

AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7

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Abstract In plant chloroplasts two superoxide dismutase (SOD) activities occur, FeSOD and Cu/ZnSOD, with reciprocal regulation in response to copper availability. This system presents a unique model to study the regulation of metal-cofactor delivery to an organelle. The *Arabidopsis thaliana* gene *AtCCS* encodes a functional homolog to yeast *Ccs1p/Lys7p*, a copper chaperone for SOD. The *AtCCS* protein was localized to chloroplasts where it may supply copper to the stromal Cu/ZnSOD. *AtCCS* mRNA expression levels are upregulated in response to Cu-feeding and senescence. We propose that *AtCCS* expression is regulated to allow the most optimal use of Cu for photosynthesis. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

The two major targets for copper (Cu) delivery within chloroplasts are plastocyanin in the thylakoid lumen and Cu/Zn superoxide dismutase (Cu/ZnSOD) in the stroma. Superoxide ions (O_2^-) are formed by the photoreduction of O_2 at photosystem-I [1]. Superoxide dismutase (SOD) detoxifies the potentially damaging superoxide to hydrogen peroxide (H_2O_2) and O_2 ; for a review see [2]. H_2O_2 is further reduced to water by other enzymes in the water–water cycle [1]. Four major SOD activities are detected in *Arabidopsis* [3]. The mitochondrial manganese SOD (MnSOD) is constitutively expressed [3]. One isoform of Cu/ZnSOD is found in the cytosol (CSD1), whereas another major isoform of Cu/ZnSOD is localized in the chloroplast stroma (CSD2) [3]. Next to Cu/ZnSOD, the stroma can also contain the activity of an Fe superoxide dismutase (FeSOD) [3], which is structurally unrelated to Cu/ZnSOD [2].

To regulate micronutrient distribution but at the same time avoid metal ion-induced damage, all living organisms have

evolved mechanisms to acquire and deliver cofactors to target proteins but at the same time avoid the accumulation of potentially damaging free metal ions in cells [4]. The *Arabidopsis COPT1* gene and its four homologs encode copper transporters that may allow the entrance of Cu into cells [5–7]. Copper is imported into the chloroplast stroma by the P-type ATPase PAA1 and mutations in *PAA1* affect both stromal Cu/ZnSOD activity and PC [8]. The *Arabidopsis* genome encodes a protein with high sequence similarity to PAA1, called PAA2, which functions to move Cu into the thylakoid lumen for delivery to plastocyanin [9].

To avoid improper interactions but also to ensure that the correct delivery pathway is used, copper ions may be directed to specific targets while bound to cysteine-containing proteins known as copper chaperones; for a review see [10]. In yeast, *Ccs1p/Lys7p* delivers copper to the Cu/ZnSOD (Sod1p) by a direct protein–protein interaction [11,12], a function which is required to maintain the activity of reactive oxygen species sensitive enzymes involved in lysine and methionine biosynthesis [13]. Since chloroplasts are a site of oxygen production it is of interest to note that O_2 and the copper chaperone for Cu/Zn superoxide dismutase (CCS) regulate posttranslational activation of Cu/ZnSOD enzymes [14] by mediating correct disulfide formation [15]. A single gene, *AtCCS* encodes for CCS in *Arabidopsis* [16]. *Arabidopsis CCS* encodes a protein with a predicted chloroplast targeting sequence but dual localization in both cytosol and plastids was predicted if transcripts of alternative length are produced [16]. Sequences encoding CCS have also been found in other higher plants such as tomato [17], potato [18] and maize [19].

The expression and regulation of SOD isoforms in plants has been a topic of a number of studies, which showed that oxidative stress, light, and electron transport activity affect plastidic SOD isoform expression [3,8,20]. It was recently shown that Cu feeding dramatically affects the activity of FeSOD and Cu/ZnSOD isoforms, whereas the activity of MnSOD is not affected by Cu availability [9]. *Arabidopsis* plants grown on media in which Cu is not limiting express Cu/ZnSOD in the cytosol and stroma. In contrast, plants grown on low Cu media do not express Cu/ZnSOD in green tissue, which may allow more efficient Cu delivery to plastocyanin under these conditions. To allow the dismutation of superoxide an FeSOD is expressed in the stroma under Cu limitation. Interestingly, in the *paa2* mutant, defective in Cu transport to the thylakoids, an increased expression and activity of Cu/ZnSOD in the stroma was observed [9]. These observations prompted us to investigate Cu delivery to stromal Cu/ZnSOD and its regulation.

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Abbreviations: CCS, copper chaperone for Cu/Zn superoxide dismutase; Cu/ZnSOD, Cu/Zn superoxide dismutase; FeSOD, Fe superoxide dismutase; SOD, superoxide dismutase; GFP, green fluorescent protein; CaMV35S, cauliflower mosaic virus 35S promoter

2. Materials and methods

2.1. Plant material

Arabidopsis plants, the wild-type ecotype Columbia-0 and the mutant lines *paa1-3* [12] and *paa2-1* [13] were grown under a 16 h light/8 h dark cycle on soil at 23 °C. For growth on agar media, seeds were surface-sterilized and sown on solid 1/2 strength Murashige and Skoog (1/2 MS) medium including 1% sucrose and 0.4% agargel [21]. The medium was supplemented with CuSO₄ as indicated.

2.2. Expression of green fluorescent reporter protein in isolated

Arabidopsis protoplasts

Messenger RNA isolation from 14-day old seedlings and cDNA synthesis are described [22]. Primers to amplify the cDNA containing the coding sequence for *AtCCS* (AGI: At1g12520) by PCR are as follows (upstream/downstream): 5' TACCGCGGTACCATGGCGACTGCTCT and 5' ATCCGCGGATCCTTAACCCTTACTGGCCAGAAA. The PCR fragments were subcloned in *KpnI/BamHI* digested pBluescript-SK (Stratagene, San Diego CA). To generate a green fluorescent protein (GFP) fusion, the full-coding sequence of *AtCCS* was amplified to introduce *Sall/NcoI* restriction sites for cloning in the GFP reporter plasmid 35S-SGFP(S65T) [23], generously provided by Dr. Norbert Rolland (Université Joseph Fourier, France). Enzymes, cellulase Onozuka R-10 and Macerozyme R-10 were obtained from Karlan Research Products (Santa Rosa, CA, USA). Protoplasts were isolated and transformed as described [24]. A confocal laser-scanning microscope (FVX-IHRT Fluoview Confocal LSM, Olympus, Melville, NY, USA) with Kr/Ar laser excitation (488 nm) was used to monitor green fluorescence (530 nm) and red chlorophyll auto fluorescence (660 nm). Images were captured and processed using Fluoview software at a 90× magnification at a scan speed of 0.45 s for 256 × 256 pixel area. Scan slices were 1.0 μm thick.

2.3. Yeast complementation

The *AtCCS* mature sequence was cloned under the control of the constitutive PGK promoter in the yeast shuttle vector pFL61 [25] after PCR amplification to introduce *NotI* restriction sites. The yeast *lys7* mutant [13] was obtained from Dr. V. Culotta (Johns Hopkins University). Strains were transformed using the Li-acetate method, transformed colonies were selected on SC minimal media lacking uracil. Complementation was tested by plating serial dilutions on SC-minimal media lacking lysine [13] or supplemented with Menadione (sigma, St Louis, Mo). Plates were incubated at 30 °C for 3 days.

2.4. Miscellaneous methods

Protein was assayed according to [26]. SOD activity assays were performed as described [8]. RNA blots were performed as described [22]. DNA constructs were sequenced in two directions at Davis Sequencing (Davis, CA, USA). Multiple protein alignments were performed with the ClustalW program [27].

3. Results

In analogy to the situation in yeast, Cu chaperones may exist, which deliver Cu to specific targets within the chloroplast. Although the *Arabidopsis* genome encodes many proteins with Cys-x-x-Cys containing possible heavy metal binding domains, our database searches revealed the presence of just two candidate genes encoding putative soluble proteins with a heavy metal binding domain and a putative chloroplast targeting sequence (AGI nos: At1g12520 and At2g28660). Of these two genes, one (At1g12520) encodes a protein similar to Ccs1p/Lys7p, the yeast Cu-chaperone for Cu/ZnSOD. Earlier database searches suggested that *AtCCS* may be the only *LYS7*-like sequence in *Arabidopsis* and it has been speculated that the gene encodes gene products in both plastids and cytosol [16]. The same protein had been identified as a potential homolog of Ccs1p/Lys7p, however that clone lacked a signifi-

cant portion of the N-terminal sequence [17]. We used RT-PCR to obtain the full-length protein-coding sequence for *AtCCS*. Sequence comparison of the cloned cDNA and the published genomic sequence (TAIR) indicated the presence of 6 exons and 5 introns (Fig. 1A), an organization that is shared with the potato homolog [18]. The cDNA sequence confirmed the exon assignment in the MIPS database (<http://mips.gsf.de/proj/thal/db/>) and indicates that it encodes a protein of 320 amino acids. A sequence alignment of *AtCCS* with other predicted CCS proteins is shown in Fig. 1B. The N-terminal region of *AtCCS* includes a predicted 66-amino acid cleavable chloroplast targeting sequence, and similar sequences are found in the other plant CCS proteins. The predicted mature *AtCCS* protein shares a three-domain structure typical of CCS proteins, including a conserved Atx1-like domain with canonical MxCxxC binding motifs (domain I), a central domain with similarity to a portion of Cu/ZnSOD (domain II) and a C-terminal region which includes two conserved cysteines (domain III).

To investigate if *AtCCS* is a functional homolog of yeast Ccs1p/Lys7p the *Arabidopsis* protein was expressed without its transit sequence in a yeast *lys7* mutant. We tested for functional complementation by assaying the growth phenotype on media that lack lysine or contain the superoxide generator menadione (Fig. 2A) and by assaying SOD activity (Fig. 2B). An isogenic wild-type strain and a *lys7* mutant transformed with an empty vector were used as controls. Expression of *AtCCS* rescues both the growth defect phenotype and SOD activity in the *lys7* mutant. We conclude that *AtCCS* is functional as a copper chaperone for SOD in yeast.

In order to investigate the intracellular localization of *AtCCS*, the coding sequence for full-length *AtCCS* protein was fused to the coding sequence for green fluorescent protein in a transient expression vector under control of the cauliflower mosaic virus 35S (CAMV35S) constitutive promoter. Isolated *Arabidopsis* protoplasts were transformed with this plasmid and analyzed for GFP expression. Protoplasts transformed with control CAMV35S:GFP exhibited GFP fluorescence in the cytosol as shown in Fig. 3. In contrast, *AtCCS*:GFP is localized in structures corresponding to chloroplasts when compared to red chlorophyll fluorescence, indicating that *AtCCS* is imported along with the GFP passenger into chloroplasts.

We analyzed the expression of *AtCCS* using RNA blots which indicated that *AtCCS* is expressed both in root and shoot tissues (Fig. 4A). A striking increase in *AtCCS* mRNA levels was seen in shoots during natural senescence (Fig. 4B). To investigate the effects of Cu feeding and plastid Cu levels we compared the mRNA expression in shoot tissue of WT and *paa1* and *paa2* mutant plants under different conditions of Cu supply (Fig. 4C). *AtCCS* mRNA levels are clearly induced by elevated Cu levels (Fig. 4C). Interestingly, in the *paa2* mutant which accumulates Cu in the stroma [9], *AtCCS* mRNA levels were induced at much lower Cu concentrations compared to the WT and *paa1* mutant. RT-PCR experiments showed that treatment with other metal ions such as Fe did not induce *AtCCS* expression.

4. Discussion

The protein encoded by At1g12520 (*AtCCS*) is most likely the metallo-chaperone that delivers Cu to CSD2 in the chloro-

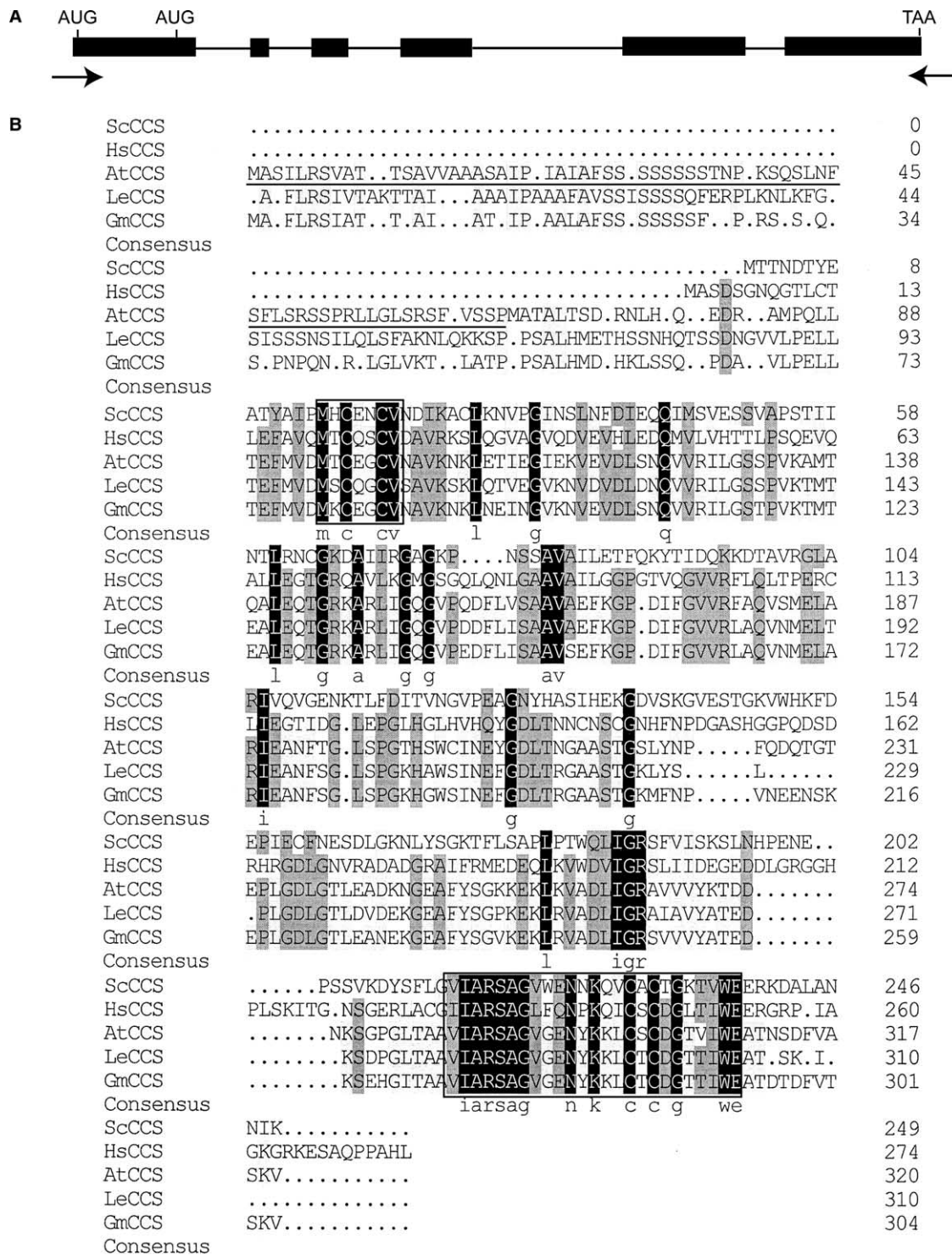


Fig. 1. Sequence alignment and domain structure of *Arabidopsis* CpCCS. (A) Genomic structure of AtCCS. Exons are indicated by solid boxes, introns by lines. Primers used to amplify AtCCS cDNA are indicated by arrows. (B) Alignment of the CCS from *Arabidopsis* (AtCCS), yeast (ScCCS), humans (HsCCS), tomato (LeCCS) and maize (GmCCS). The predicted chloroplast transit sequence of AtCCS is underlined. Conserved regions implied in metal binding are indicated by boxes.

plast stroma, in view of its sequence, the complementation data and the observed localization in plant cells. However, AtCCS is the only candidate in the *Arabidopsis* genome with high conservation as a copper chaperone for superoxide dismutase. It is therefore possible that AtCCS delivers copper

to both CSD1 and CSD2, perhaps utilizing an alternative translation start site that skips the chloroplast targeting peptide as was suggested based on bioinformatic data [16]. The expression of chloroplastic and CSD2 as well as cytosolic CSD1 is dramatically upregulated by Cu [9] and AtCCS is

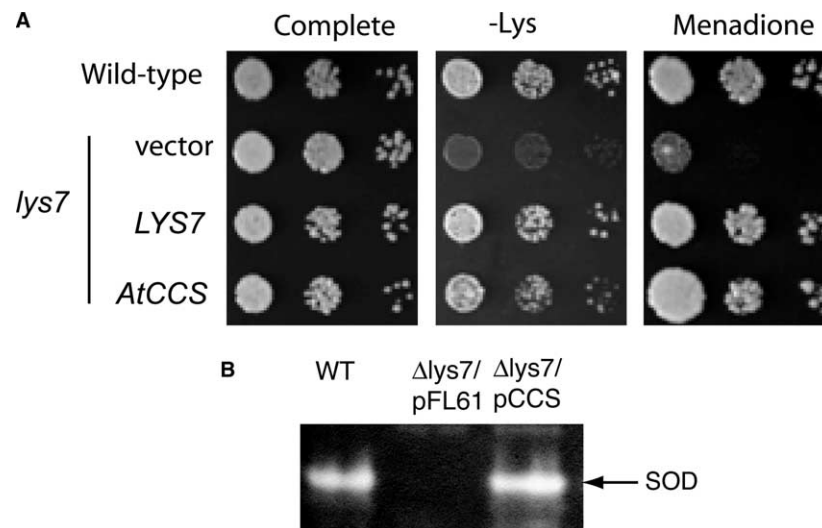


Fig. 2. Functional complementation of the yeast *lys7/ccc1* mutant by *AtCCS*. (A) Complementation of the growth phenotype of *lys7*. *S. cerevisiae* wild type and *lys7* mutant cells transformed with empty plasmid (vector), or vector-containing yeast *LYS7/CCS1* or mature *AtCCS* were assayed for growth on SD (Complete), SD without lysine (-Lys), and YPD with 25 μ M menadione. Cells were grown in SD lacking uracil to exponential phase ($\sim A_{600} = 1.0$), spotted in 10-fold serial dilutions starting at $A_{600} = 0.1$ and incubated at 30 °C for 3 days. (B) Native gel assay for Cu/ZnSOD activity in yeast. Wild-type or mutant cells transformed with the indicated plasmids were grown in SD media and cell extracts (10 μ g protein) tested for SOD activity.

co-regulated with the *CSD1* and *CSD2* targets indicating an important role of delivery Cu in the regulation of oxidative stress protection. Interestingly and consistent with our findings, *AtCCS*, *CSD1* and *CSD2* were found to be downregulated together in response to both Zn and Cu deficiency in a transcript profiling study using micro-arrays [28]. The regulation of *AtCCS* in response to Cu and senescence may also reflect the need for protection from oxidative stress and the need to buffer excess Cu.

It is of interest to compare the reported phenotypes of plants that are deficient in stromal Cu/ZnSOD [29] and plastocyanin [30]. *Arabidopsis* expresses two plastocyanin genes, that are

closely related in sequence [31,32]. Silencing of both copies of plastocyanin leads to a very severe growth phenotype [33] and plants with insertions in both plastocyanin genes cannot be maintained on soil [30]. Thus, in higher plants, plastocyanin is an essential protein and it can be expected that Cu delivery to plastocyanin is a priority for plants. *Arabidopsis* knock-down mutants for *csd2* were reported to display a very severe and light-dependent growth phenotype [29]. Photo-reduction of O_2 at PSI is thought to be an important mechanism of superoxide ion formation [1]. In *paal*, the reduced electron transport rate may diminish the need for photo-protection via an active water–water cycle because photosystem-I will

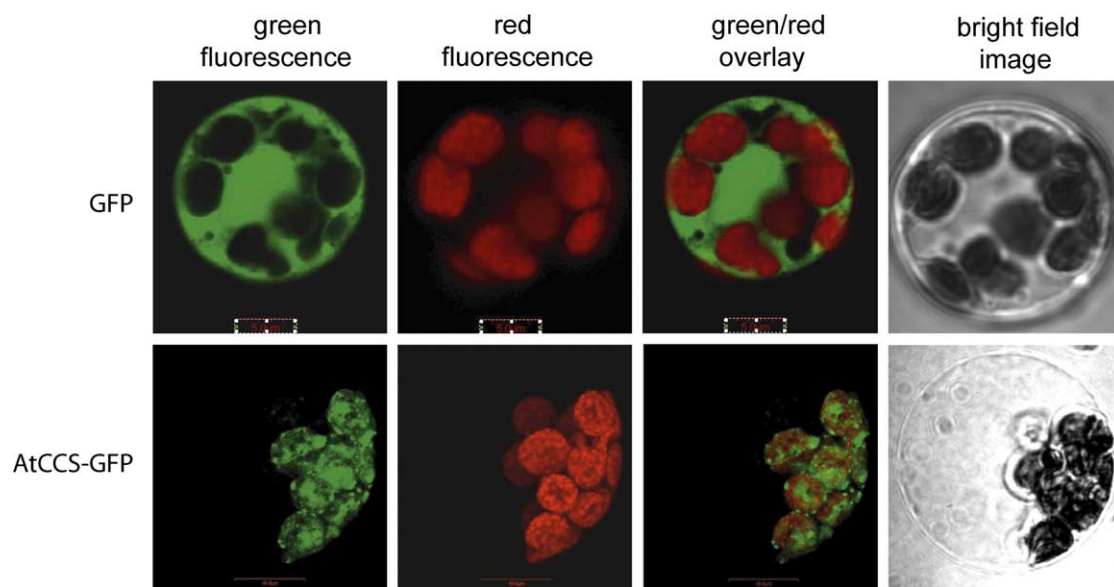


Fig. 3. CpCCS is localized in chloroplasts. The coding sequence of the *AtCCS* precursor was fused to GFP and expressed in protoplasts. Plasmid expressing GFP alone was used as a control. Cells were analyzed 16 h after transformation by confocal microscopy.

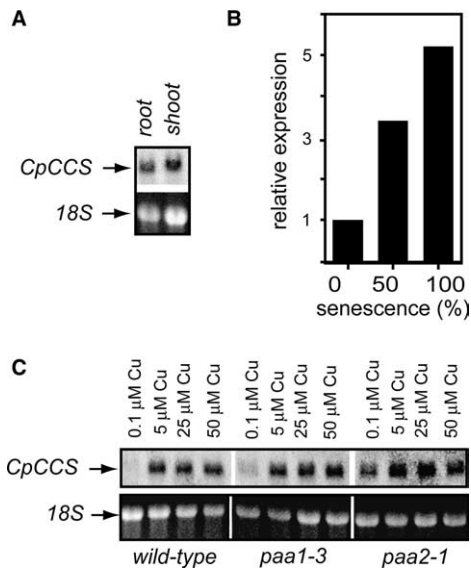


Fig. 4. mRNA expression analysis of CpCSS. (A) Expression in roots and shoots. (B) *AtCCS* expression during senescence. RNA was prepared from the leaves of adult plants. CCS expression was analyzed at different senescence stages, indicated by the percentage of yellow leaf surface. (C) Expression in shoots in response to Cu feeding and in response to mutations in the chloroplast Cu transporters *paa1* and *paa2*. Equal amounts of total RNA were loaded and separated on agarose gels, blotted to Hybond membranes and probed with a *AtCCS* probe. All experiments were performed in duplicate. Quantitative data are the average of two measurements normalized to the control.

be more oxidized [8]. We hypothesize that for optimal photosynthesis, the chloroplast needs to have a Cu delivery system that balances the activity of luminal plastocyanin and stromal SOD enzymes under variable metal supply, ensuring that sufficient SOD activity is present to prevent oxidative damage if plastocyanin is present and PSI can be reduced (see Fig. 5). The available data suggest that this is achieved by a reciprocal regulation of FeSOD and CSD2 expression [9] and regulation of the copper chaperone for SOD in response to Cu in the chloroplast (Fig. 4C). On low Cu media WT plants still produce active plastocyanin, whereas SOD activity is provided by FeSOD alone. The reduced activity of CSD2 under these conditions may help save Cu for delivery to plastocyanin in the lumen. Under Cu sufficient conditions CSD2 is transcribed and a balanced delivery of Cu to stromal CSD2 (likely via *AtCCS*) and to plastocyanin must take place. Under high Cu conditions in the plastid both plastocyanin and in particular stromal Cu/ZnSOD may help to buffer Cu concentrations.

We observed the switching between a Cu-enzyme (CSD2) and an Fe-enzyme (FeSOD) in chloroplasts in response to nutrient status. A Cu/ZnSOD is not found in cyanobacteria or in the eukaryotic green algae *Chlamydomonas reinhardtii*, which depend on FeSOD activities and therefore this switching does not occur in these organisms. Plastocyanin is indispensable in plants [30] and therefore a priority for Cu delivery. However in many algae, including *Chlamydomonas* a cytochrome-*c*(6) can functionally replace plastocyanin under low Cu conditions [34]. This presumably saves Cu for other essential functions such as respiration, which take priority under Cu starvation. Thus, Cu delivery pathways in higher plants and algae may have adapted differently to ensure delivery of Cu to the most essential Cu proteins in each organism.

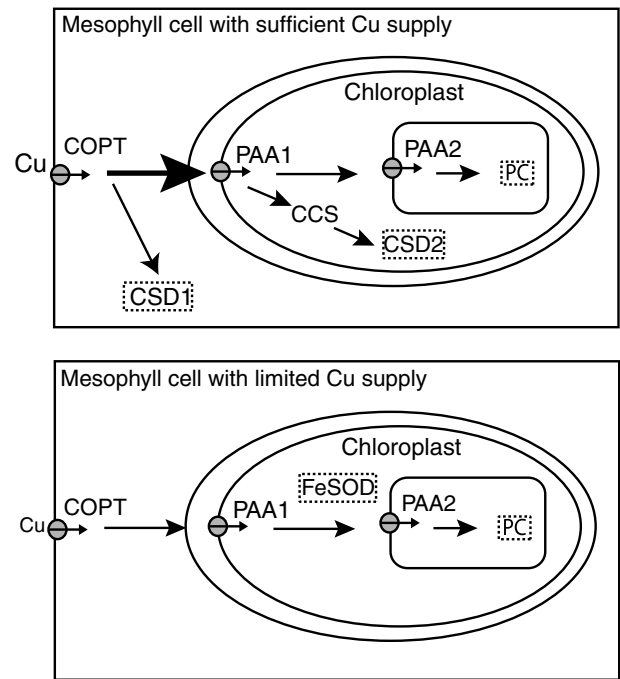


Fig. 5. A model for SOD regulation and Cu delivery in chloroplasts. Cu enters the chloroplast by the PAA1 transporter. When Cu supply is sufficiently high, *CSD1* and *CSD2* and the Cu-chaperone gene *CCS* are transcribed. The *CSD2* protein functions to detoxify the superoxide produced as a result of photosynthetic electron transport. Under these conditions PAA2 can deliver Cu to plastocyanin and both *CSD1* and *CSD2* may help to absorb excess available Cu and prevent toxicity. When Cu supply is limited, the mRNA and protein levels for *CSD1*, *CSD2* and *CCS* are reduced while the FeSOD mRNA and protein become abundant. This regulation allows PAA2 to effectively deliver Cu to plastocyanin without the need to compete with *CCS* for Cu under these limiting conditions.

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