**Regulation of the chloroplastic copper chaperone (CCS) and cuprozinc superoxide
dismutase (CSD2) by alternative splicing and copper excess in *Glycine max***

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Summary text:

- Copper ($\text{Cu}^{++}/\text{Cu}^{+}$) is an essential micronutrient for all living organisms. Both deficiency and excess can cause severe nutritional and environmental damages.
- In the present work we study the regulation of gene and protein expressions of two important cuproproteins, CCS and CSD2, in soybean under environmental copper excess.
- The obtained results allow a better understanding of the copper homeostasis in higher plants.

Abstract. Metal homeostasis is an important aspect of plant physiology, and the copper transport into the chloroplast and its fate after delivery is of special relevance for plants. In this work, the regulation of the chloroplastic copper chaperone for the cuprozinc superoxide dismutase (*GmCCS*) and its target, the cuprozinc superoxide dismutase (*GmCSD2*), was investigated in photosynthetic cell suspensions and entire plants from *Glycine max*. Both genes were expressed in cell suspensions and in all plant tissues analysed, and their RNAs matured by alternative splicing with intron retention (IntronR). This mechanism generated a spliced and three non-spliced mRNAs in the case of *GmCCS* but only a spliced and a non-spliced mRNAs in *GmCSD2*. Copper excess strongly upregulated the expression of both fully spliced mRNAs but mostly unaffected the non-spliced forms. In entire plants, some tissue specificity was also observed depending on copper content status. At protein level, the *GmCCS* was mostly unaffected but the *GmCSD2* was strongly induced under copper excess in all subcellular fractions analysed suggesting a post-transcriptional regulation for the former. This different protein regulation of the chaperone and its target may indicate some additional function for the CSD2 protein. In addition to its well-known SOD activity, it may well function as a metal sink in copper excess availability to avoid metal cell damage. Furthermore, the *GmCCS* seems to be present in the stroma only but the *GmCSD2* was present in both stroma and thylakoids despite the general idea that the SOD enzymes are typically soluble stroma proteins. The presence of the SOD enzyme on the surface of the thylakoid membranes is reasonable considering that the superoxide radical (O_2^-) is preferentially formed at the acceptor side of the photosystem I.

Introduction

Copper (Cu) is an essential micronutrient for plant growth and development and it can be found as Cu (II) and Cu (I) oxidation states. Because of its redox properties, Cu plays a pivotal role in cellular biochemical and physiological processes as a catalytic cofactor in the redox chemistry of enzymes involved in a broad spectrum of biological activities, including photosynthetic and mitochondrial electron transport, oxidative stress responses, and hormone perception, among others (Himmelblau and Amasino 2000; Burkhead *et al.* 2009). Despite being essential, Cu is highly toxic to plants at elevated concentrations. The reactivity of Cu ions can lead to generation of harmful reactive oxygen species (ROS) *via* Fenton and Haber-Weiss reactions that cause severe oxidative damage to the cells (Halliwell and Gutteridge 1984). Cu excess causes phytotoxicity by inhibiting key cellular processes, including photosynthesis, lipid peroxidation and disruption of protein functions due to Cu-binding to sulphydryl groups (Sandmann and Böger 1980; Yruela *et al.* 1993). Thus to prevent the damage of Cu excess, plants have developed cellular and molecular mechanisms to maintain Cu homeostasis at physiological level, which include regulation of Cu-uptake in root cells (Sancenon *et al.* 2004), Cu-trafficking *via* P-type ATPases and Cu chaperones (Wintz and Vulpe 2002; Abdel-Ghany *et al.* 2005*b*; Sagasti *et al.* 2011), and regulation of the levels of cuproproteins in response to change in metal availability (Yamasaki *et al.* 2007; Abdel-Ghany and Pilon 2008; Yamasaki *et al.* 2009; Beauclair *et al.* 2010).

Several proteins or protein families involved in plant Cu homeostasis are largely conserved through the diversity of living organisms (Pilon *et al.* 2006). A class of abundant intracellular cuproproteins is formed by the cuprozinc superoxide dismutase (CuZnSOD or CSD) enzyme, whose function is to catalyze the dismutation of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (Asada 1999). Plant CSDs are normally 30-33 kDa homo-dimeric proteins *in vivo* (Mauro *et al.* 2005). Three genes encode for the CSDs in the Arabidopsis genome (Kliebenstein *et al.* 1998): CSD1 is active in the cytosol, CSD2 in the chloroplast and CSD3 in the peroxisome. CSD1 and CSD2 are the major isoforms in green tissue. Plants also contain a functional homolog to the yeast Cu chaperone (CCS) that functions in the maturation of CSD1 and CSD2 (Abdel-Ghany *et al.* 2005*a*; Chu *et al.* 2005). Since the first description of the

CCS in yeast and human (Culotta *et al.* 1997), its identification and physiological significance have been defined in multiple species including some plants, as for instance, in tomato (Zhu *et al.* 2000), potato (Trindade *et al.* 2003), maize (Ruzsa and Scandalios 2003), Arabidopsis (Abdel-Ghany *et al.* 2005a; Cohu *et al.* 2009) and soybean (Sagasti *et al.* 2011).

Biochemical and crystallographic studies of the CCS protein in yeast and humans allowed identifying some structural and functional aspects of this chaperone family (Lamb *et al.* 1999; Schmidt *et al.* 1999a,-b; Stasser *et al.* 2007). CCS is a 26-30 kDa protein with three main domains: domain I at the N-terminal region binds Cu(I) at the MXCXXC metal binding-motif; domain II in the central region is a key domain for the interaction with CSD2 (Lamb *et al.* 2000); and domain III at the C-terminal region is a highly disordered short tail. The short tail contains a CXC Cu(I)-binding site that can interact with domain I, and that interaction is thought to be essential for Cu delivery to the apo-CSD *in vivo*. Several studies using a recombinant yCCS show that its apo-form is a monomer, while the Cu-yCCS form can exist in monomer/dimer equilibrium (Schmidt *et al.* 1999a,-b). In Arabidopsis, CCS is encoded by one gene containing two in-frame ATG start sites that flank a chloroplast targeting sequence. Therefore, there are both a cytosolic and chloroplastic versions of CCS that may function in Cu delivery and activation of CSD1 and CSD2, respectively (Chu *et al.* 2005). Cohu *et al.* (2009) and Huang *et al.* (2012) found that CCS-independent activation of CSD in plants is insignificant, demonstrating the essentiality of this chaperone for CSD maturation.

Recent findings in Arabidopsis show that Cu availability is the major factor that determines whether iron superoxide dismutase (*FSD1*), *CCS* or *CSD1* and *CSD2*, among other genes, are expressed (Abdel-Ghany *et al.* 2005a,-b; Yamasaki *et al.* 2007; Cohu and Pilon 2007; Dugas and Bartel 2008). In Arabidopsis, *CCS*, *CSD1* and *CSD2* regulation is mediated by a microRNA, miR398, which targets mRNA for degradation during growth on low Cu environments (Sunkar *et al.* 2006; Yamasaki *et al.* 2007; Cohu *et al.* 2009; Beauclair *et al.* 2010). miR398 is regulated by a transcription factor called SPL7 (Yamasaki *et al.* 2009). This Cu-microRNA is one of several Cu regulated microRNAs that downregulate transcripts for a number of cuproproteins during Cu-limiting growth conditions (Abdel-Ghany and Pilon 2008). It was proposed that the mechanism of cuproprotein downregulation during Cu limiting growth allows for preferential delivery of Cu to plastocyanin, an essential component for plant photosynthetic electron transport and survival (Burkhead *et al.* 2009). Indeed, the first

step after supplementation with Cu in this metal limiting growth conditions was to re-establish the normal content of plastocyanin before the synthesis of any other cuproprotein (Tapken *et al.* 2012).

Suboptimal concentrations of Cu cause severe growth and fertility defects, while excess amounts of Cu can be highly toxic, and plants have to cope with both extreme situations. To that end, plants have developed complex mechanisms to control tightly the homeostasis of this essential micronutrient to ensure the plant survival in Cu limiting and excess conditions. Most of the previous studies were focused on plant response in limiting Cu availability making the present work more interesting to further understand the plant behaviour under metal excess conditions. In the present study, we report the identification of soybean Cu chaperone (*GmCCS*) and the superoxide dismutase (*GmCSD2*)-related mRNAs, their maturation by alternative splicing, and their response to Cu excess. Here we also show the identification, localization and response to Cu excess of *GmCCS* and *GmCSD2* proteins. The results will improve our understanding of Cu homeostasis regulation within chloroplasts in Cu excess growth conditions.

Materials and methods

Growth conditions of photosynthetic soybean cell suspensions

Photosynthetic cell suspensions from soybean (*Glycine max* var. Corsoy SB-P line) were grown as described by Rogers *et al.* (1987) with some modifications (Bernal *et al.* 2006). Cell suspensions were grown in photomixotrophic (KN¹) medium under continuous light ($30 \pm 5 \mu\text{E m}^{-2} \text{s}^{-1}$) and air with 5% CO₂ supplementation at 24°C on a rotatory shaker (TEQ Model OSFT-LS-R, Massamá, Portugal) at 110 rpm. Control cell suspensions (C) were grown in the presence of 0.16 μM CuSO₄. To investigate the effect of different metals in cell suspension growth, the medium was supplemented with 0.5 μM CuSO₄, 10 μM CuSO₄, 10 μM FeSO₄, and 10 μM ZnSO₄, respectively. Cells used for experiments were collected from liquid cultures after 21 days of growth by filtration through Miracloth paper (Hoechst, Calbiochem, EMD Biosciences Inc, San Diego, CA, USA) and stored at -80°C.

Plant material, growth conditions and experimental treatments

Soybean plants (*Glycine max* cv. Volania) were grown hydroponically in a 0.5x Hoagland nutrient solution (Arnon 1950) in a climate-controlled growth chamber with a

photoperiod of 16 h light/8 h dark, at $350 \mu\text{mol m}^{-2} \text{s}^{-1}$, 70% relative humidity, and day and night temperatures of 24°C. The control plant (C) medium contained 0.16 μM CuSO_4 . To examine the effect of Cu on soybean plant growth, two different treatments were performed: 1) the nutrient solution was supplemented with 10 μM CuSO_4 (root Cu treatment, CuR). The hydroponic medium was changed once per week during 7 days (young plants) and during 20 days (mature plants) of growth; and 2) the leaves were painted with 10 μM CuSO_4 (Cu leaf treatment, CuL). Painting was every other day during 7 days (young leaves) and every 4 days during 20 days (mature leaves) of growth. The hydroponic medium was changed once per week.

RNA preparation and cDNA synthesis

Cell suspensions (0.3 g fresh weight) and leaves (0.1 g fresh weight) were washed once with fresh growth medium or distilled H_2O , respectively, and homogenized using a pestle and mortar in liquid nitrogen. Total RNA was isolated and purified using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Quality and quantity of RNA was analysed by spectrophotometric analysis (A_{260} and A_{280}). Genomic DNA contamination was checked by PCR. cDNA was synthesized from total RNA (5 μg) using 200 units of reverse transcriptase (M-MLV reverse transcriptase, Promega, Madison, WI, USA) and 1 μM oligo(dT)₁₂₋₁₈ (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Transcript analysis

To examine the effect of Cu in gene expression patterns, equal amounts of RNA were used for semi-quantitative PCR. Equal amounts of specific primers pairs were used as follows: i) F-CCS and R-CCS to amplify *GmCCS*; ii) F-NSP1CCS and R-CCS to detect *NSP1-GmCCS*; iii) F-NSP2CCS and R-CCS to detect *NSP2-GmCCS*; iv) F-CCS and R-NSP3CCS to detect *NSP3-GmCCS*; v) F-CSD2 and R-CSD2 to detect *GmCSD2*; vi) F-CSD2 and R-NSPCSD2 to detect *NSP-GmCSD2*. Actin primers (F-ACT and R-ACT) were used as controls (Supplementary Table 1). PCR amplification was performed in a Perkin-Elmer thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, Foster City, CA, USA). The resulting PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced (CNIO, Madrid, Spain) to check their identity.

Sequence analysis

The alignment of the sequences was performed using ClustalW program (Thompson *et al.* 1994). The identification of putative introns within the sequences was done with GenScan Web Server (Burge and Karlin 1997). The identities and similarities were calculated using the BLAST program.

Determination of copper concentrations

Tissues (root, leaf, stem) from 4-week-old plants were washed with 2 mM EDTA solution and distilled H₂O. The samples were then dried out in a ventilated oven at 60°C for 6 days and subsequently grinded with a ball mill (Retsch, Haan, Germany). Cu concentration was determined by inductive coupled plasma - mass spectrometry (ICP-MS) at the Ionomica service of CEBAS-CSIC (Murcia, Spain).

Isolation of chloroplasts, thylakoids and stroma

Soybean cell suspensions were collected, filtered through a layer of Miracloth and weighted. Cells were then suspended in a buffer containing 400 mM NaCl, 2 mM MgCl₂, 0.2% (w/v) sucrose, and 20 mM TRICINE, pH 8.0, at a cell to buffer ratio of 1:2 (w/v). Cells were broken with a motor-powered Teflon homogenizer during 10 min with 1 min delay every 2 min homogenization to avoid sample heating. The resultant suspension was gently stirred for 10 min and centrifuged at 300 x g for 2 min. The supernatant was centrifuged at 13,000 x g for 10 min, and the resultant sediment was resuspended in buffer containing 150 mM NaCl, 5 mM MgCl₂, and 20 mM TRICINE, pH 8.0, and centrifuged at 13,000 x g for 10 min. The pellet (thylakoid fraction) was resuspended in buffer containing 15 mM NaCl, 5 mM MgCl₂, 400 mM sucrose, and 50 mM MES, pH 6.0, and stored at -80°C, and the supernatant (stroma fraction) was concentrated in Centriprep-10 and Centricon-10 tubes (Amicon, Billerica, MA, USA) and also stored at -80°C. Young and mature soybean leaves from 4-week-old plants were collected, washed twice with distilled H₂O, and cut into small pieces. Leaf pieces were ground in a buffer containing 400 mM NaCl, 2 mM MgCl₂, 0.2% (w/v) bovine serum albumin (BSA), 40 mM ascorbate, and 20 mM TRICINE, pH 8.0, at a leaves to buffer ratio of 1:2 (w/v). The mixture was filtered with a layer of Miracloth paper and centrifuged at 300 x g for 2 min. The following steps were the same as described for soybean cell suspensions. To verify the quality of the sub-chloroplast fractionation, commercial anti-RbcL and anti-PsbA (D1) antibodies (Agrisera AB, Vännäs, Sweden) were used as stroma and thylakoid membrane specific markers, respectively.

Isolation of chloroplasts was performed according to van Wijk *et al.* (1995) with some modifications (Andreasson *et al.* 1988), adapted to soybean cell suspensions and

plants. Leaves were cut and washed with distilled H₂O, and then homogenized in a buffer containing 330 mM sorbitol, 5 mM ascorbate, 2 mM EDTA, 20 mM NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 0.5 mM KH₂PO₄, 5 mM MES-KOH, pH 6.1, filtered with a layer of Miracloth paper and centrifuged for 2 min at 1000 x g. Cells were then homogenized as described above, and the supernatant was centrifuged for 1 min at 300 x g. Both supernatants were centrifuged for 2 min at 1000 x g. The resultant pellets were suspended in a minimum volume of a buffer containing 330 mM sorbitol, 5 mM ascorbate, 2 mM EDTA, 20 mM NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 50 mM HEPES-KOH, pH 7.6, loaded on a Percoll cushion (35%, w/v) and centrifuged at 5000 x g for 10 min. A band located approximately 1 cm down the air-liquid interface was collected as chloroplasts and washed with the last buffer, centrifuged at 1300 x g for 5 min and resuspended carefully in a buffer containing 330 mM sorbitol, 5 mM MgCl₂, 50 mM HEPES-KOH, pH 7.6.

All solutions used in the fractionation procedure contained 100 µg ml⁻¹ pefablock, 1 µg ml⁻¹ leupeptin and 1 µg ml⁻¹ antipain as protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). All steps were carried out at 4°C under dim light. Final samples (chloroplasts, thylakoids, stroma) were first frozen in liquid nitrogen and then stored at -80°C until use.

Production of GmCCS and GmCSD2 antibodies

Rabbit polyclonal antibodies anti-*GmCCS* (Genosphere Biotechnologies, Paris, France) were produced from a 15 amino acid synthetic peptide --CVPEDFLISA AVSEF-- from the domain I of the *GmCCS*. The antibodies were designed from the deduced amino acid sequence of soybean CCS (AF329816). The antibody anti-*GmCCS* was subsequently purified by affinity chromatography using the produced synthetic peptide. This antibody was already described and positively tested in Sagasti *et al.* (2011). An antibody against the entire CSD2 from barley (a generous gift from Prof. B. Sabater, Plant Physiology Department, Alcalá de Henares University, Alcalá de Henares, Spain) and was used to detect the presence of that polypeptide.

Western blotting analysis

Protein concentration was determined according to Bradford (1976) using BSA as standard. Denaturing procedure was carried out at room temperature for 1 hour using a sample buffer containing 2.3% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol, and 62 mM TRIS-HCl, pH 6.8. Samples were separated by SDS-PAGE and transferred onto nitrocellulose or

polyvinylidene fluoride membranes (BioTrace-PVDF; Pall Corporation, Ann Arbor, MI, USA) using a Mini Trans-Blot transfer system (Bio-Rad, Palo Alto, CA, USA). After blocking the membrane with 5% (w/v) skimmed milk, blots were incubated with the corresponding antibody. Western blot detection was carried out by two different methods: *i*) amplified alkaline phosphatase GAR kit (Bio-Rad); and *ii*) SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, NH, USA).

Antioxidant enzyme assays

SOD enzyme activities were assayed in the chloroplast stroma fraction. Samples were loaded on a native PAGE gel, essentially as described by Laemmli (1970) except that SDS was omitted. Electrophoretic separation was performed at 4°C in a 15% (w/v) PAGE gel. SOD enzymes were detected in the gels by the photochemical nitroblue tetrazolium (NBT) method as described by Beauchamp and Fridovich (1971). The different types of SODs were identified by incubating with specific inhibitors (KCN or H₂O₂); H₂O₂ inhibits the activity of both CuZnSOD and FeSOD, KCN only inhibits the activity of CuZnSOD and the MnSOD is insensitive to both inhibitors. After electrophoresis, gels were incubated with 50 mM potassium phosphate buffer, pH 7.8, containing 3 mM KCN or 5 mM H₂O₂ for 1 hour at 25°C in darkness. Gels were then incubated with 50 mM potassium phosphate buffer, pH 7.8, containing 0.48 mM NBT for 30 min and then with 30 µM riboflavin, 0.02% (v/v) TEMED, 50 mM potassium phosphate buffer, pH 7.8 for 30 min. Gels were subsequently washed with 50 mM potassium phosphate buffer, pH 7.8, for 5 min and illuminated for 10 min.

Results

GmCCS gene is regulated by alternative splicing and Cu availability

To characterize the expression profile of the *GmCCS* transcript, semi-quantitative PCR experiments were performed in soybean cell suspensions as well as in entire soybean plants using two specific primers (F-CCS and R-CCS) (Supplementary Table 1, Fig. 3). Surprisingly, three groups of cDNA bands were reproducibly amplified in control (C) cell suspensions (Fig. 1) and entire plants (Fig. 2). Sequence analysis revealed that clones obtained from the lower band (747 bp) corresponded to *GmCCS*, as expected due to its size. The sequence information obtained for the intermediate band yielded two different sequences of 856 bp and 880 bp, respectively. These bands corresponded to CCS with two different introns retained (IntronR), the first one is Intron 2 of 109 bp and the second one is Intron 3 of 133 bp. Finally, the highest band (1880 bp) also retained

an intron (Intron 5) (for more information see Fig. 3) of 1133 bp in its sequence. These sequences were named as *NSP1-GmCCS*, *NSP2-GmCCS* and *NSP3-GmCCS* (NS, non-spliced), respectively. Based on the alternative splicing classification (Itoh *et al.* 2004), the putative regulation observed for *GmCCS* corresponds with the IntronR type, characterized by the alternative intron inclusion or intron splicing in RNA. In fact, IntronR is the most prevalent alternative splicing type in both Arabidopsis and rice (Wang and Brendel 2006). All these *NSP-mRNAs* contain a premature termination codon at codon position 20, 50, and 183, respectively, which leads to putative truncated polypeptides with theoretical molecular masses of 2.1 kDa for *NSP1-GmCCS*, 5.4 kDa for *NSP2-GmCCS* and 19.9 kDa for *NSP3-GmCCS*.

Toward a further understanding of this regulation, we have analysed the CCS regulation in a simpler system, the soybean cell suspensions. In Cu-treated cells, the fully spliced transcript (*GmCCS*) was strongly upregulated whereas the non-spliced ones (*NSP1-GmCCS*, *NSP2-GmCCS* and *NSP3-GmCCS*) appeared downregulated compared to control cells (Fig. 1A, upper part). However, after using specific primers for each NSP transcript (Fig. 1A, lower part), we observed that the NSP forms were all present and were not regulated by copper excess as the upper part of Fig. 1 firstly suggested.

Based on the study of Stasser *et al.* (2005), who reported the *hCCS* contains 2 Cu(I) and 1 Zn(II) ions per protein, and Sagasti *et al.* (2011), who suggested the same metal content in soybean plants, we decided to analyse the influence of both metals (Cu and Zn), individually and combined, on *GmCCS* gene expression in cell suspensions (Fig. 1B). Cu excess alone or in combination with Zn, strongly induced the fully spliced *GmCCS* transcript expression, but no effect was observed by Zn excess (Fig. 1B).

In order to determine whether Cu status controls *GmCCS* gene expression in the different vegetative parts of the plant and during leaf development, semi-quantitative PCR experiments were carried out in entire plants grown in control conditions (C) and treated with Cu excess at leaf (CuL) and root levels (CuR) (see Materials and methods, for a detail description of the Cu treatments). The fully spliced *GmCCS*, *NSP2-GmCCS* and *NSP3-GmCCS* transcripts were detected in all plant tissues analysed but the *NSP1-GmCCS* transcript was absent (Fig. 2, panel A). The presence or absence of the NSP forms was confirmed using specific primers for each transcript (Supplementary Table 1, Fig. 3), designed within the intron sequences of *NSP1-GmCCS* (F-NSP1CCS), *NSP2-*

GmCCS (F-NSP2CCS) and *NSP3-GmCCS* (R-NSP3CCS) (Fig. 2, panels B, C, D, respectively).

The most abundant transcript in any conditions in both cell suspensions and entire plants seems to be the fully spliced *GmCCS* (Fig. 1 and 2, panels A). Young and mature leaves showed similar expression pattern, suggesting that *CCS* is expressed independently of the leaf maturation status. In CuR plants, the fully spliced *GmCCS* was clearly upregulated and apparently mask the expression of other transcripts (Fig. 2, panel A). Interestingly, in roots no clear Cu upregulation of the *GmCCS* transcript was observed because in this particular tissue its expression was already high in C conditions (Fig. 2, panel A), most probably because roots were directly in contact with the hydroponic medium and stored higher Cu content than the other tissues analysed. That suggestion was confirmed by ICP-MS analysis where a higher Cu concentration was observed in roots in all experimental conditions, especially in CuR (S. Sagasti, 2009, Ph. D. Thesis, Department of Biochemistry and Molecular and Cell Biology, University of Zaragoza, Zaragoza, Spain).

Schematic representation of the four identified transcripts is shown in the Fig. 3.

GmCSD2 gene is regulated by alternative splicing and Cu availability

To characterize the expression profile of *GmCSD2* transcripts in soybean, semi-quantitative PCR experiments were performed in cell suspensions and entire plants for comparison by using two specific primers (F-CSD2 and R-CSD2), designed within the UTR regions of the gene (Supplementary Table 1, Fig. 5). Two groups of bands were reproducibly amplified in control cell suspensions (Fig. 4A). The lower band was much more abundant than the higher one, and migrated at position corresponding to 791 bp, as expected for *GmCSD2*. Sequence information of clones obtained from the higher band (891 bp) showed that the sequence retained the intron 4 of 100 bp. This transcript with the intron 4, named *NSP-GmCSD2*, contains a premature termination codon at codon position 185 that leads to a putative truncated polypeptide with a theoretical molecular mass of 19.4 kDa. As in the case of the *GmCCS* gene, the alternative splicing observed here corresponded to an IntronR type (Itoh *et al.* 2004). In order to determine whether Cu status regulates *GmCSD2* expression, we carried out semi-quantitative PCR experiments in photosynthetic cell suspensions and entire plants grown under control and Cu-treated conditions (Fig. 4). In Cu-treated cells, *GmCSD2* was upregulated

whereas the expression of *NSP-GmCSD2* did not significantly change compared to control cells (Fig. 4A).

Since the CSD2 enzyme contains Cu and Zn ions as cofactors, we proposed to analyse the influence of both metals, individually and combined, on *GmCSD2* gene expression in cell suspensions, likewise we did with the *GmCCS* gene. Cu excess alone or in combination with Zn, strongly induced the *GmCSD2* expression compared to control cells, but no effect was observed in the presence of Zn alone (Fig. 4B). These results were identical with those observed for *GmCCS* (Fig. 1B).

In soybean plants, one group of bands with the expected size of *GmCSD2* was also reproducibly amplified in all tissue analyzed: young leaf, mature leaf, stem, root and flower (Fig. 4C, panel a). Although *NSP-GmCSD2* was not visualized in the gel (Fig. 4C, panel a), its presence was demonstrated in all growth conditions (C, CuL, CuR) and all tissues analysed using the specific primer R-NSPCSD2, designed within the intron sequence of the non-spliced transcript in combination with F-CSD2 primer (Supplementary Table 1, Fig. 5). This primer pair also confirmed the presence of *NSP-GmCSD2* in control and Cu-treated cell suspensions (Fig. 4A, lower part).

In young leaves, *GmCSD2* was clearly present and upregulated by Cu treatments compared to control plants (Fig. 4C, panel a). However, in mature leaves *GmCSD2* was hardly detected in both C and CuL leaves (Fig. 4C, panel a). This is in agreement with a lower Cu content in mature leaves compared to young leaves as determined by ICP-MS (S. Sagasti, 2009, Ph. D. Thesis, Department of Biochemistry and Molecular and Cell Biology, University of Zaragoza, Zaragoza, Spain). However, the upregulation by Cu was not clearly observed in roots because in this particular tissue the *GmCSD2* expression was already high even in control growth conditions (Fig. 4C, panel a), as in the case of the *GmCCS*. As mentioned above, this was most probably due to the high Cu content detected in root tissue in all growth conditions. The stem shows an expression pattern similar to young leaf in both control and Cu-treated conditions (Fig. 4C, panel a).

Schematic representation of the two identified transcripts is shown in the Fig. 5.

Identification, localization and Cu regulation of CCS and CSD2 proteins

The GmCCS chaperone protein

The polyclonal antibody developed against a synthetic peptide of the *GmCCS* (see Materials and methods, for further details) was validated against the purified

recombinant protein (Sagasti *et al.* 2011). Indeed, a band at around 37 kDa, corresponding with the *GmCCS* protein as proved by MS, specifically reacted with this antibody. In the present work, western blot analysis with this antibody was conducted with protein extracts from chloroplasts, thylakoids and stroma from control and Cu-treated cell suspensions (grown in the presence of 10 μ M CuSO₄) and plant leaves (Fig. 6). Two commercial antibodies, anti-RbcL (large subunit of the RUBISCO enzyme) and anti-PsbA (D1 protein, a PSII reaction center subunit), were used as specific markers for stroma and thylakoid fractions, respectively. The D1 protein was only detectable in chloroplast and thylakoid fractions, and the RUBISCO was only present in chloroplast and stroma fractions (Supplementary Fig. 1), indicating the good quality of the fractionation. The antibody against the *GmCCS* chaperone revealed three main bands of apparent molecular masses around 94, 37, and 23 kDa in different samples from control and Cu-treated cell and leaf fractionation (Fig. 6). The middle band corresponds with the monomeric form of *GmCCS* (Sagasti *et al.* 2011), the lower band might be due to the NSP3-*GmCCS* and the highest band might be an oligomeric form. However, it turned out that the highest molecular weight band remained after blotting with no primary or secondary antibodies or without both, demonstrating that this band was an artefact. Note that the middle band due to *GmCCS* was absent in the thylakoid samples (Fig. 6), confirming that CCS is a soluble stroma protein. Surprisingly, copper treatment seemed to have no significant effect on the *GmCCS* protein expression compared to controls in contrast to what was observed with the gene expression (Fig. 1 and 2), suggesting a post-transcriptional regulation of the *GmCCS* protein.

The GmCSD2 protein

To investigate the *GmCSD2*, western blot analysis was conducted with a polyclonal antibody developed against the entire CSD2 protein from barley. The antibody immunoreacted with several bands around 25 kDa, as expected, but it also cross-reacted with bands corresponding with the *GmCCS* forms (Fig. 7). Note that the CSD has a high homology with the domain II of the CCS, and, therefore, this immunocross-reaction is expected. Indeed, the domain II of the CCS interacts with the CSD during the Cu delivery (Stasser *et al.* 2007). The blots also showed that copper treatment strongly increased the three bands of apparent molecular masses around 25 kDa, demonstrating that copper clearly induced the *GmCSD2* isoforms in soybean (Fig. 7, right panel). On the contrary, the *GmCCS* was again not affected by copper excess (Fig. 7, left panel), confirming the previous results of the Fig. 6. Interestingly, all three isoforms of the

GmCSD2 seemed to be localized in both stroma and thylakoid fractions (Fig. 8A), though the chaperone *GmCCS* appeared only in the stroma fraction (Fig. 6). The results of Fig. 7 and 8A indicated that *GmCSD2* proteins were not very abundant in control cell suspensions in our experimental growth conditions. This was confirmed by the SOD enzyme gel activity assays where the activity was again hardly detectable in the stroma of control cell suspensions (Fig. 8B). However, under Cu-treatment that activity clearly appeared (Fig. 8B). The effects of other essential metals such as Fe and Zn were also tested. Iron treatment increased *GmFeSOD* enzyme activity (Fig. 8B) with no apparent effect on *GmCSD2* activity, and Zn-treatment had no effect on neither ones (Fig. 8B). Note that MnSOD activity most probably from mitochondria was clearly detected in all conditions due to the high abundance and robust activity of this enzyme, and thus it is a common contaminant during chloroplast fractionation. The different SODs were identified using specific inhibitors (not shown). Our western blot and activity analysis on the *GmCSD2* protein in response to Cu excess followed the results of the RT-PCR analysis, where the spliced *GmCSD2* mRNA was also strongly upregulated in Cu-treated cell suspensions and entire plants. Note that very small amounts of *GmCSD2* isoforms in terms both of protein (Fig. 8A) and activity (Fig. 8B) were detected in control cell suspensions. The same can be said for control plants whose *GmCSD2* activity was very low in our experimental growth conditions (data not shown). This strongly suggests that FeSOD is the principal responsible for the chloroplastic SOD activity in our control growth conditions, and the concentration of Cu used in our medium (0.16 μ M) can be considered as limiting although plant growth was not significantly affected. It was actually confirmed by growing soybean cell suspensions and entire plants in media containing 0.5 μ M CuSO₄. In these new growth conditions, the *GmCSD2* activity was highly increased (data not shown).

Discussion

In natural environments both metal excess and limiting availability conditions may exist, and plants have to cope with both situations. The strategies that plants use to cope with these extreme situations should be, in principle, different. In general, under Cu limiting conditions, most of the available Cu is used to mature and maintain the function of the plastocyanin, a cuproprotein essential for the photosynthetic electron transport that shuttles electrons from cytochrome *b₆f* to PSI in the thylakoid lumen (Redinbo *et al.* 1994). Therefore, other less essential cuproproteins are reduced drastically in such Cu

limiting availability to economize on Cu (Abdel-Ghany and Pilon 2008). Our present study focussed on the chloroplastic Cu chaperone, the CCS, and its target, the CSD2, under Cu excess. It has been shown that under Cu limiting availability, plants downregulate the abundance of both proteins by highly reducing the transcript levels through an increase of a Cu microRNA, miR398, which is controlled by the transcription factor SPL7 (Dugas and Bartel 2008; Yamasaki *et al.* 2009; Beauclair *et al.* 2010). In those conditions, CSD2 is replaced by FeSOD acting thus as the main SOD form in the chloroplast. In the present work, the chloroplastic CCS and CSD2 gene and protein expressions have been analysed under control and Cu excess growth conditions in soybean cell suspensions and entire plants. Our study demonstrated that both genes are subjected to alternative splicing with IntronR mechanism producing four different transcripts in the case of *GmCCS* (a fully spliced and three NSP forms), and two transcripts for *GmCSD2* (a fully spliced and a non-spliced forms). Alternative splicing has been studied extensively in mammalian systems but much less in plants. The great majority of alternatively spliced genes in *A. thaliana* encode proteins with regulatory functions. Additionally, genes associated with various stress (biotic, water, light, salt, wounding, heavy metal, heat) responses seem to be particularly prone to alternative splicing regulation in both plants and animals (Kazan 2003). Among those, *AtCutA* mRNA that encodes a chloroplast protein involved in Cu tolerance is regulated by alternative splicing (Burkhead *et al.* 2003).

Under Cu excess, the fully spliced *GmCCS* form was strongly upregulated but the NSP forms were not affected by the Cu-treatment (Fig. 1A, lower part); the regulation of the fully spliced *GmCCS* is in agreement with previous publications in Arabidopsis (Abdel-Ghany *et al.* 2005a; Cohu *et al.* 2009; del Pozo *et al.* 2010). In the case of the *GmCSD2*, the fully spliced form was also strongly upregulated by Cu excess compared to control conditions but Cu excess had no effect on the NSP form (Fig. 4A). Surprisingly, despite the strong induction of the spliced *GmCCS* transcript (Fig. 1 and 2), its corresponding protein product was not significantly affected by Cu excess treatment (Fig. 6). Such discrepancy did not exist in the case of the *GmCSD2* where both the spliced transcript (Fig. 4) and the proteins (Fig. 7 and 8) were strongly upregulated. This may indicate that the CSD2 proteins have another function besides acting as an antioxidant enzyme. It may well serve as a Cu sink in metal excess availability to avoid cell damage. This is consistent with the idea that the CSD2 is an abundant chloroplastic protein and thus it would be able to chelate important amounts of

Cu ions. This idea was nicely developed in yeast by Culotta's work using specific mutants and growth conditions. They claimed that one subset of the cytosolic Cu/Zn SOD1 was involved in copper detoxification and other subset in superoxide scavenging (Culotta et al., 1995). In contrast, the CCS should not play that role and its known function is to deliver Cu in order to mature its target, the CSD2. As a chaperone, the CCS may also help to the proper reorganization of the three-dimensional structure of its target.

Although gene expression patterns were somewhat different depending on tissue and treatment, a remarkable difference of the *GmCSD2* gene expression between young and mature leaves was apparent. In young leaves, *GmCSD2* gene showed good expression level both in C and CuL plants (Fig. 4C, panel a) but its expression became downregulated in mature leaves (Fig. 4C, panel a). This observed downregulation during leaf development is at odd with previous publication in Arabidopsis, where the expression was relatively lower in younger plants but it increased after the onset of the flowering (Pilon *et al.* 2010). This discrepancy could be due to the different species studied. Although less pronounced, the expression level of the *GmCCS* gene was also somewhat lower in mature leaves compared to young leaves (Fig. 2A). As mentioned in the Results, these observations may be due to a lower Cu content observed in mature leaves as compared to young leaves (S. Sagasti, 2009, Ph. D. Thesis, Department of Biochemistry and Molecular and Cell Biology, University of Zaragoza, Zaragoza, Spain). A reproducible low expression of the *NSP2-GmCCS* transcript in CuL mature leaves was also observed but we do not have any clue for such behaviour.

Another interesting observation is the localization of the *GmCSD2* in thylakoid membranes in addition to the stroma, though all SOD enzymes are normally considered as globular soluble proteins. A similar observation was shown a few years ago using the TEM immunogold particle technique in spinach (Ogawa *et al.* 1995), but in contrast with a more recent publication, which identified the CSD2 rotein only in the stroma (Mauro *et al.* 2005). These discrepancies may well be due to the different protocols to obtain the material analysed. In our opinion, it is reasonable to think that the SOD enzymes are mostly needed nearby where the superoxide radical (O_2^-) is produced, *i.e.*, mainly at the acceptor side of the PSI.

In short, in the present study we have described some of the responses of soybean cell suspensions and entire plants under Cu excess. In such conditions, some plant tissue specificities were observed depending on the Cu status. Copper content seems to

decrease during leaves development in soybean being more abundant in young leaves compared to mature leaves close to plant flowering stage (S. Sagasti, 2009, Ph. D. Thesis, Department of Biochemistry and Molecular and Cell Biology, University of Zaragoza, Zaragoza, Spain). This correlates well with the fact that young leaves showed much higher *GmCSD2* gene expression and, to a lesser extent, higher *GmCCS* gene expression. Besides, *GmCCS* protein expression pattern did not follow the Cu excess upregulation of the corresponding gene expression pattern due to a yet unknown post-transcriptional regulation in contrast to the situation with the *GmCSD2*, where both gene and protein upregulations were correlated. These observations let us to suggest that the CSD2 protein may have another function besides its antioxidant role. We propose that this protein may act as a sink for Cu to avoid cell damage in Cu excess availability conditions. Moreover, the location of CSD2 on the surface of the thylakoid membranes and close to the acceptor side of the PSI is certainly a beneficial strategy for plants because the superoxide radical (O_2^-) has a short half-life and possesses potential damage for various biological structures. Although more future work is needed, our present data would help to have a more complete picture of Cu homeostasis in plant chloroplasts.

Accession numbers

Sequence data from this article can be found in the Phytozome data libraries under accession numbers Glyma05g04170 in the case of *GmCCS* and Glyma12g08650 in the case of *GmCSD2* (see <http://www.phytozome.net/soybean>).

Supplementary data

Supplementary Table 1. Primers used for RT-PCR.

Supplementary Fig. 1. Western blots using anti-RbcL and anti-PsbA.

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Fig. 1. Expression analysis of *GmCCS* transcripts in soybean cell suspensions by semi-quantitative PCR. (A) Soybean cell suspensions were grown in the presence of 0.16 μ M CuSO_4 (C, control cells) and 10 μ M CuSO_4 (Cu) for 21 days. (B) To investigate the effect of different metals on the expression of the different *GmCCS* transcripts, the medium was supplemented with 10 μ M CuSO_4 (Cu), 10 μ M FeSO_4 (Fe), and 10 μ M ZnSO_4 (Zn), respectively. B, blank; M, 1.0 kb plus DNA ladder.

Fig. 2. (A-E) Expression analysis of *GmCCS* transcripts in soybean plant tissues by semi-quantitative PCR. Soybean plants were grown hydroponically in a 0.5x Hoagland nutrient solution. To examine the effect of Cu on soybean plant growth, the nutrient solution was supplemented with 10 μ M CuSO_4 (CuR) and the hydroponic medium was changed once per week during 7 days (young plants) and 20 days (mature plants) of growth. Additionally, leaves from control plants were painted with 10 μ M CuSO_4 solution (CuL) every other day during 7 days (young leaves) or every 4 days during 20 days (mature leaves) of growth. M, 1.0 kb plus DNA ladder.

Fig. 3. Schematic representation of the *GmCCS* mRNAs transcripts identified in soybean. The differently sized exons are illustrated as boxes and the introns as lines. Primers for RT-PCR are shown with arrows indicating the polarity.

Fig. 4. Expression analysis of *GmCSD2* transcripts in soybean cell suspensions and plants by semi-quantitative PCR. (A) Soybean cell suspensions were grown in the presence of 0.16 μM CuSO_4 (C, control cells) and 10 μM CuSO_4 (Cu) for 21 days. (B) To investigate the effect of different metals on the expression of the different *CSD2* transcripts, the cell suspension medium was supplemented with 10 μM CuSO_4 (Cu), 10 μM FeSO_4 (Fe), and 10 μM ZnSO_4 (Zn), respectively, and the cells were grown for 21 days. (C) Soybean plants were grown hydroponically in a 0.5x Hoagland nutrient solution. To examine the effect of Cu on soybean plants growth, the nutrient solution was supplemented with 10 μM CuSO_4 (CuR) and the hydroponic medium was changed once per week during 7 days (young plants) and 20 days (mature plants) of growth. Additionally, leaves from control plants were painted with 10 μM CuSO_4 solution (CuL) every other day during 7 days (young leaves) and every 4 days during 20 days (mature leaves) of growth. B, blank; C, control cells; Cu, 10 μM Cu-treated cells. M, 1.0 kb plus DNA ladder.

Fig. 5. Schematic representation of *GmCSD2* transcripts identified in soybean. The differently sized exons are shown as boxes and the introns as lines. Primers for RT-PCR are shown with arrows indicating the polarity.

Fig. 6. Immunoblot analysis of soybean cell suspensions and plant leaves using a specific anti-*GmCCS* antibody (Sagasti *et al.* 2011). The amount of protein loaded was 30 μ g of stroma from control cells (C cells); 30 μ g of stroma from Cu-treated cells (Cu cells); 30 μ g of chloroplasts from control cells; 18 μ g of chloroplasts from Cu-treated cells; 30 μ g of stroma from control leaves (C leaves); 13 μ g of thylakoids from Cu-treated cells; and 20 μ g of thylakoids from control leaves. M, molecular weight markers.

Fig. 7. Comparison of western blots of *GmCCS* and *GmCSD2* proteins in the stroma fraction from soybean cell suspensions. Left panel, antibody against the soybean *GmCCS*; Right panel, antibody against the barley CSD2. The amount of protein loaded was 30 μ g per well. M, molecular weight markers.

Fig. 8. (A) Western blot of soybean cell suspensions using a specific antibody against the entire CSD2 protein from barley. The amount of protein loaded was 30 μ g of chloroplasts from control cells; 18 μ g of chloroplasts from Cu-treated cells; 13 μ g of thylakoids from Cu-treated cells; and 30 μ g of stroma from Cu-treated cells. (B) Native gel assay for the activity of the major SOD isoenzymes of the stroma fraction from soybean cell suspensions. The amount of protein loaded was 15 μ g per well.

Supplementary Table 1: Primers used for RT-PCR.

Gene name	Primer name	Primer sequence (5' - 3')
<i>GmCCS</i>	F-CCS	gagctcATGGACCACAAACTCT
	R-CCS	ggtaccCTATCAGACCTTGCTA
<i>GmCSD2</i>	F-CSD2	GTGGCTCTGTGAGTGAGTGAG
	R-CSD2	GCTTCACAGAAGACATAACATCAG
<i>NSP1-GmCCS</i>	F-NSP1CCS	GTTAGTTCCTTGTTGTCTG
<i>NSP2-GmCCS</i>	F-NSP2CCS	GCATGCCATGGATTTCCGTC
<i>NSP3-GmCCS</i>	R-NSP3CCS	GTCAGCCATCATGATGCATC
<i>NSP-GmCSD2</i>	R-NSPCSD2	CATAAGCAGGTCCAGGATCCAG
<i>Actin</i>	F-ACT	ATTGTAGGTCGTCCTCGTC
	R-ACT	TTGCATAAAGTGAAAGAACAG

Supplementary Figure 1. Western blots of soybean cell suspensions and plant leaves. Anti-RbcL and anti-PsbA were used as specific markers for stroma and thylakoid fractions, respectively. The amount of protein loaded was 18 μ g of chloroplasts from Cu-treated cells, 30 μ g of stroma from Cu-treated cells, 13 μ g of thylakoids from Cu-treated cells, 9 μ g of stroma from CuL leaves and 20 μ g of thylakoids from CuL leaves.