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Evidence for the plant-specific intercellular transport of the Arabidopsis copper chaperone CCH

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Summary

Arabidopsis copper chaperone (CCH) belongs to a family of eukaryotic proteins that participates in intracellular copper homeostasis by delivering this metal to the secretory pathway. In this work we show that the CCH protein is mainly located along the vascular bundles of senescing leaves and petioles, as shown by tissue prints and immunohistochemical detection. CCH protein also accumulates in stem sieve elements and is collected in phloem exudates. Accordingly, Arabidopsis CCH is the only member of the metallochaperone family described to function intercellularly to date. Moreover, the CCH protein remains stable when plants are subjected to excess copper that causes a rapid and specific decrease in its mRNA. These facts point to a role for CCH in copper mobilization from decaying organs towards reproductive structures, as a result of metalloprotein breakdown.

Keywords: ATX1-homologue, CCH, copper homeostasis, metallochaperone, phloem transport, senescence.

Introduction

Intracellular free copper levels must be tightly regulated through homeostatic networks to ensure proper function of key metalloproteins while avoiding toxic excess. Complementation studies performed in *Saccharomyces cerevisiae* indicate that similar copper homeostatic pathways operate in all eukaryotes (for reviews see Koch *et al.*, 1997; Peña *et al.*, 1999). In plants, copper homeostasis has been brought to centre stage (Himelblau and Amasino, 2000) since ethylene perception has been shown to require the proper flow of copper through the secretory pathway (Hirayama *et al.*, 1999).

One of the copper network components in *Arabidopsis thaliana* is the copper chaperone CCH, a member of an emerging family of proteins that binds copper ion within the cytosol and delivers it to the appropriate subcellular destinations (for review, see Harrison *et al.*, 2000). CCH is the homologue of *S. cerevisiae* cytoplasmic copper chaperone Antioxidant 1 (Atx1), that delivers Cu(I) to Ccc2, a P-type ATPase that pumps the metal ion into the lumen of a post-Golgi compartment (Lin *et al.*, 1997). The Arabidopsis Ccc2 homologue, RAN1, is involved in ethylene signalling by transporting copper to the secretory pathway (Hirayama *et al.*, 1999; Woeste and Kieber, 2000), where it is required for the creation of functional ethylene receptors (Rodriguez *et al.*, 1999). By analogy with the homeostatic model from yeast, CCH could be involved in directing Cu(I) to RAN1. Thus, ethylene receptors and other secreted copper-binding proteins (e.g. extracellular multicopper oxidases) might also depend on the CCH/RAN1 copper route to acquire the copper cofactor.

In spite of the fact that these data indicate a role for CCH in the homeostatic regulation of copper within plant cells, little is known about the function that this protein might have in the physiology and development of plants. Dealing with this, it has been recently demonstrated that *CCH* mRNA is up-regulated during natural and ozone-accelerated leaf senescence (Himelblau *et al.*, 1998; Miller *et al.*, 1999), although the role of the copper chaperone during these processes is unclear. Leaf senescence is characterized by an orderly loss of structures and metabolic functions collectively referred to as the *senescence syndrome*, a progression of changes that ultimately lead to cell death (Bleecker, 1998; Noodén and Guiamet, 1996; Weaver *et al.*, 1997). Leaf senescence involves degradation of lipids, proteins and nucleic acids, and mobilization of the resulting nutrients to other parts of the plant, where the vascular

system plays a pivotal role in the translocation of resources, therefore contributing to growth of developing organs.

In this paper, we analyse some of the exclusive features that the Arabidopsis copper chaperone CCH presents in comparison to its homologues from all other organisms studied, with the aim of gaining clues regarding the role of copper chaperones in plants. Special attention has been paid to the location and protein stability of this plant metallochaperone and its implication in senescence.

Results

CCH accumulation and distribution during senescence

A plant-specific characteristic of copper homeostasis is the increase in the mRNA levels during senescence of several described homeostatic components such as *CCH* (Himelblau *et al.*, 1998) and *RAN1* (Himelblau and Amasino, 2000). We studied CCH protein accumulation during natural leaf senescence in Arabidopsis by Western blot analysis. Leaves at different stages of development were chosen from the rosette by visual estimation, taking into consideration the percentage of yellow leaf surface. In mature green, 25%, 50% and 100% yellow senescing leaves, both mRNA and protein followed the same accumulation pattern: progressively rising from mature green to 50% yellow leaves and decreasing thereafter (Figure 1a,b). In order to examine the spatial distribution of the CCH protein within the leaf, tissue prints of senescing rosette leaves at the above indicated stages were performed. Figure 1(c) shows that CCH protein is mainly restricted to the midrib vascular vein (class I vein) and to the petiole of 50% yellow senescing leaves. A parallel experiment where Rubisco antibodies were used to develop leaf tissue prints, as a control for non-specific protein release, did not show any preferential Rubisco distribution in the midrib vein (result not shown).

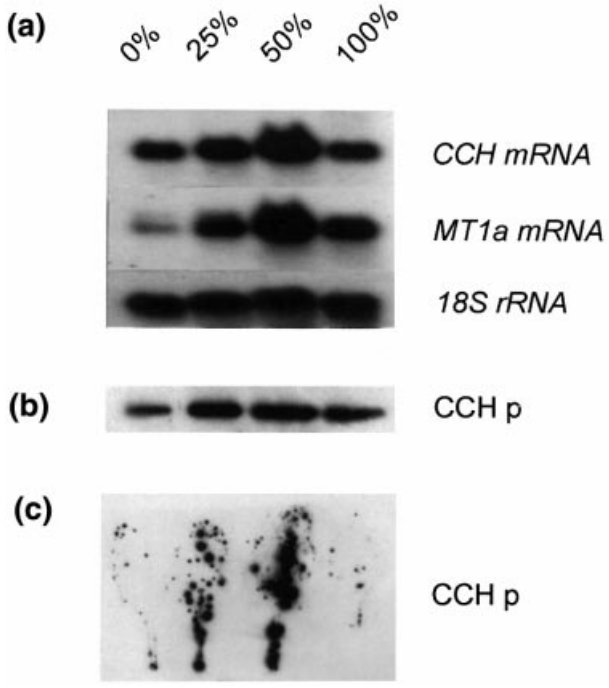

Figure 1. *CCH* expression and protein levels during senescence.
Arabidopsis leaves were selected by visual estimation as fully expanded green (0%) or approximately 25%, 50% and 100% of yellow surface. (a) Total RNA extracted from leaves and Northern blot hybridized with *CCH*, *MT1a and 18S* rRNA probes. (b) Western immunoblot of leaf protein extracts incubated with the CCH-antiserum. (c) Leaf tissue prints immunodetected using antiserum against CCH as indicated in Experimental procedures.

Tissue location of CCH protein

In order to further address the question of cellular location of the CCH protein during leaf ageing, immunohistochemical detection of CCH was carried out in transverse sections of mature green and 50% yellow leaves. Preimmune serum used as a control virtually gave no signal both in green (Figure 2a) and in senescing (not shown) leaf sections. Immunohistochemical labelling was mainly detected in the class I and class II phloem veins from both green leaves (Figure 2b) and the green portion of 50% yellow leaves (Figure 2c), although the signal dramatically increased in senescing leaves.

After a closer examination of the vascular bundles of green leaves where CCH is mainly located, we observed CCH protein accumulation in specific phloem cells (Figure 2e), whereas other cell types displayed undetectable labelling (comparable to the preimmune control, Figure 2d). In contrast, CCH accumulated at high levels all over the phloem of senescing leaves (Figure 2f). In addition, immunoreactivity was also observed in phloem-associated parenchyma cells and in the apoplast regions surrounding phloem and xylem elements (arrows in Figure 2f). Transverse sections of green (Figure 2g) and senescing (Figure 2h) petioles displayed a similar CCH distribution pattern.

In order to check phloem tissues other than leaf and petiole, protein location studies were also undertaken in stem transverse (Figure 3a,c,d) and longitudinal sections (Figure 3e). After immunodetection, samples were stained with DAPI for nuclei (Figure 3b,c,d,f) and with water blue for callose detection (results not shown). The use of both stains helped to discriminate between phloem companion cells and sieve elements, revealing that accumulation of the CCH protein occurs in stem sieve elements but not in their associated companion cells.

Presence of the CCH protein in phloem exudates

CCH protein location along the phloem was further assessed by Western blot analysis of leaf phloem exudates

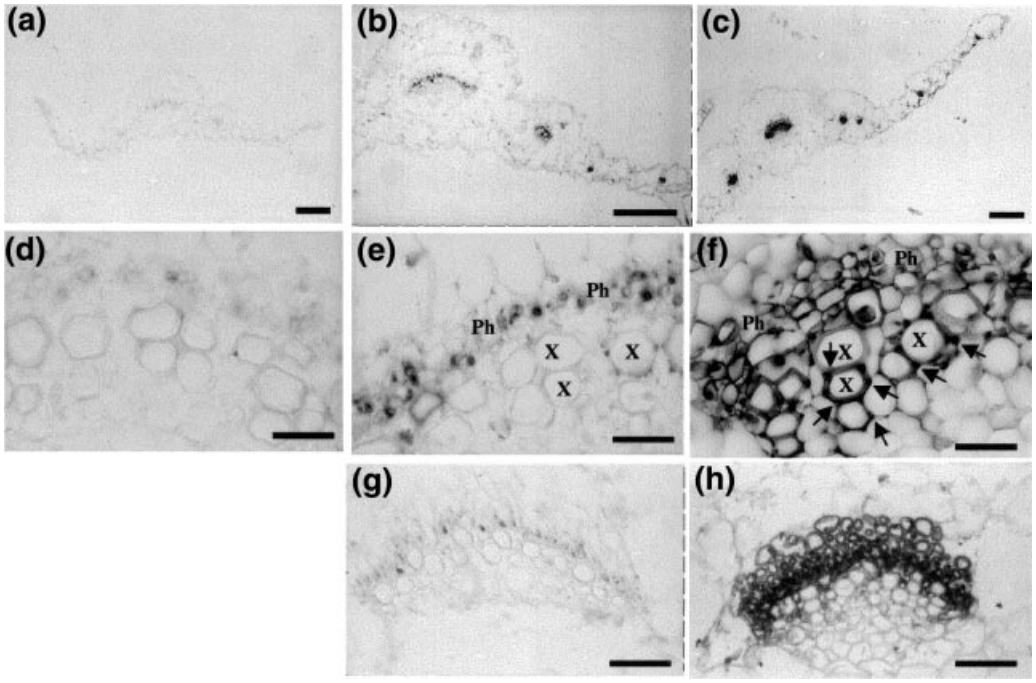

Figure 2. Histochemical location of the CCH protein in green and senescing Arabidopsis leaves and petioles during development.
General view of green (a,b) and senescing (c) leaf cross sections incubated with preimmune (a) and anti-CCH (b,c) serum. Detailed view of the vascular system of green (d,e) and senescing (f,h) leaves (d,e,f) and green (g) and senescing (h) petioles incubated with preimmune (d) and anti-CCH (e–h) serum. X = xylem; Ph = phloem; arrows = apoplast. Scale bar = 200 μm (a–c); 16 μm (d–f) and 40 μm (g,h)

that were obtained by incubating detached leaves in EDTA solutions (Weibull *et al.*, 1990). While the CCH protein almost disappeared in the leaf after 48 h, it became detectable in the exudate, most likely due to unloading of the phloem sap (Figure 4). In order to check if extensive protein degradation is taking place in leaves under our experimental conditions, or if exudates are contaminated by non-phloem proteins, the same samples were also analysed using a Rubisco antiserum. The Rubisco signal was detected in detached leaves before and after incubation, but was not detected in their exudates (Figure 4).

Effects of metal treatment on CCH expression and protein stability

Another fact that differentiates the Arabidopsis metallochaperone CCH is that its mRNA is down-regulated by copper treatment (Himelblau *et al.*, 1998), whereas homologues in other organisms are not susceptible to copper status, but rather subjected to iron-regulated gene expression (Lin *et al.*, 1997). With the aim of checking whether the *CCH* mRNA decay in plants is specifically regulated by copper ions, the mRNA was quantified by Northern blot analysis after 30 min treatment of whole plants with different metals such as copper, iron, zinc and cadmium. As shown in Figure 5(a), only copper treatment induced a decrease in *CCH* mRNA.

Since copper levels have been shown to diminish during senescence in Arabidopsis (Himelblau and Amasino, 2000) and to further address if protein location is related to copper status in the plant, Arabidopsis excised leaves were treated with copper or with the copper chelator BCS. The effectiveness of the treatments was assessed by measuring the enzymatic activity of the copper-binding protein ascorbate oxidase. When compared to the control, ascorbate oxidase activity increased and decreased after copper and BCS treatments, respectively (Figure 5b). Northern and Western blots revealed that, under conditions where *CCH* mRNA levels were undetectable and expression of copper detoxifying systems such as metallothionein *MT1a* was induced, the amount of CCH protein remained unaltered in Arabidopsis leaves (Figure 5c).

Discussion

The *CCH* gene appears to be in a single copy in the Arabidopsis genome based on a database search. The CCH protein is ubiquitously detected by Western blot throughout all the plant organs analysed (results not shown), which is in accordance with the gene transcription profile (Himelblau *et al.*, 1998). The protein is also detected in leaf protoplasts mainly obtained from mesophyll cells (results not shown), in agreement with the intracellular housekeeping function associated with metallochaperones

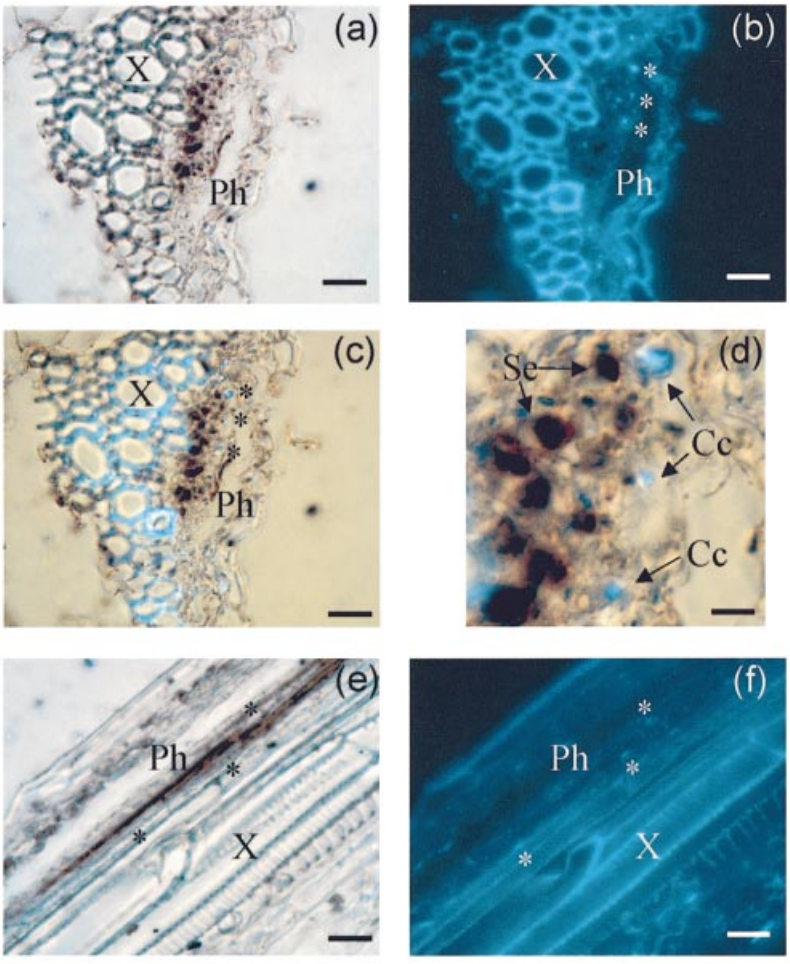

Figure 3. Details of CCH immunolocation in cross and longitudinal vascular system stem sections.
Cross-section of a stem vascular bundle stained with the CCH antibody (a), DAPI (b) or both (c). Detail of the phloem tissue shown in c (d). Longitudinal section of a stem vascular bundle incubated with anti-CCH serum (e) or DAPI (f). X = xylem; Ph = phloem; * = nuclei. Scale bar: 20 μm (a-c,e,f), 5 μm (d).

(Harrison *et al.*, 2000). In this sense, the apparent absence of signal in mesophyll cells, shown in Figures 2 and 3, can be explained by the staining conditions used for microscopy experiments that emphasize the high abundance of CCH protein in sieve elements of green leaves and stems, but do not allow detection of low levels of protein present in other cells from the same sample.

Based on the *CCH* transcript and protein levels observed during senescence (Figure 1a,b), *CCH* can be classified as a senescence-associated gene (SAG), a group of diverse genes that are mainly involved in salvage functions (Gan and Amasino, 1997). However, since total protein decays during senescence (Brady, 1988; Lohman *et al.*, 1994), the increase in relative abundance of the CCH protein could simply reflect the maintenance of its levels during this period. Nevertheless, considering results obtained from tissue print experiments, it seems the concentration of CCH protein indeed rises during senescence on a per leaf area basis and in leaf vascular bundles (Figure 1c), suggesting a role in nutrient transport. Our results show that the CCH protein belongs to the pool of soluble

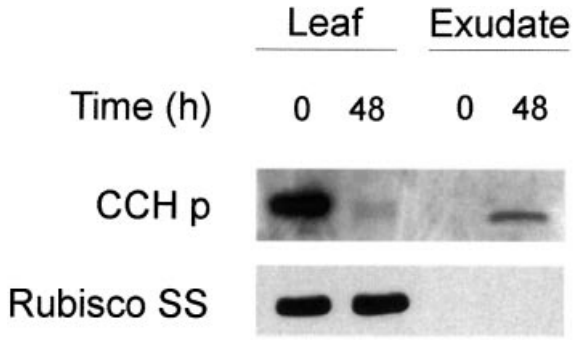

Figure 4. Presence of the CCH protein in phloem exudate from the leaf.
Fully expanded leaves were excised from the plant and incubated in 5 mM EDTA for 48 h to collect exudates. Total protein was extracted from leaves before and after incubation and subjected to a Western blot analysis developed with CCH and Rubisco antibodies (see Experimental procedures).

polypeptides found in phloem sap, as it is detected by Western blot analysis of Arabidopsis phloem exudates (Figure 4), which further indicates that CCH could participate in long-distance copper transport in vascular plants.

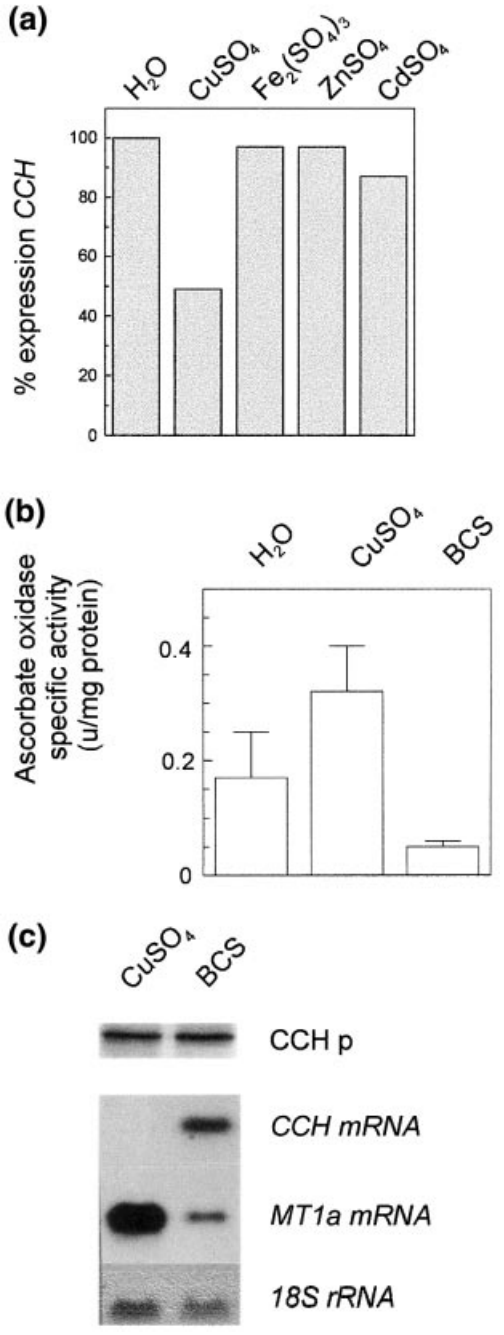

Figure 5. *CCH* mRNA, ascorbate oxidase activity and CCH protein levels in Arabidopsis under different metal status.
(a) *CCH* expression (%) under treatment with different metals. Results of Northern quantification (see Experimental procedures) were normalized versus the 18S rRNA. 100% corresponds to water treatment *CCH* mRNA levels. (b) Ascorbate oxidase specific activity (u/mg protein) in Arabidopsis leaves treated with water, 1 mM copper or 1 mM BCS solutions during 18 h. The mean and standard deviation from two independent measurements are represented. (c) Western and Northern analyses of Arabidopsis leaves treated with copper (1 mM, 18 h) and BCS (1 mM, 18 h). Total protein and mRNA were extracted after treatments. Western blot was developed with anti-CCH serum (pCCH) and the Northern filter was hybridized with *CCH, MT1a and* 18S rRNA probes.

During sieve element differentiation, cells undergo a selective degradation of internal structures. As a result, sieve elements of flowering plants loose their capacity for transcription, translation and protein maturation, and depend upon associated companion cells for much of their metabolic functions (Oparka and Turgeon, 1999; Thompson and Schulz, 1999). Thus, like other proteins found in sieve elements, CCH must be synthesized in companion cells and imported to the sieve elements through plasmodesmata connecting both cell types, and once inside the sieve tube, the protein would flow from source to sink organs in an osmotically driven translocation stream (Fischer *et al.*, 1992). Therefore, our results demonstrate that CCH crosses the sieve element/companion cell interface. Because in organisms other than plants copper chaperones have been ascribed to function exclusively inside the cells, this CCH intercellular trafficking between companion cells and sieve elements represents a differential feature of plant chaperones. It is therefore tempting to speculate that the exclusive CCH protein C-terminal domain of unknown function (Himelblau *et al.*, 1998) could be involved in this transport.

It has been described that plants recycle metal ions from senescing leaves to the growing organs (Drossopoulos *et al.*, 1994; Drossopoulos *et al.*, 1996; Hocking, 1994; Mauk and Noodén, 1992). Organic compounds such as nicotianamine and metal-binding proteins have been implicated in the phloem-mediated copper redistribution, at least in certain species (García-Hernández *et al.*, 1998; Miller *et al.*, 1999; Schmidke and Stephan, 1995; Stephan and Scholtz, 1993). In this sense, the Arabidopsis *MT1a* gene is highly expressed in tissues involved in nutrient transport and its mRNA has been located to the vascular bundle (García-Hernández *et al.*, 1998). However, metallothioneins such as MT1a presumably act as chelators that both bind copper in a nonexchangeable form and are up-regulated by copper once toxic metal concentrations are achieved inside the cell (Zhou and Goldsbrough, 1994). On the contrary, *CCH* expression is specifically repressed by copper (Figure 5) and the CCH protein has the ability to bind and deliver copper to a specific target, at least when produced in yeast (Himelblau *et al.*, 1998), thus indicating that MT1a and CCH function might be different. It is possible that the high levels of CCH in the phloem are necessary to compete with other molecules for copper and to guarantee its delivery to the secretory pathway, mediating the loading of extracellular copper-requiring proteins en route from senescing tissues to developing organs. Moreover, due to the antioxidant role demonstrated for other non-plant CCH homologues (Lin and Culotta, 1995; Portnoy *et al.*, 1999) and for CCH in yeast (Mira and Peñarrubia, unpublished results) a nonexcluding possibility is that CCH may participate in the protection of the vascular system from oxidant toxic molecules produced during senescence,

preventing the damage of these structures and keeping them functional for the proper and organized dismantling of other cells in a senescing organ.

We have found that *CCH* mRNA is specifically down-regulated by copper when compared to treatments with other metals (Figure 5a). After severe copper exposure of detached leaves to 1 mM copper solutions, used by other authors to check the molecular effects of copper on plants (Mehta *et al.*, 1992), whereas *CCH* mRNA levels are undetectable and *MT1a* expression is already induced, CCH protein levels remain constant (Figure 5b). *CCH* mRNA instability in Arabidopsis under copper treatment suggests that its product is not required in the presence of excess copper. However, the protein is stable under these conditions that usually induce degradation of other proteins such as Rubisco (Mehta *et al.*, 1992). CCH protein stability under copper-induced oxidative stress could be based on its amino acid composition. Cysteine residues have been proposed as the main targets of protein oxidative-induced turnover (Moreno *et al.*, 1995; Stadtman, 1990). In CCH, there are only two Cysteine residues and they are likely to be involved in copper binding (Himelblau *et al.*, 1998). This probably blocks the sensitivity of the protein to environmental redox conditions. Thus, these apparently contradictory effects of copper on *CCH* mRNA and protein levels could simply reflect differences in stability of both molecules under oxidative stress caused by copper excess and probably indicate that under these conditions the CCH protein present in cells is enough to perform its function.

These results add new aspects to the discussion on the role played by CCH during senescence. First, the stability of CCH protein under enhanced oxidative conditions, which also characterize senescence, would agree with CCH operating during this physiological process. Moreover, as senescence progresses, different leaf cells would suffer from copper excess or defect, depending on the path and kinetics that metal follows to exit these organs. In this sense, the role of CCH remains still unsolved, since CCH could participate either when copper is abundant (as discussed above) or, on the other hand, CCH could function by delivering copper, when scarce, once the bulk of copper has left the organ, to cells that have to be functional in order to guarantee a thorough leaf dismantling. Although further experimental support would be necessary to explore this possibility, the decrease in *CCH* mRNA levels under copper excess (Figure 5) seems to point to a preponderant role of CCH under copper defect.

Briefly, our results indicate a specific cell-to-cell role of metallochaperones in plants and the implication of CCH in phloem-mediated copper reallocation during senescence-associated nutrient salvage.

Experimental procedures

Plant growth conditions

Seeds of *Arabidopsis thaliana*, ecotype Columbia (Col 0), were grown in pots and covered with a clear plastic dome. The covered pots were placed in a cold room at 4°C for 2 days to synchronize germination, and then moved to a growth cabinet at 23°C with a 16 h photoperiod (65 mmol m^{-2} of cool-white fluorescent light). Plants were harvested after indicated times.

Metal and BCS treatments

For metal and bathocuproinedisulphonic acid (BCS, Sigma, St. Louis, MO, USA) treatments, either plants or rosette leaves from 4- to 5-week-old Arabidopsis were excised and then immediately submerged in 1 mM copper, iron, zinc or cadmium sulphates or 1 mM BCS solutions for 30 min or 18 h.

Exudate collection

To obtain phloem exudates, rosette leaves from 4- to 5-week-old Arabidopsis were excised and their petioles immediately submerged in 5 mM EDTA solution for 48 h, and placed in a moist chamber as described elsewhere (Weibull *et al.*, 1990).

Protein extraction, gel electrophoresis, Western blot analysis, tissue prints and ascorbate oxidase determinations

Plant tissue was extracted using 2 volumes of extraction buffer [50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 20 mM $MgCl_2$, 2 mM PMSF, 2 g l^{-1} leupeptin, aprotinin]. After 1 min of vortexing followed by centrifugation twice at 5000 ***g*** for 10 min, the total protein in the supernatants was determined by Lowry's method following the protocol of Ausubel *et al.* (1995).

Ten μg of total protein were separated using reducing 14% SDS-PAGE. Gels were either stained with Coomassie blue or electroblotted onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) for immunodetection with antiserum against CCH recombinant protein (H. Mira and L. Peñarrubia, unpublished results) or against Rubisco from *Euglena gracilis* (García-Ferris and Moreno, 1994). Detection of antigen-antibody complexes was performed using the ECL System (Amersham, Buckinghamshire, UK). Tissue prints were performed on nitrocellulose membranes and developed as Western immunoblots.

Ascorbate oxidase (EC 1.10.3.3) activity was measured on total protein extracts from Arabidopsis leaves utilizing the protocol from Diallinas *et al.* (1997).

RNA isolation and Northern blot analysis

Total RNA was isolated from Arabidopsis tissues as described by Prescott and Martin (1987). RNA was quantified by UV spectrophotometry and its integrity was visually assessed on ethidium bromide-stained agarose gels. For RNA blots, 5 μg of total RNA were separated by denaturing-agarose gel electrophoresis and transferred onto a nylon membrane as described by Sambrook *et al.* (1989). RNA blots were hybridized overnight at 65°C with [α-^{32}P]dATP-labelled cDNA probes which were previously purified in a Sephadex G-10 spin column (Pharmacia Biotech AB, Uppsala,

Sweden). After washing, the filters were analysed using a radioanalytical imaging system (InstantImager 2024, Packard, Canberra, Australia) or were exposed to X-ray films at –80°C using an intensifying screen.

Immunohistochemistry

Arabidopsis leaves, petioles and stems were sectioned and incubated overnight in phosphate buffer pH 7.4 with paraformaldehide 4% (w/v). Samples were rinsed thoroughly in the same buffer, dehydrated in graded alcohols, cleared in xylene and embedded in low temperature-melting paraplast wax (Sigma). Ten-micron transverse or longitudinal sections of leaves, petioles and stems, obtained with a rotating microtome, were mounted onto slides using TESPA (Sigma) as the adhesive medium. After de-waxing and hydrating the slides, the avidin-biotin-complex (ABC) indirect immunoperoxidase detection of CCH was performed. Where indicated, nuclear DNA was stained with 3 μg ml^{-1} 4′-6-diamidino-2-phenylindol (DAPI, Sigma) or with 0.01% aniline blue prepared in 67 mM K_2HPO_4, pH 12 buffer.

Prior to the immunohistochemical protocol, endogenous peroxidase activity was inhibited by bathing the sections in 2% H_2O_2 prepared in 100 mM Tris, pH 7.5, 0.9% (w/v) NaCl (TBS) for 30 min at room temperature. Samples were incubated overnight at 4°C in a moist chamber with primary antibody (1 : 2000 dilution) followed by incubation at room temperature for 2 h with 1 : 200 diluted biotinylated IgG goat antirabbit (Vector Labs, Burlingame, CA, USA). Immunodetection was carried out with the avidin/biotin ABC (Vector Labs) system. The resulting peroxidase label was histochemically developed with 0.025% (w/v) 3,3′-diaminobenzidine (DAB; Sigma) and 0.01% H_2O_2 for 20–30 min at room temperature. Finally, slides were dehydrated in graded ethanol, cleared with xylene and coverslipped using Permount (Fisher Scientific, NJ, USA). For comparison, all samples were treated in parallel with the same solutions and reagents and controls with preimmune serum were performed in consecutive sections.

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References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) *Current Protocols in Molecular Biology*. New York: John Wiley and Sons.

Bleecker, A.B. (1998) The evolutionary basis of leaf senescence: method to the madness? *Curr. Op. Plant Biol.* **1**, 73–78.

Brady, C.J. (1988) Nucleic acid and protein synthesis. In *Senescence and Aging in Plants* (Noodén, L.D. and Leopold, A.C., eds). London: Academic Press, pp. 147–181.

Diallinas, G., Pateraki, I., Sanmartin, M., Scossa, A., Stilianou, E., Panopoulos, N.J. and Kanellis, A.K. (1997) Melon ascorbate oxidase: cloning of a multigene family, induction during fruit development and repression by wounding. *Plant Mol Biol.* **34**, 759–770.

Drossopoulos, J.B., Bouranis, D.L. and Bairaktari, B.D. (1994) Patterns of mineral nutrient fluctuations in soybean leaves in relation to their position. *J. Plant Nutr.* **17**, 1017–1035.

Drossopoulos, J.B., Kouchaji, G.G. and Bouranis, D.L. (1996) Seasonal dynamics of mineral nutrients by walnut tree reproductive organs. *J. Plant Nutr.* **19**, 421–434.

Fischer, D.B., Wu, Y. and Ku, M.S.B. (1992) Turnover of soluble proteins in the wheat sieve tube. *Plant Physiol.* **100**, 1433–1441.

Gan, S. and Amasino, R.M. (1997) Making sense of senescence. Molecular genetic regulation and manipulation of leaf senescence. *Plant Physiol.* **113**, 313–319.

García-Ferris, C. and Moreno, J. (1994) Oxidative modification and breakdown of ribulose-1,5-bisphosphate carboxylase/oxygenase induced in *Euglena gracilis* by nitrogen starvation. *Planta*, **193**, 208–215.

García-Hernández, M., Murphy, A. and Taiz, L. (1998) Metallothioneins 1 and 2 have distinct but overlapping expression patterns in Arabidopsis. *Plant Physiol.* **118**, 387–397.

Harrison, M.D., Jones, C.E., Solioz, M. and Dameron, C.T. (2000) Intracellular copper routing: the role of copper chaperones. *Trends Biochem. Sci.* **25**, 29–32.

Himelblau, E. and Amasino, R.M. (2000) Delivering copper within plant cells. *Curr. Op. Plant Biol.* **3**, 205–210.

Himelblau, E., Mira, H., Lin, S.J., Culotta, V.C., Peñarrubia, L. and Amasino, R.M. (1998) Identification of a functional homolog of the yeast copper homeostasis gene *ATX1* from Arabidopsis. *Plant Physiol.* **117**, 1227–1234.

Hirayama, T., Kieber, J.J., Hirayama, N. *et al.* (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. *Cell*, **97**, 383–393.

Hocking, P.J. (1994) Dry-matter production, mineral nutrient concentrations, and nutrient distribution and redistribution in irrigated spring wheat. *J. Plant Nutr.* **17**, 1289–1308.

Koch, K.A., Peña, M.M.O. and Thiele, D.J. (1997) Copper-binding motifs in catalysis, transport, detoxification and signaling. *Chem. &. Biol.* **4**, 549–560.

Lin, S.J. and Culotta, V.C. (1995) The *ATX1* gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proc. Natl Acad. Sci. USA*, **92**, 3784–3788.

Lin, S.J., Pufahl, R.A., Dancis, A., O'Halloran, T.V. and Culotta, V.C. (1997) A role for the *Saccharomyces cerevisiae ATX1* gene in copper trafficking and iron transport. *J. Biol. Chem.* **272**, 9215–9220.

Lohman, K.N., Gan, S., John, M.C. and Amasino, R.M. (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiol. Plant.* **92**, 322–328.

Mauk, C.S. and Noodén, L.D. (1992) Regulation of mineral redistribution in pod-bearing soybean explants. *J. Exp. Bot.* **43**, 1429–1440.

Mehta, R.A., Fawcett, T.W., Porath, D. and Mattoo, A.K. (1992) Oxidative stress causes rapid membrane translocation and *in vivo* degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase. *J. Biol. Chem.* **267**, 2810–2816.

Miller, J.D., Arteca, R.N. and Pell, E.J. (1999) Senescence-associated gene expression during ozone-induced leaf senescence in Arabidopsis. *Plant Physiol.* **120**, 1015–1024.

Moreno, J., Peñarrubia, L. and García-Ferris, C. (1995) The mechanism of redox regulation of ribulose-1,5-bisphosphate

carboxylase/oxygenase turnover. A hypothesis. *Plant Physiol. Biochem.* **33**, 121–127.

Noodén, L.D. and Guiamet, J.J. (1996) Genetic control of senescence and aging in plants. In *Handbook of the Biology of Aging* 4th edn. (Schneider, E.L. and Rowe, J.W., eds). San Diego: Academic Press, pp. 94–118.

Oparka, K.J. and Turgeon, R. (1999) Sieve elements and companion cells – Traffic control centers of the phloem. *Plant Cell*, **11**, 739–750.

Peña, M.M.O., Lee, J. and Thiele, D.J. (1999) A delicate balance: homeostatic control of copper uptake and distribution. *J. Nutr.* **129**, 1251–1260.

Portnoy, M.E., Rosenzweig, A.C., Rae, T., Huffman, D.L., O'Halloran, T.V. and Culotta, V.C. (1999) Structure-function analyses of the Atx1 metallochaperone. *J. Biol. Chem.* **274**, 15041–15045.

Prescott, A. and Martin, C. (1987) A rapid method for the quantitative assessment of levels of specific mRNAs in plants. *Plant Mol. Biol. Report*, **4**, 219–224.

Rodriguez, F.I., Esch, J.J., Hall, A.E., Binder, B.M., Schaller, G.E. and Bleecker, A.B. (1999) A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. *Science*, **283**, 996–998.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Schmidke, I. and Stephan, U.W. (1995) Transport of metal micronutrients in the phloem of castor bean (*Ricinus communis*) seedlings. *Physiol. Plant.* **95**, 147–153.

Stadtman, E.R. (1990) Covalent modification reactions are marking steps in protein turnover. *Biochemistry*, **29**, 6323–6331.

Stephan, U.W. and Scholtz, G. (1993) Nicotianamine: mediator of transport of iron and heavy metals in the phloem? *Physiol. Plant.* **88**, 522–529.

Thompson, G.A. and Schulz, A. (1999) Macromolecular trafficking in the phloem. *Trends Plant Sci.* **4**, 354–359.

Weaver, L.M., Himelblau, E. and Amasino, R.M. (1997) Leaf senescence: gene expression and regulation. In *Genetic Engineering* Volume 19 (Setlow, J.K., ed.) New York: Plenum Press, pp. 215–234.

Weibull, J., Ronquist, F. and Brishammar, S. (1990) Free amino acid composition of leaf exudates and phloem sap. *Plant Physiol.* **92**, 222–226.

Woeste, K.E. and Kieber, J.J. (2000) A strong loss-of-function mutation in RAN1 results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. *Plant Cell*, **12**, 443–455.

Zhou, J. and Goldsbrough, P.B. (1994) Functional homologs of fungal metallothionein genes from Arabidopsis. *Plant Cell*, **6**, 875–884.

GenBank accession number U88711.