys auxotrophy of *sod1*Δ (Corson et al., 1998). Because ySOD1 activation depends totally on yCCS function, endogenous ySOD1 activity is lost in *ccs*Δ (Carroll et al., 2004), and heterologously expressed Arabidopsis CSDs could be responsible for the Lys biosynthesis ability in both *sod1*Δ and *ccs*Δ. In our experiments, neither *sod1*Δ nor *ccs*Δ was able to grow under Lys-depleted conditions, as expected (Fig. 1, B and C), which confirms the absence of ySOD1 activity in these two mutants. Lys biosynthesis ability was recovered in both *sod1*Δ and *ccs*Δ with *CSD1* and *CSD3* expression. With the expression of *CSD2*, the phenotype of *sod1*Δ was partially rescued, but that of *ccs*Δ was not restored at all, which demonstrates a weak expression and activity of CSD2 in *sod1*Δ. Therefore, both CSD1 and CSD3 can be activated via the CCS-independent pathway in yeast. However, the failure of CSD2 to rescue Lys biosynthesis might be related to the heterologous nature of the expression system used.

CSD2 Activity Depends on CCS Only in Chloroplasts but Is CCS Independent in Cytoplasm

To address the question of how CSD2 is activated, *CSD2* and *AtCCS* genes were transiently overexpressed in *Atccs* protoplasts (Fig. 2, A and B). Use of yellow fluorescent protein (YFP) fused to CSD2 revealed that the full-length CSD2 localized in chloroplasts, whereas the transit peptide-deleted CSD2 (ΔTP-CSD2) localized to cytoplasm (Fig. 2A). Full-length CSD2 was activated in the presence of AtCCS (Fig. 2B, lane 2) but was undetectable in the absence of AtCCS (lane 3). However, ΔTP-CSD2 showed CCS-independent activity (lane 7), which indicates that CSD2 can be activated via the CCS-independent pathway when localized in cytoplasm.

CCS-Independent Activation of CSD1 Is Lost When Localized in Chloroplasts

To gain an understanding of CSD activation in chloroplasts, we constructed a chloroplast-directed CSD1

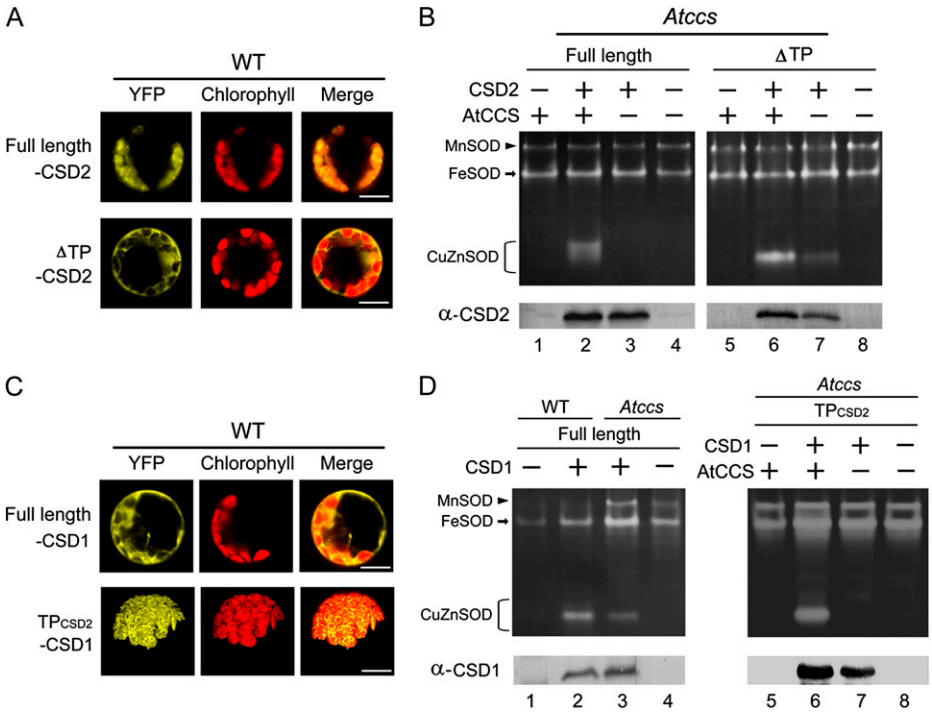

Figure 2. Localization and SOD activity of CSD1 and CSD2 overexpressed in *Atccs* protoplasts. A and C, YFP fusion of full-length and chloroplast transit peptide-deleted CSD2 (ΔTP-CSD2; A) and YFP fusion of full-length CSD1 and that fused to the chloroplastic transit peptide of CSD2 (TP_{CSD2}-CSD1; C) were expressed in Arabidopsis wild-type (WT) protoplasts for localization analysis. The chloroplasts are visualized as red autofluorescence. Bars = 10 μm. B and D, Full-length CSD1 and ΔTP-CSD2 (B) and full-length CSD1 and TP_{CSD2}-CSD1 (D) were overexpressed with or without AtCCS coexpression in wild-type or *Atccs* protoplasts, then SOD activity (top) and protein level (bottom) were analyzed. Because CSD protein expression was reduced in *Atccs*, we loaded 3-fold more *Atccs* protoplasts (approximately 7.5×10^5 cells) than wild-type protoplasts (approximately 2.5×10^5 cells) to present similar protein levels in lanes 2 and 3. The *CSD* genes used here contained no YFP fusion.

to observe its behavior in this compartment (Fig. 2, C and D). We used YFP fusion proteins to confirm the localization of a CSD1 fused with the chloroplast transit peptide of CSD2 (TP_{CSD2}-CSD1) in chloroplasts (Fig. 2C). We found CCS-independent CSD1 activity in *Atccs* (Fig. 2D), and the efficiency of CSD1 activation (activity μg^{-1} protein) in *Atccs* was approximately 36% of that in the wild type (compare lanes 2 and 3). In contrast, chloroplast-localized CSD1 was active in the presence of AtCCS (lane 6), and its activity was undetectable in the absence of AtCCS (lane 7). This result is consistent with the observation that CCS-independent CSD activation occurs in the cytoplasm but not in the chloroplast.

No Detection of Peroxisome-Localized CSD3 and Cytoplasm-Directed CSD3 Activities

A similar experiment was also performed with CSD3 (Fig. 3). GFP fused to full-length CSD3 showed it localized in peroxisomes and localized in cytoplasm with deletion of the C-terminal peroxisome-targeting sequence AKL (CSD3-ΔAKL; Fig. 3A). Unlike CSD1 or CSD2, CSD3 activities were undetectable even when it was overexpressed in *Atccs* with *AtCCS* coexpression (Fig. 3B) or in wild-type protoplasts (Supplemental Fig. S3).

CSD1 Mutants Show Different Activity Levels in Yeast

A previous study by Carroll et al. (2004) demonstrated the importance of amino acid residues 142S and 144L of human hSOD1 for CCS-independent activation. As well, replacing these amino acids with Pro, as with dual Pro residues in yeast ySOD1, prevents activation by this pathway. We aligned CSD sequences of Arabidopsis, human, yeast, and nematode and found the corresponding residues in these three Arabidopsis CSDs to be 141G/143V in CSD1, 143G/145L in CSD2, and 147S/149V in CSD3, similar to residues 142S/144L in hSOD1 (Fig. 4A). Here, we tested only CSD1, because only CSD1 activity could be detected by in-gel SOD activity assay (Fig. 1A). We generated a series of CSD1 mutants, G141A/V143A ($CSD1_{AA}$; nematode form), G141S/V143L ($CSD1_{SL}$; human form), and G141P/V143P ($CSD1_{PP}$; yeast form), to examine the effect on CCS-independent CSD1 activity in yeast *sod1*Δ and *ccs*Δ strains.

We detected activities of wild-type CSD1 ($CSD1_{GV}$) and $CSD1_{SL}$ in both *sod1*Δ and *ccs*Δ, but $CSD1_{AA}$ activity was detected only in *sod1*Δ (Fig. 4B, lanes 1–6). $CSD1_{GV}$ and $CSD1_{SL}$ could restore the Lys-auxotrophic phenotype of *sod1*Δ and *ccs*Δ under the Lys-depleted condition (Fig. 4, C and D). $CSD1_{AA}$ could also restore the viability of *sod1*Δ, but that of *ccs*Δ was only partially rescued. Although the in-gel $CSD1_{PP}$ activity was undetectable in both *sod1*Δ and *ccs*Δ (Fig. 4B, lanes 7 and 8), the activity of this mutant was discernible in *sod1*Δ but not *ccs*Δ by use of the Lys biosynthesis assay (Fig. 4, C and D). Therefore, the Arabidopsis

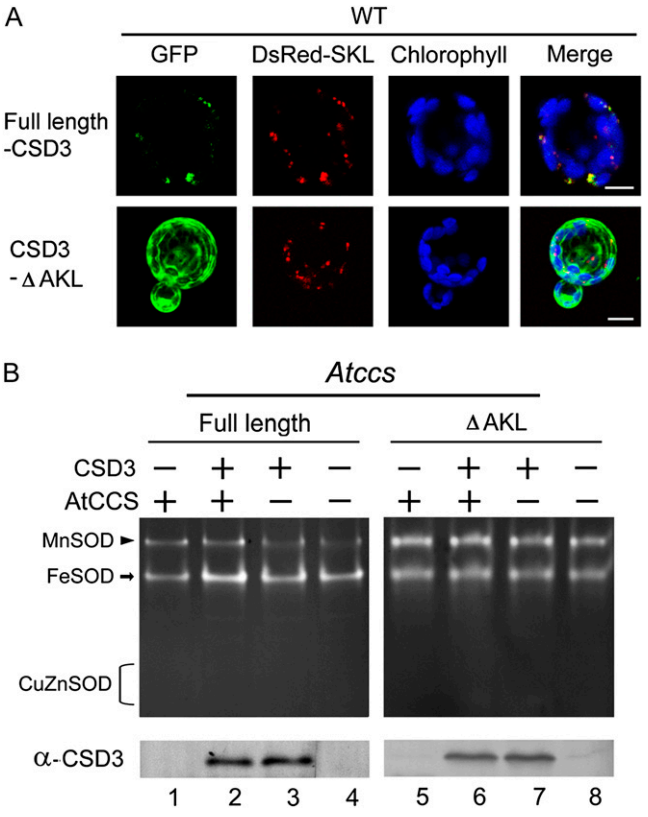

Figure 3. Localization and SOD activity of full-length and AKL-deleted CSD3 overexpressed in Arabidopsis protoplasts. A, GFP fusion proteins of full-length and C-terminal AKL-deleted CSD3 (CSD3-ΔAKL) were expressed in Arabidopsis wild-type (WT) protoplasts for localization analysis. DsRed-SKL is a peroxisomal marker. The autofluorescence of chlorophyll is blue. Bars = 10 μm. B, CSD3 full length and ΔAKL were overexpressed with or without AtCCS coexpression in *Atccs* or wild-type protoplasts, then CSD3 activity (top) and protein level (bottom) were analyzed. The *CSD3* used here contained no GFP fusion.

$CSD1_{GV}$, human form $CSD1_{SL}$, and nematode form $CSD1_{AA}$ but not yeast form $CSD1_{PP}$ could be activated by the CCS-independent pathway. The CCS-independent activation in Arabidopsis and human CSDs shared the highest functional similarity.

Physiological Function of CCS-Independent CSD Activity

We used seed germination rate in *Atccs* and *CSD1*-knockout lines (*Atcsd1*) to determine whether these residual CSD activities were physiologically functional in vivo.

In the *Atcsd1* mutant (Fig. 5A), *CSD1* mRNA and protein were absent, but the expression levels of *CSD2* and *CSD3* were unchanged (Fig. 5B). The total CSD activity in *Atcsd1* (with functional CSD2 and CSD3) was lower than that in the wild type but was higher than that in *Atccs* (Fig. 5C). When *Atccs* and *Atcsd1* mutants were grown on half-strength Murashige and Skoog (1/2 MS) plates, their seed germination rates

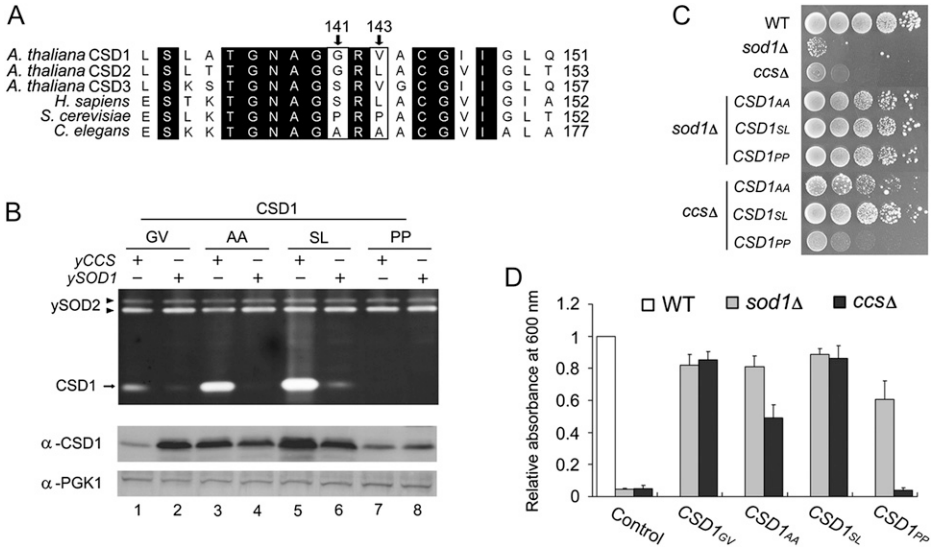

Figure 4. Activity of Arabidopsis *CSD1* variants in yeast *sod1*Δ and *ccs*Δ. A, Sequence alignment of CuZnSOD in Arabidopsis (CSD1, CSD2, and CSD3), human (hSOD1), *S. cerevisiae* (ySOD1), and *C. elegans* (wSod-1). Residues corresponding to 141G and 143V of CSD1 are indicated in white boxes with arrows. Identical residues are shown in black. B, Yeast *sod1*Δ and *ccs*Δ expressing the *CSD1* variants were analyzed as described in Figure 1A for SOD activity (top) and CSD1 protein level (bottom). C and D, Viability of yeast *sod1*Δ and *ccs*Δ expressing the *CSD1* variants under Lys-lacking conditions. Plate (C) and liquid (D) assays were as described in Figure 1, B and C. The data are from four independent tests (means ± SD). WT, Wild type.

were only 4% lower then that of the wild type (Fig. 5D, control), which indicated that the CCS-independent activities of CSD in *Atccs* were sufficient for normal growth. We used treatment with reactive oxygen species (ROS)-generating reagents, methyl viologen (MV) and *tert*-butyl hydroperoxide (BH), to induce oxidative stress to magnify the phenotypic effects due to the decreased CSD activity. With 0.008 or 0.04 μM MV, the seed germination rate of *Atccs* was significantly lower than that of the wild type but higher than that of *Atcsd1* (Fig. 5D, MV). Similar results were obtained with 100 μM BH treatment (Fig. 5D, BH). Hence, although the total CSD activity in *Atccs* (resulting from the CCS-independent CSD activity) was lower than that in *Atcsd1* (resulting from CSD2 and CSD3 activities), the phenotype of inhibition of seed germination was less pronounced in *Atccs* than in *Atcsd1*.

Glutathione Plays a Role in CCS-Independent CSD Activity

A previous study of human hSOD1 implicated a strict dependence on GSH for CCS-independent activation (Carroll et al., 2004). To investigate the possible involvement of GSH in the activation of Arabidopsis CSD, we tested the effect of GSH on *Atccs* protoplasts overexpressing *CSD1* (Fig. 6A). The cellular concentrations of total glutathione and GSH were slightly enhanced by GSH treatment, by 1.23- and 1.13-fold, respectively, whereas the CSD1 activity did not increase noticeably. On treatment with a glutathione chelator, 1-chloro-2,4-dinitrobenzene (CDNB), both the concentrations of total glutathione and GSH decreased (to 0.42- and 0.87-fold), as did the level of CSD1 and its activity. The relatively slight change in internal GSH concentration after exogenous GSH treatments might be due to an in vivo mechanism that maintains the homeostasis of reducing power, too much of which could be potentially damaging to cells (Lockwood, 2003; Pasternak et al., 2008).

In a further experiment, we manipulated GSH concentration by overexpressing glutaredoxin (Xing et al., 2005; Cheng et al., 2006; Rouhier, 2010), *ROXY1* (a cytoplasmic glutaredoxin) or *GRXcp* (a chloroplastic glutaredoxin), together with *CSD1*, in *Atccs* protoplasts (Fig. 6B). GSH concentration decreased with *ROXY1* or *GRXcp* overexpression, whereas total glutathione concentration was not significantly changed. When *ROXY1* was coexpressed with *CSD1*, both the level and activity of CSD1 were decreased; the coexpression of *GRXcp* reduced CSD1 activity, with no change in protein level. Thus, we show an overall decrease in CSD1 activity with reduced intracellular GSH concentration.

Cu Ion and GSH Greatly Enhance the Activation of Apo-CSD1 in the Presence of *Atccs* Cellular Extracts in Vitro

To gain a better understanding of GSH involvement in the CCS-independent pathway, we performed in vitro analyses using affinity-purified proteins. We purified the glutathione *S*-transferase (GST)-tagged CSD1 (GST-CSD1), then removed the GST (Holo-CSD1) by using PreScission protease (Supplemental Fig. S2, A and B). We then prepared the inactive apo-

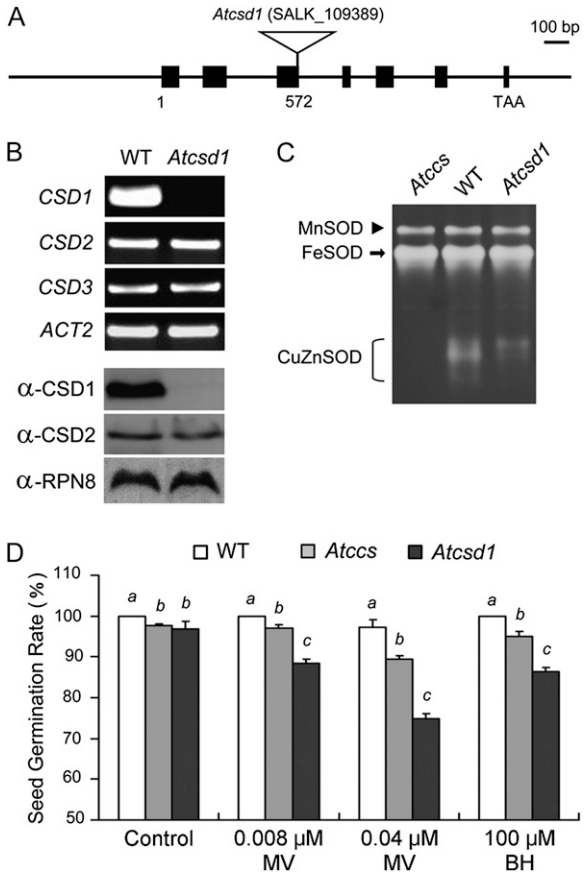

Figure 5. Characterization of CSD activity and seed germination rate of the wild type (WT), *Atccs*, and *Atcsd1*. A, The T-DNA insertion of the *CSD1*-knockout plant, *Atcsd1*, is in the third exon of *CSD1* (nucleotide 572 of the genomic sequence). B and C, Analysis of mRNA expression of *CSD1*, *CSD2*, and *CSD3* (B, top), protein levels of CSD1 and CSD2 (B, bottom), and SOD activities (C) in wild-type, *Atcsd1*, and *Atccs* 7-d-old seedlings. CSD3 protein was undetectable, so those data are not presented, and the CuZnSOD activity level is lower in *Atccs* young seedlings, which explains the undetectable residual activity. D, Comparison of the seed germination rates of wild-type, *Atccs*, and *Atcsd1* plants grown on 1/2 MS plates with the indicated treatments. Data represent results from five independent experiments (means ± SD). Letters (*a*, *b*, and *c*) indicate difference between the wild type, *Atccs*, and *Atcsd1* for each treatment at $P < 0.05$.

form of CSD1 (Apo-CSD1) by an acid treatment method (Lepock et al., 1981). We tested the effect of pH on this assay system and found the recovery of Apo-CSD1 activity to be maximal at pH 7 (Supplemental Fig. S2C), so we used this pH in subsequent experiments (Fig. 7). When Apo-CSD1 was treated with 0.1 μM $CuSO_4$, CSD1 itself could spontaneously bind Cu and recover its own activity (Fig. 7, lane 2). Recovery of Apo-CSD1 activity was unsuccessful with 1 mM GSH alone (lane 3). However, CSD1 activity was not enhanced with GSH plus Cu than with Cu alone (compare lanes 2 and 4), which indicates that the Cu-GSH complex itself was not sufficient to facilitate CSD1 activation. The same experiments were performed with *Atccs* cellular extract (lanes 6–9). With the extract plus GSH (lane 8), the activity of CSD1 increased slightly relative to the extract alone (lane 6). However, CSD1 activity was greatly enhanced with the extract, GSH, and Cu in the reaction mixture (lane 9) as compared with only extract and Cu (lane 7). Therefore, GSH activated CSD1 efficiently under these conditions. Because the *Atccs* cellular extract could greatly enhance the level of CSD1 activation by GSH (compare lanes 4 and 9), the interaction between GSH and CSD1 was facilitated by an as yet unidentified cellular factor.

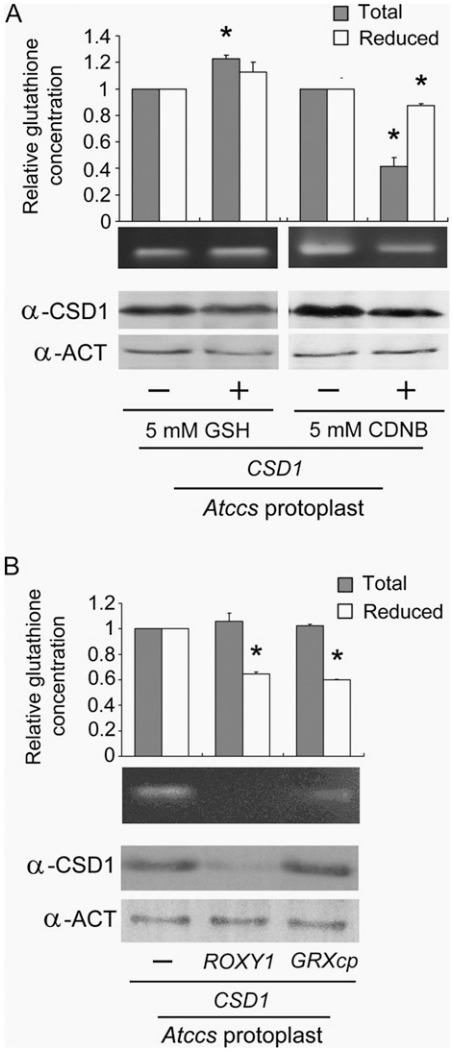

Figure 6. Effect of glutathione concentration on the activity of CSD1 transiently expressed in Arabidopsis protoplasts. A, *Atccs* protoplasts overexpressing *CSD1* were treated with GSH or CDNB for 2 h as indicated. B, *CSD1* was transiently expressed in *Atccs* protoplasts together with *ROXY1* or *GRXcp*. For both A and B, cellular extracts of the indicated protoplasts were analyzed for total glutathione and GSH level, CSD1 activity, or amount of CSD1 protein. Data represent results of three independent experiments (means ± SD). * $P < 0.05$.

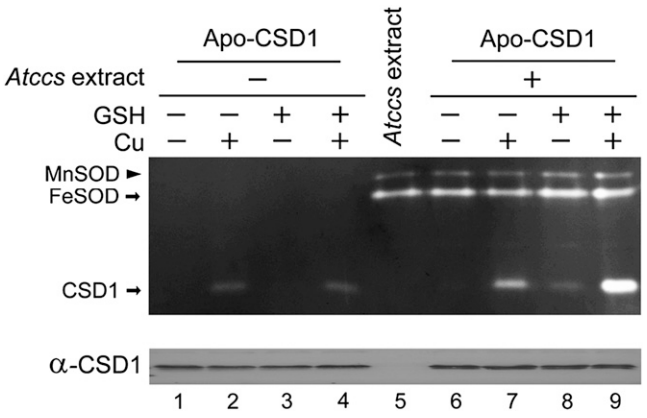

Figure 7. Activation of Apo-CSD1 by Cu, GSH, and *Atccs* cellular extract. Top, SOD activity assay. Each reaction contained 870 ng of Apo-CSD1 protein and 20 μM $ZnSO_4$, to which 0.1 μM $CuSO_4$, 1 mM GSH, or 15 μg *Atccs* cellular extract was added as indicated. Lane 5 represents 15 μg of *Atccs* leaf cellular extract used as a control. Bottom, a replicate of 50 ng of Apo-CSD1 was subjected to the same treatments and then analyzed for protein level by immunoblotting.

DISCUSSION

In this study, we have demonstrated the existence of both CCS-dependent and -independent activation pathways for CSD in plant cells. CSD1 can be activated by both pathways in yeast and Arabidopsis protoplasts (Figs. 1 and 2D). In contrast, CSD2 could not be activated by a CCS-independent pathway in either organism (Figs. 1 and 2B). Thus, ySOD1 and CSD2 might be activated by a similar mechanism. However, further investigation revealed significant differences between the two orthologs. The C-terminal amino acid residues 142P/144P of ySOD1 prevented its activation by the alternative pathway (Carroll et al., 2004), and this effect is mediated primarily by 144P (Leitch et al., 2009a); when 144P was mutated to 144L, this ySOD1 variant showed clear activity in the absence of CCS. For CSD2, the amino acid residues corresponding to 142P/144P of ySOD1 are 143G/145L (Fig. 4A), which are typical for a CSD protein capable of being activated by the CCS-independent pathway (Carroll et al., 2004; Leitch et al., 2009a). The CSD2 protein can be activated by this pathway because the ΔTP-CSD2 (cytoplasm-localized CSD2) was active in *Atccs* (Fig. 2B). Thus, the lack of CCS-independent activity of CSD2 may not result from the protein structure but rather from the presence of factor(s) in the chloroplast that inhibit the CCS-independent activation pathway. This hypothesis is supported by the results obtained with the TP_{CSD2}-CSD1 (chloroplast-directed CSD1), which also lost CCS-independent activity (Fig. 2D). The inhibitory effect on CCS-independent CSD activity in chloroplasts may be due to the absence of chloroplastic factor (s) that are required for CCS-independent activation or to the presence of inhibitory factor(s) in the chloroplast, a novel phenomenon that has not been reported so far for any other species.

Oxygen may be a factor explaining the inhibition of the CCS-independent pathway in chloroplasts. In CCS-dependent activation, oxygen is required during the disulfide formation between the interacting CCS and CSD proteins (Brown et al., 2004; Furukawa et al., 2004): activation cannot occur under hypoxic conditions. Thus, activation by CCS in the chloroplast requires a constant supply of oxygen, which was produced by a properly functioning electron transport chain of the photosynthetic machinery. Such a mechanism can ensure the presence of CSD activity to prevent a buildup of ROS by photosynthesis. In contrast, CCS-independent activation of CSD does not require oxygen (Leitch et al., 2009a). Numerous photosynthetic enzymes require Cu as their cofactor, which could be conferred by chloroplastic CSD (Shcolnick and Keren, 2006). Abundant oxygen, produced from photosynthetic reactions, might be a signal indicating that the photosynthetic enzymes are saturated with Cu cofactors. An antioxidant system in chloroplasts regulated by oxygen could guarantee priority of the distribution of Cu to photosynthetic enzymes.

Although the CSD3 activity was undetectable by the in-gel SOD activity assay in yeast, it could complement the phenotypes of both yeast *sod1*Δ and *ccs*Δ (Fig. 1), which indicates the presence of a physiologically significant level of CSD activity. In these experiments, the activity in *ccs*Δ could have occurred only through the CCS-independent pathway. However, *sod1*Δ contains factors that can facilitate both CCS-dependent and -independent activation, so phenotype recovery by CSD3 in this system may have occurred by either or both pathways. Thus, these results demonstrate only that CCS-independent activation of CSD3 has occurred, not the level of CSD3 activity that can be conferred by CCS.

To further characterize the activation of CSD3 in a homologous plant system, we did not observe activation with overexpression of the full-length CSD3 in Arabidopsis protoplasts. Considering the possible effects of localization similar to CSD2, we deleted its peroxisome-targeting sequence but still found no activity even with *AtCCS* coexpression (Fig. 3; Supplemental Fig. S3). Kliebenstein et al. (1998) demonstrated similar immunoblotting signals with the same amount (12 ng) of recombinant CSD1, CSD2, and CSD3 protein when probed with α-CSD1, α-CSD2, and α-CSD3 antibodies, respectively. Thus, the amount of overexpressed CSD3 protein in protoplasts should be similar to that of overexpressed CSD1 and CSD2 (Supplemental Fig. S3) seen in Figure 2. Hence, our failure to detect CSD3 activity suggests that its level of activation by CCS is much lower than that of CSD1 and CSD2 (Supplemental Fig. S3). In addition, the level of CSD3 activation associated with both pathways (expression in *Atccs* with *AtCCS* coexpression or in wild-type protoplasts) seems not to be significantly higher than the levels associated with the CCS-independent pathway only (expression in *Atccs*). Therefore, CSD3 may be activated primarily by the CCS-independent path-

way. Notably, the activation of nematode wSod-1 in the presence of CCS did not result in a higher activity than that by the CCS-independent pathway (Jensen and Culotta, 2005). Thus, CSD3 seems to be similar to the nematode wSod-1 in this respect.

To date, studies of CSDs in yeast, nematode, mouse, and human have shown different preferences for CCS-dependent and -independent activation (Carroll et al., 2004; Jensen and Culotta, 2005; Leitch et al., 2009b). In Arabidopsis, different types of CSDs are present in a single cell: cytoplasmic CSD1 is activated mainly depending on CCS and partially by the CCS-independent pathway (Fig. 2D); chloroplastic CSD2 activation depends completely on CCS, with inhibition of the alternative pathway in chloroplasts (Fig. 2B); and peroxisomal CSD3 may be activated mainly by the CCS-independent pathway (see above). The species studied thus far showed high variation in CSD activation. Why different organisms have evolved different preferences for CCS-dependent and -independent activation is unclear but may reflect unique lifestyle requirements for Cu and oxygen (Culotta et al., 2006). Because plants cannot move to avoid environmental stresses, the complex activation mechanisms for plant CSDs might be a solution to cope with such varied and stressful surroundings.

In investigating the physiological effects of the residual CCS-independent CSD activity (Fig. 5), we obtained a higher level of seed germination rate in *Atccs* than in *Atcsd1* when treated with ROS-generating reagents. In *Atcsd1*, CSD1 activity was completely lost, whereas residual CSD1 activity was present in *Atccs*. Because phospholipid membranes are impermeable to O_2^- (Takahashi and Asada, 1983), protection of different organelles can be achieved by variable distribution of SODs in different cellular compartments. In other words, the SOD activities in one compartment cannot complement functional deficiencies in another. Therefore, the lower germination rate of *Atcsd1* was possibly due to the complete absence of CSD1 activity in the cytoplasm. In addition, current studies of SODs indicate that the level of SOD activity required for normal growth is much lower than the actual activity level measured (Cohu et al., 2009; C.-H. Huang, W.-Y. Kuo, C. Weiss, and T.-L. Jinn, unpublished data). In fact, *Atccs* showed no obvious phenotype under normal and high-light stress conditions as compared with the wild type (Cohu et al., 2009; C.-H. Huang, W.-Y. Kuo, C. Weiss, and T.-L. Jinn, unpublished data). Therefore, loss of most of the CSD activity did not seriously compromise plant viability, so CCS-independent CSD activities are physiologically functional and sufficient to support the growth of plant cells. The high expression of CSD proteins in wild-type plants may explain their role in Cu buffering (Cohu et al., 2009).

Previous studies in yeast, human, and nematode have shown GSH to be involved in the alternative activation pathway (Carroll et al., 2004; Jensen and Culotta, 2005). Evidence of this involvement includes analyses of a yeast knockout line for GSH biosynthesis in which the major CCS-independent activity was abolished. We tested this hypothesis in Arabidopsis by overexpressing a glutaredoxin in *Atccs* protoplasts to interfere with internal GSH concentrations. However, *ROXY1* expression resulted in a greatly reduced amount of CSD1, with reduced activity (Fig. 6B). Because ROXY1 colocalizes with CSD1, it can readily reduce its disulfide bond, which would lead to degradation of the disulfide-reduced CSD1 (Borchelt et al., 1994; Wang et al., 2003; Carroll et al., 2006; Rouhier, 2010). In contrast, when we overexpressed *GRXcp*, the GSH concentration was decreased, along with CSD1 activity, without a corresponding change in the level of CSD1 protein (Fig. 6B). GRXcp localizes in chloroplasts and cannot interact with CSD1 (Cheng et al., 2006); therefore, the effect of GRXcp on CSD1 activity is due to decreased GSH concentration only. Our result also confirmed the involvement of GSH in this pathway.

However, the results of our in vitro experiments showed that GSH was not sufficient to activate CSD1 unless it was added together with cellular extract (Fig. 7). Therefore, GSH was involved in the CCS-independent pathway in Arabidopsis in the presence of an unidentified factor. Further investigation is required to characterize the unidentified factor and elucidate the complete mechanism.

MATERIALS AND METHODS

Plants, Yeast Strains, and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) *AtCCS* (At1g12520) and *AtCSD1* (At1g08830) knockout lines, *Atccs* (SALK_025986) and *Atcsd1* (SALK_109389), were obtained from the Arabidopsis Biological Research Center (http://abrc.osu.edu;). Seeds were incubated at 4°C for 3 d in the dark before sowing, then plants were grown in a growth chamber at 23°C with 16 h of light at an intensity of 60 to 100 μmol m^{-2} s^{-1}. *Saccharomyces cerevisiae* BY4741 (*MAT*a, *his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*) was used as the yeast wild type, and *sod1*Δ (*sod1*::*kanMX4*) and *ccs*Δ (*ccs*::*kanMX4*) are derivatives of BY4741. Yeast was incubated at 30°C under aerobic conditions without shaking, and G418 was used at 200 μg mL^{-1} to maintain the *ySOD1* and *yCCS* deletions in the yeast as required.

Lys-Independent Aerobic Growth

Yeast strains were cultured in appropriate medium overnight at 30°C without shaking. Then, cells were centrifuged, washed, and resuspended in sterile water, and the A_{600} was measured. For plate assays, the yeast cultures were diluted serially from optical density at 600 nm = 1 to 10^{-4}, plated on synthetic dropout Lys medium, and incubated at 30°C under aerobic conditions for 3 d. For the liquid assay, the yeast cultures were seeded from optical density at 600 nm = 0.01 in the Lys-lacking medium, incubated at 30°C for 24 h under aerobic conditions, and then cell density was determined by measuring the A_{600} (Corson et al., 1998; Wallace et al., 2004).

Seed Germination Rate

Sterilized seeds of the wild type, *Atccs*, and *Atcsd1* were plated on 1/2 MS medium with 1% (w/v) Suc, supplemented with MV or BH at the indicated concentrations, and incubated at 23°C with 16 h of light for 3 d, then seed germination rates were measured.

Constructs and Gene Cloning

For yeast expression, all cDNAs from ATG to the stop codon were inserted at the *Hin*dIII site of yeast expression vector pADNS (Colicelli et al., 1989),

with the exception of *CSD3* (At5g18100), which was inserted at the *Not*I site. The 35S promoter of pPE1000 vector (Hancock et al., 1997) was inserted between the *Xho*I/*Hin*dIII sites of pEYFP vectors (Clontech; http://www.clontech.com) to create p35S-EYFP. For gene overexpression in Arabidopsis protoplasts, cDNA of *CSD1*, *CSD2* (At2g28190), and *CSD3* with a stop codon was subcloned into p35S-EYFP at *Eco*RI/*Bam*HI, *Hin*dIII, and *Eco*RI sites, respectively. For *ΔTP-CSD2*, the 61 amino acids at the N terminus of *CSD2* were deleted and then inserted into p35S-EYFP at the *Hin*dIII site. The chloroplast transit peptides of *CSD2* (TP_{CSD2}) with *Eco*RI/*Nco*I sites and of *CSD1* with *Nco*I/*Sal*I sites were ligated into p35S-EYFP at *Eco*I/*Sal*I sites to create a chloroplast-localized TP_{CSD2}-CSD1. *CSD3* and *CSD3-ΔAKL* (peroxisome-targeting sequence deleted) were inserted into the 326-GFPnt vector (Lee et al., 2001) at the *Sma*I/*Sal*I sites. Both *ROXY1* (At3g02000; Xing et al., 2005) and *GRXcp* (At3g54900; Cheng et al., 2006) cDNAs with a stop codon were inserted into p35S-EYFP at *Sma*I/*Bam*HI sites. Point mutations of *CSD1* were created by the megapriming method (Landt et al., 1990). For GST-tagged protein, *CSD1* was inserted into pGEX-6P-1 (Amersham Pharmacia Biotech; http://www.gelifesciences.com) at the *Eco*RI site. All gene fragments were amplified by PCR and sequenced before making the constructs. Primers used in this study are shown in Supplemental Table S1.

RNA Extraction and Reverse Transcription-PCR

Total RNA was prepared with TRIZOL reagent (Invitrogen; http://www.invitrogen.com) and the TURBO DNA-free Kit (Applied Biosystems; http://www.appliedbiosystems.com). cDNA synthesis involved the use of high-capacity cDNA Reverse Transcription Kits (Applied Biosystems). Reverse transcription-PCR involved the use of the primers shown in Supplemental Table S1.

Protein Extraction, SOD Activity Assay, and Immunoblotting

Arabidopsis leaf cellular extracts were prepared with 150 mM Tris, pH 7.2, grinding buffer (Chu et al., 2005). Yeast crude protein was extracted by the glass bead lysis protocol (Culotta et al., 1997). Protein concentration was determined by the method of Bradford (1976). SOD activity assay and immunoblotting were performed on nondenaturing and denaturing gels, respectively (Chu et al., 2005). SOD activities and protein signals were quantified by analyzing the activity gels and immunoblotting membranes with the use of LAS-3000 (Fuji Film; http://www.fujifilm.com) and Image-Quant software (Molecular Dynamics; http://www.mdyn.com).

Protoplast Preparation and Transfection

Protoplast preparation and transfection were as described (Yoo et al., 2007). About 10^6 protoplasts was transfected with 100 to 300 μg of plasmid DNA for each construct, then incubated for 16 to 24 h. For protein extract, protoplasts were collected and resuspended in 150 mM Tris buffer (pH 7.2), then vortexed five times for 5 s each. Cell lysates were then subjected to the following experiments.

Glutathione Treatment and Quantification

Atccs protoplasts transfected with CSD1 were incubated at room temperature for 16 h, then 1 mM GSH or CDNB (dissolved in 95% ethanol) was added for 15 min. Total glutathione and GSH concentrations were quantified by the use of the Total Glutathione Quantification Kit (Dojindo Laboratories; http://www.dojindo.com) and the QuantiChrom Glutathione Assay Kit (BioAssay Systems; http://www.bioassaysys.com). The resulting concentrations were normalized to the protein concentration of the same sample, and the glutathione concentration values are expressed relative to those with mock treatment.

Apo-CSD1 Protein Preparation and in Vitro Treatments

GST-tagged proteins were affinity purified according to the Glutathione-Agarose user manual (Sigma; http://www.sigmaaldrich.com). GST was removed by the use of PreScission protease (Sigma), and the protein mixture was passed through a GST agarose column to remove free GST. Preparation of inactivated Apo-CSD1 was as described (Lepock et al., 1981). For the in vitro treatments, each reaction contained 870 ng of Apo-CSD1 (equals 2.9 μM) and 20 μM $ZnSO_4$, to which 0.1 μM $CuSO_4$, 1 mM GSH, or 15 μg of *Atccs* cellular extract was added as indicated. After incubation at room temperature for 30 min, 1.5 mM EDTA was added to the samples, and then native-PAGE was performed with 0.1 mM EDTA on both the nondenaturing gels and running buffer. In the treatments with both Cu and GSH, the two solutions were mixed and incubated at 4°C for 16 h before the experiment.

Statistical Analysis

All experiments were independently repeated at least three times. Statistical analysis involved the use of Student's *t* test (two-tailed, unpaired). $P < 0.05$ was considered statistically significant.

Antibodies

α-CSD1, α-CSD2, and α-CSD3 antisera were generous gifts from D.J. Kliebenstein (Kliebenstein et al., 1998), and an Arabidopsis internal control antibody, α-RPN8, was kindly provided by H.-Y. Fu (Yang et al., 2004). We used α-ACT (Chemicon; http://www.chemicon.com) and yeast α-PGK1 (Molecular Probes; http://probes.invitrogen.com) antibodies as internal controls.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Residual CSD activities analyzed in Arabidopsis *Atccs*.

Supplemental Figure S2. Affinity purification of CSD1 and its activity under various pH treatments.

Supplemental Figure S3. Characterization of individual CSD activities in Arabidopsis protoplasts.

Supplemental Table S1. Primers used in this study.

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