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Defective Copper Transport in the *copt5* Mutant Affects Cadmium Tolerance

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Cadmium toxicity interferes with essential metal homeostasis, which is a problem for both plant nutrition and the consumption of healthy food by humans. Copper uptake is performed by the members of the Arabidopsis high affinity copper transporter (COPT) family. One of the members, COPT5, is involved in copper recycling from the vacuole toward the cytosolic compartment. We show herein that *copt5* mutants are more sensitive to cadmium stress than wild-type plants, as indicated by reduced growth. Exacerbated cadmium toxicity in *copt5* mutants is due specifically to altered copper traffic through the COPT5 transporter. Three different processes which have been shown to affect cadmium tolerance are altered in *copt5* mutants. First, ethylene biosynthesis diminishes under copper deficiency and, in the presence of cadmium, ethylene production diminishes further. Copper deficiency responses are also attenuated under cadmium treatment. Remarkably, while *copt5* roots present higher oxidative stress toxicity symptoms than controls, aerial *copt5* parts display lower oxidative stress, as seen by reduced cadmium delivery to shoots. Taken together, these results demonstrate that copper transport plays a key role in cadmium resistance, and suggest that oxidative stress triggers an NADPH oxidase-mediated signaling pathway, which contributes to cadmium translocation and basal plant resistance. The slightly lower cadmium levels that reach aerial parts in the *copt5* mutants, irrespective of the copper content in the media, suggest a new biotechnological approach to minimize toxic cadmium entry into food chains.

Keywords: *Arabidopsis thaliana* • Cadmium toxicity • Copper transport • *copt5* mutants • Ethylene production • Oxidative stress.

Abbreviations: AGR, absolute growth rate; Cd, cadmium; Cu, copper; COPT, copper transporter; CSD, Cu/Zn superoxide dismutase; ETR1, ethylene receptor 1; Fe, iron; FID, flame ionization detector; FSD1, iron superoxide dismutase 1; HMA4, heavy metal ATPase 4; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; MS, Murashige and Skoog; NAS2, nicotianamine synthase 2; qPCR, quantitative PCR; RBOH, respiratory burst oxidase homolog; ROS, reactive oxgen species; SOD, superoxide dismutase; SPL7, SQUAMOSA-binding promoter-like 7; WT, wild type; Zn, zinc.

Introduction

Cadmium (Cd) is a transition metal found naturally in the earth's crust at trace levels, but its concentrations in the environment are rising due to human activities. Although Cd is highly toxic, plants are more tolerant and constitute the main entry of Cd into trophic chains, which can contaminate human food (Agency for Toxic Substances and Disease Registry). The limit set for Cd in agricultural soils is $<3\ \mu g\ g^{-1}$ dry soil (Clemens et al. 2013). Plant exposure to large amounts of Cd is especially toxic in roots, where it can be sequestered within vacuoles or incorporated into the xylem, along which it is redistributed throughout the plant (Lux et al. 2011). Cd concentrations are usually higher in roots than in aerial parts (Rubio et al. 1994, Fargasova 2001), which not only suggests that the transport of this metal via the xylem is restricted in many plants, but also explains why it is found in very small quantities in seeds, fruits and tubers (Lux et al. 2011). In roots, Cd acts as a potent rhizotoxin and affects overall plant growth (Rodríguez-Serrano et al. 2009). Photosynthesis is also sensitive to Cd exposure since Chl and the enzymes involved in CO_2 fixation are Cd targets (Herbette et al. 2006), and it also inhibits PSII photoactivation. Cd toxicity is associated with alterations in the entry and distribution of macro- and micronutrients (Rubio et al. 1994, Tsyganov et al. 2007) as it competes with other cations at protein-binding sites and transporters (Clemens 2006). It is, therefore, important to study how plants are able to adapt to and survive differences in the supply of different nutrients when Cd is present.

Considerable natural variation exists in Cd accumulation in plants, which allows dissection of the processes mediating Cd translocation and accumulation in grains, mainly in rice (Clemens et al. 2013). The study of two zinc (Zn)- and Cd-hyperaccumulators, *Arabidopsis halleri* and *Noccaea caerulescens*, has shed light on the genetic basis of Cd hyperaccumulation. Two genes which are highly expressed in *A. halleri* roots are responsible for efficient root to shoot Cd translocation: *HMA4* (*Heavy Metal ATPase 4*) and *NAS2* (*Nicotianamine*

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Synthase 2) (Hanikenne et al. 2008, Deinlein et al. 2012). Low xylem loading of Cd in roots has been reported to be responsible for the reduced root to shoot translocation of Cd and its accumulation in grains (Uraguchi et al. 2009).

Among molecular responses in low Cd-accumulating plants, *Solanum torvum* induces expression of the *COPT5* homolog under Cd stress in roots (Yamaguchi et al. 2010). The COPT5 protein belongs to the high affinity CTR-like copper (Cu) transporters, denoted COPTs in Arabidopsis (Sancenón et al. 2003). Based on cellular localization, these transporters have been classified as not only plasma membrane transporters (COPT1, COPT2 and COPT6), which fully complement the corresponding yeast mutants, but also as those transporters that partially complement the yeast phenotypes (COPT3 and COPT5) (Sancenón et al. 2003, Peñarrubia et al. 2010). COPT5 localizes to the tonoplast and/or the membrane of the pre-vacuolar compartment in Arabidopsis cells. The *copt5* mutant exhibits chlorosis, impaired photosynthetic electron transfer, root elongation defects and compromised vegetative growth under severe Cu starvation conditions (Garcia-Molina et al. 2011, Klaumann et al. 2011). *COPT5* is expressed mostly in root vascular tissues and siliques. Nonetheless, it has also been detected, but to a much lesser extent, in trichomes, meristems, the vascular conduits of cotyledons, leaves and reproductive organs, but not in pollen (Garcia-Molina et al. 2011). Biochemical studies have demonstrated that *copt5* plants display reduced vacuolar Cu export, which leads to Cu accumulation in roots and low Cu in siliques and seeds, and strongly suggests that COPT5 functions in Cu distribution from roots to reproductive tissues (Klaumann et al. 2011). By checking the metal specificity of the *copt5* phenotype under severe Cu deficiency conditions, sensitivity to several metals, including Cd, is enhanced (Garcia-Molina et al. 2011).

Cd has recently been shown to modify Cu deficiency responses in yeast (Heo et al. 2012) and in *Arabidopsis thaliana* SPL7 (SQUAMOSA-binding Promoter-Like 7)-dependent Cu deficiency responses. They include Cu uptake by the three plasma membrane-localized Cu transporters, COPT1, COPT2 and COPT6, which are required for basal Cd tolerance (Gayomba et al. 2013). In *A. thaliana*, the transcription factor SPL7 recognizes the GTAC boxes present in the promoters of target genes, such as *COPT2*, *FSD1* (iron superoxide dismutase 1) and miRNA398 b/c, to activate their transcription (Yamasaki et al. 2009, Bernal et al. 2012). One of the SPL7-dependent strategies to save Cu under limiting conditions is to replace cuproproteins with other metalloproteins that bind another metal [usually iron (Fe)] to perform a similar function, which is the case of the switch of superoxide dismutases (SODs; Yamasaki et al. 2007). To conserve Cu, *FSD1* expression replaces *CSD1* and *CSD2* (encoding cytosolic and chloroplastic Cu/Zn SODs) under Cu-deficient conditions, so Cu is saved for other essential proteins such as plastocyanin (Yamasaki et al. 2007).

Once inside the cell, Cu is distributed to the final acceptors located in diverse subcellular compartments through different metallochaperones and heavy metal P-type ATPases (Pilon 2012). Ethylene receptors are cuproproteins (Rodríguez et al. 1999), and RAN1 is the P-type ATPase responsible for supplying Cu to ethylene receptor 1 (ETR1) (Hirayama et al. 1999). Since Cd binding to the Atx1 metallochaperone affects its interaction and provokes defects in Cu delivery to cuproproteins in yeast (Heo et al. 2012), a similar effect in Arabidopsis would affect ethylene perception. Among the different effects induced by heavy metal stress in plants, increased ethylene biosynthesis is well documented. Both Cu and Cd have been shown to enhance ethylene release in different species (Arteca and Arteca 2007, Rodríguez-Serrano et al. 2009), and it has been suggested that it may be used as a marker of stress-tolerant genotypes (Lu and Kirkham 1991). However, other species, in particular, but not exclusively, semi-aquatic plants, show diminished ethylene biosynthesis when subjected to stress by heavy metals or to other adverse environmental conditions (Poschenrieder et al. 1993, Lin et al. 2002). Ethylene has been recently proposed to promote soil salinity tolerance via improved Na/K homeostasis. This effect is associated with an increased accumulation of RESPIRATORY BURST OXIDASE HOMOLOG F (RBOHF)-dependent reactive oxygen species (ROS) in the root stele (Jiang et al. 2013).

NADPH oxidases have been suggested to play a key role in Cd-induced ROS production (Smeets et al. 2009, Cuypers et al. 2010), and one of the first effects observed in plants exposed to Cd is ROS production, mainly hydrogen peroxide (H_2O_2) (Martínez-Peñalver et al. 2012). Depending on the levels, H_2O_2 can either serve as a component in signaling processes or induce a response to oxidative stress damage (Rodríguez-Serrano et al. 2009). Cd also regulates the expression of those genes that encode proteins and are involved in defense against oxidative stress (Cuypers et al. 2011, Keunen et al. 2013, Smeets et al. 2013). Cd-induced changes vary according to the concentration of the metal, plant age, exposure time and the tissue or organ being studied (Llamas et al. 2000, Rodríguez-Serrano et al. 2009, Sanz et al. 2009).

Since present Cd exposure levels already surpass the threshold for adverse effects on human health, mostly through plant-derived food, inhibition of the processes controlling the passage of Cd from soil to roots, or its translocation to edible plant organs, is urgent (Clemens et al. 2013). To this end, the Cd-sensitive *copt5* mutant was used to gain further insights into both Cd interference with Cu deficiency responses and Cd distribution mechanisms throughout the plant to develop biotechnological strategies in the near future which aim to diminish Cd translocation to aerial plant organs.

Results

Effect of cadmium on the root growth of the *copt5* mutant seedlings and its interaction with copper

The *copt5* mutants exhibit altered metal sensitivity under severe Cu deficiency conditions (Garcia-Molina et al. 2011). Half-strength Murashige and Skoog (1/2 MS) medium has been shown to be slightly Cu deficient (Abdel-Ghany et al. 2005, Yamasaki et al. 2009, Andrés-Colás et al. 2013). In order to characterize the *copt5* phenotype further in the commonly

used 1/2 MS medium, we checked the root length of 7-day-old wild-type (WT) and *copt5-2* mutant seedlings supplemented with various Zn and Cd concentrations (**Supplementary Fig. S1A, B**). Under low Cu (1/2 MS medium), the *copt5-2* mutant showed a significant differential effect on root length by Cd, but not by Zn treatments (**Supplementary Fig. S1A, B**). In order to exacerbate the Cu deficiency conditions, and to confirm further the metal sensitivity displayed by the *copt5* mutant (Garcia-Molina et al. 2011), the experiment was performed in the presence of the Cu chelator bathocuproinedisulfonic acid (BCS) at 50 μM at different Zn concentrations (**Supplementary Fig. S2**). Indeed, under severe Cu-deficient conditions (50 μM BCS), the *copt5* mutant was sensitive to the presence of metals, as previously reported (Garcia-Molina et al. 2011). In order to understand the role of COPT5 in metal toxicity under mild conditions, we selected the low Cu 1/2 MS medium for further experiments, as well as a Cd concentration which, without being excessively toxic, had a differential effect on the WT and *copt5* plants. Thus unless otherwise stated, the chosen Cd concentration hereinafter was 30 μM.

Under the slightly Cu-deficient conditions of the 1/2 MS medium, the knock-out mutant lines (*copt5-2* and *copt5-3*) did not show significant differences in root growth if compared with the WT, while the root growth of the *COPT*-overexpressing (*COPT5*^{OE}) line was greater (**Fig. 1A**). In order to ensure Cu-sufficient conditions, 1 μM Cu was added to the 1/2 MS medium, which had no influence on root growth (**Fig. 1A**). Cd toxicity was also tested under these two Cu conditions (1/2 MS medium and the same medium with 1 μM Cu) (**Fig. 1A**). Whereas both *copt5* mutant lines show increased sensitivity to 30 μM Cd under low Cu (1/2 MS) when compared with the WT, the *COPT5*^{OE} line maintained the opposite

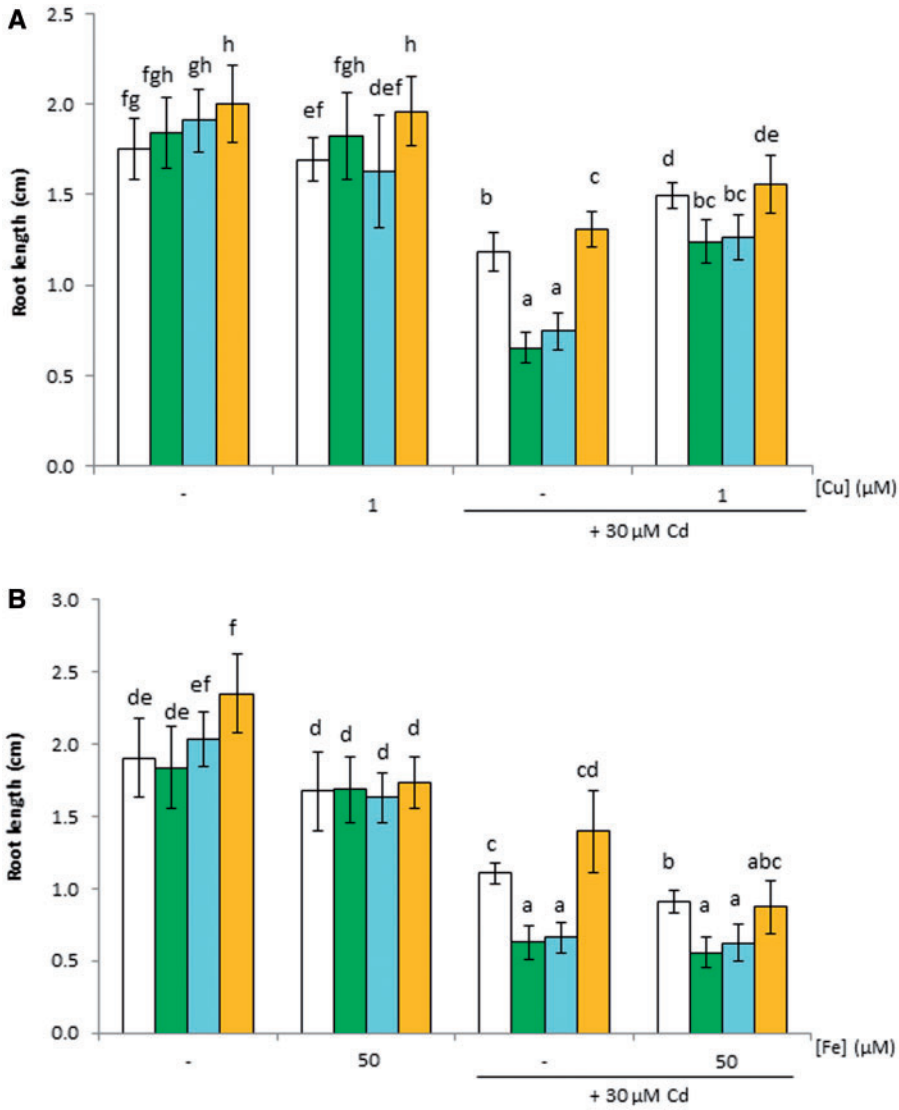

Fig. 1 Interaction of Cu and Fe with Cd toxicity. Root length of 7-day-old wild-type (white bars), *copt5-2* (green bars), *copt5-3* (blue bars) and *COPT5*^{OE} (yellow bars) seedlings grown in 1/2 MS medium (−) and the same medium supplemented with 1 μM Cu, containing or not Cd (30 μM) (A), or in 1/2 MS medium (−) and the same medium supplemented with 50 μM Fe, containing or not Cd (30 μM) (B). Different letters indicate significant differences ($P < 0.05$, $n = 21–30$).

phenotype of their roots, which grew slightly longer than those of the controls (Fig. 1A). Strikingly, by adding only 1 μM Cu to the growth medium, root growth notably increased in all four lines and Cd sensitivity was almost reversed, which indicates the strong Cu effect on Cd sensitivity (Fig. 1A). The 15 μM Cu concentration, which fell within the upper sufficiency range, fully reverted the root growth defects of the *copt5-2* mutant at two Cd concentrations (10 and 30 μM) (Supplementary Fig. S3A). The *copt5* line complemented with *COPT5* under its own promoter (Garcia-Molina et al. 2011) also significantly reverted root growth inhibition, indicating partial phenotype restoration (Supplementary Fig. S3B). We also checked the Cd sensitivity of the plasma membrane COPT transporters under our experimental conditions by analyzing the root length of the corresponding single mutants (*copt1*, *copt2* and *copt6*). None showed significant differences compared with the WT controls (Supplementary Fig. S4). To check if this effect was Cu specific, the same experiment shown in Fig. 1A was carried out in the presence of 50 μM Fe instead of Cu. The results obtained revealed that Fe was unable to reverse Cd-induced root length inhibition or the increased Cd toxicity in the knock-out mutant (Fig. 1B), which implies that the Cd sensitivity exhibited by the *copt5* mutants is Cu specific. Taken together, these results indicate that the Cd sensitivity phenotype is indeed *COPT5* dependent and is due to the COPT5 function in Cu homeostasis.

Ethylene perception in the *copt5* mutant seedlings

Since ethylene has been implicated in plant responses to stress by heavy metals (Arteca and Arteca 2007), and as Cu is involved in ethylene perception (Hirayama et al. 1999), the possibility of the observed responses to Cd treatments in the *copt5* mutant being mediated by changes in ethylene perception was studied in etiolated seedlings, where ethylene is well known to promote hypocotyl shortening (Guzmán and Ecker 1990). The hypocotyls and root length in the WT, the two *copt5* mutants and the $COPT5^{OE}$ lines were measured in etiolated seedlings under these four conditions: (i) 1/2 MS; (ii) 1/2 MS with 1 μM Cu; (iii) 1/2 MS with 30 μM Cd; and (iv) 1/2 MS with 1 μM Cu and 30 μM Cd. The results obtained from these experiments are presented in Fig. 2. As observed, hypocotyl length became significantly shorter under Cd treatment, irrespective of the presence of Cu in the medium, and no consistent differences were observed in the *copt5* mutants (Fig. 2A). The greater sensitivity of the *copt5* mutants to Cd in 1/2 MS was particularly evident in roots given the reduction in length under these conditions (Fig. 2B), and was even greater than in plants grown in light/dark cycles at the same Cd concentration (Fig. 1). $COPT5^{OE}$ plants behaved like the controls under all the tested conditions (Fig. 2).

Root length in the presence of ethylene is greatly reduced, which precluded us from measuring this. However, Cd-induced hypocotyl growth inhibition in the etiolated plants, a well-known parameter affected by ethylene (Guzmán and Ecker 1990), was drastically reduced by around half, as measured in both the *copt5* lines and the WT plants (Fig. 3). Mutants showed greater sensitivity to Cd than the WT, even in the presence of ethylene. Moreover, the WT plants were not significantly affected by Cd when the hormone was present, while a further reduction in hypocotyl length was measured in the *copt5* seedlings (Fig. 3).

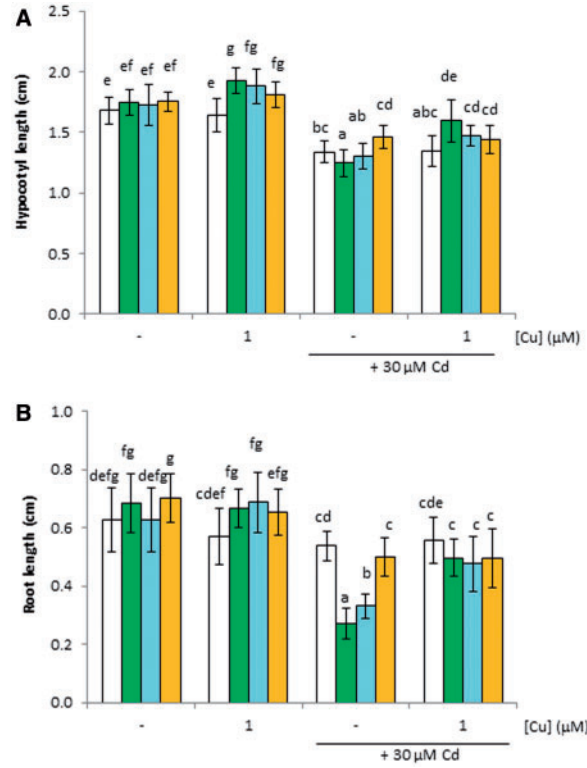

Fig. 2 Effect of Cd on etiolated seedlings. Hypocotyl (A) and root (B) length of the WT (white bars), *copt5-2* (green bars), *copt5-3* (blue bars) and $COPT5^{OE}$ (yellow bars) seedlings grown for 7 d in the absence of light in 1/2 MS medium (−), and in the same medium supplemented with 1 μM Cu, containing or not Cd (30 μM). Mean values ± SD are shown ($n = 18$). Different letters indicate significant differences ($P < 0.05$).

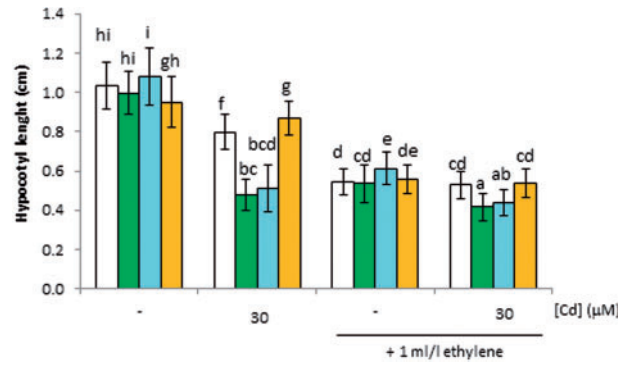

Fig. 3 Effect of ethylene on hypocotyl elongation of etiolated seedlings under Cd stress. Hypocotyl length of etiolated 7-day-old WT (white bars), *copt5-2* (green bars), *copt5-3* (blue bars) and $COPT5^{OE}$ (yellow bars) seedlings grown in sealed pots containing 1/2 MS medium either without or with 30 μM Cd, and in the absence or presence of 1 ml l^{-1} ethylene (+ ethylene). Mean values ± SD are shown ($n = 40$). Different letters indicate significant differences ($P < 0.05$).

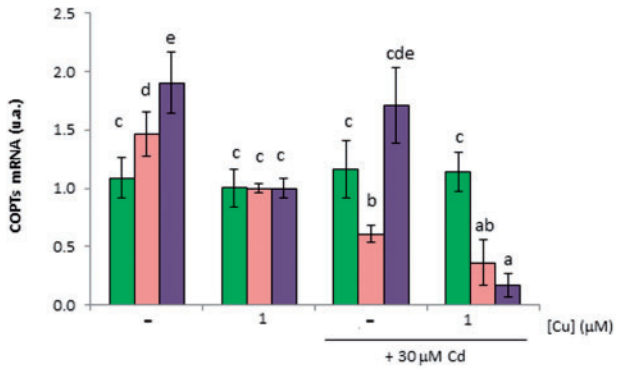

Fig. 4 Expression of COPTs under Cd stress. Relative mRNA levels of *COPT1* (green bars), *COPT2* (pink bars) and *COPT5* (purple bars) in the 7-day-old WT seedlings grown in 1/2 MS (−), supplemented with 1 μM Cu, 30 μM Cd or 1 μM Cu plus 30 μM Cd. Total RNA was extracted and analyzed by qRT-PCR. Values correspond to arithmetic means ($2^{-\Delta\Delta Ct}$) ± SD ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$).

Effect of cadmium on gene expression in the wild-type and *copt5* mutant seedlings

The effect of Cd on COPT gene expression was checked in the WT seedlings germinated on plates under the four above-defined different Cu and Cd conditions. The obtained results indicate that, unlike *COPT1* and *COPT2*, *COPT5* was not regulated by Cu, which agrees with previously reported data (Sancenón et al. 2003). Thus the relative expression of *COPT1* and *COPT2* was reduced significantly in the 1 μM Cu medium, while *COPT5* expression remained constant. At the concentrations used, its expression remained unaltered in the presence of Cd, while the regulation of *COPT1* and *COPT2* by Cu was still observed, or even further reduced, in this medium (**Fig. 4**).

The expression of *FSD1*, a Cu deficiency target regulated by the SPL7 transcription factor (Yamasaki et al. 2009, Andrés-Colás et al. 2013), was also determined. The analysis of *FSD1* expression in 7-day-old WT, *copt5-2*, *copt5-3* and $COPT5^{OE}$ seedlings, grown under the four experimental conditions, revealed that it was decreased by >75% in the WT seedlings when Cu was added to the 1/2 MS medium (**Fig. 5A**), as also occurred in the *copt5-2* and *copt5-3* seedlings. However, under low Cu conditions, it was significantly lower in the mutants than in the WT (**Fig. 5A**). In addition to being regulated by Cu, *FSD1* expression was also decreased under Cd treatment, and a further reduction was noted in the presence of both metals (**Fig. 5A**), similar to the effect observed on *COPT1* and *COPT2* expression (**Fig. 4**).

As the SPL7 transcription factor also regulates *MIR398b/c* under Cu deficiency which, in turn, regulates *CSD1* expression at the post-transcriptional level (Yamasaki et al. 2007), *CSD1* expression was also checked in the same samples (**Fig. 5B**). Unlike *FSD1*, *CSD1* expression in the WT more than doubled when Cu was added to the medium, and it became exacerbated in the presence of Cd (**Fig. 5B**). These findings further indicate that Cd attenuates Cu deficiency responses, which agrees with the results obtained for plasma membrane-regulated transporters *COPT1* and *COPT2* (**Fig. 4**) and *FSD1* (**Fig. 5A**).

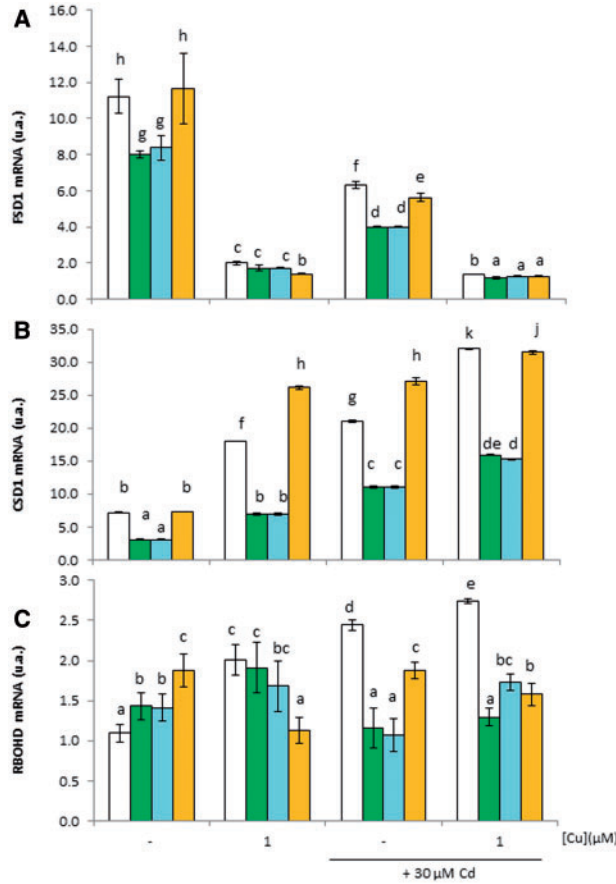

Fig. 5 Expression of oxidative stress markers. Relative expression of *FSD1* (A), *CSD1* (B) and *RbohD* (C) mRNA in the WT (white bars), *copt5-2* (green bars), *copt5-3* (blue bars) and $COPT5^{OE}$ (yellow bars) seedlings grown for 7 d in 1/2 MS medium (−), supplemented with 1 μM Cu, containing or not Cd (30 μM). Total RNA was extracted and analyzed by qRT-PCR. Values correspond to arithmetic means ($2^{-\Delta\Delta Ct}$) ± SD ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$).

The *CSD1* expression values in the *copt5-2* and *copt5-3* seedlings were also significantly lower than in the WT under all the studied conditions, whereas $COPT5^{OE}$ seedlings behaved mostly like the WT (**Fig. 5B**).

The expression of a ROS-generating enzyme encoded by *RBOHD*, which has been used as a control for gene expression under Cd toxicity (Keunen et al. 2013), was employed to study the effect of both metals on gene expression under the above-described four conditions. As shown in **Fig. 5C**, the WT seedlings grown in 1/2 MS showed a 50% lower *RBOHD* expression than when grown under Cu sufficiency conditions. The same effect was observed when Cd was present in the medium as the *RBOHD* expression levels increased significantly in the 1 μM Cu medium, in addition to the effect caused by Cd alone. Conversely, in the *copt5-2* and *copt5-3* mutant plants, the relative *RBOHD* expression remained almost constant under the four different conditions (**Fig. 5C**). $COPT5^{OE}$ lines showed the opposite *RBOHD* pattern of expression since the presence of Cu reduced it and it remained unaffected by Cd under low Cu conditions, but slightly increased when Cu was present (**Fig. 5C**).

The expression pattern of these three genes in the *spl7* mutant under the same four experimental conditions (**Supplementary Fig. S5**) allowed us to identify the Cd effects deriving from the altered Cu deficiency responses in the *copt5* mutants. As expected, the characteristic Cu regulation of *FSD1* and *CSD1* (**Fig. 5A**, **B**) was not observed in the *spl7* seedlings (**Supplementary Fig. S5A**, **B**). However, *FSD1* attenuation and *CSD1* induction observed in the presence of Cd were maintained in the *spl7* mutant, which indicates that the Cd effects on Cu deficiency markers are at least partially independent of the master regulator SPL7. In contrast, the responses of the *RBOHD* expression pattern to Cu were altered in the *spl7* mutant, where no activation was observed by either Cu or Cd. This indicates that oxidative stress is a putative mediator of Cd effects and alters SPL7-dependent responses (**Supplementary Fig. S5C**).

Longer term effects of cadmium on plant growth and oxidative stress

In order to study the possible distinctive effects of Cd on roots and shoots, the WT and *copt5* plants were grown for 31 d in hydroponic culture in modified Hoagland without Cu. Afterward, both the WT and *copt5-2* mutants were subjected to 16 d Cd treatment by adding different concentrations to the nutrient solution (2.5–10 μM; **Supplementary Fig. S6**). Root growth was very sensitive to the presence of Cd in the medium. Thus at the end of the 16 d treatment, both plant types showed a progressively lower absolute growth rate (AGR) with an increased Cd concentration. The largest root length difference between the WT and *copt5-2* mutant plants was measured at 2.5 μM (16% lower in the mutant). Both plant types revealed an almost total cessation of root growth at 10 μM Cd (**Supplementary Fig. S6A**). Growth of aerial parts (**Supplementary Fig. S6B**), measured as increased fresh weight, was impaired by the longer term Cd treatments and, as indicated for seedlings (**Supplementary Fig. S1**), the *copt5-2* plants showed greater sensitivity than the WT at the 5 μM Cd concentration. At this concentration, the AGR was 18% lower in the mutant than in the WT, which is a similar difference to that found for root growth at a lower concentration (2.5 μM Cd).

To investigate the functional role of COPT5 in Cd translocation to shoots, lipid peroxidation [measured as malondialdehyde (MDA) content] was quantified in leaves and roots (**Fig. 6A**, **B**) under 0, 5 and 10 μM Cd to check whether putative changes in Cd transport can be revealed by differences in oxidative stress levels. Despite MDA contents being similar in the absence of Cd in the WT and *copt5-2* mutant plants in leaves and roots, lipid peroxidation increased in the WT leaves when Cd was added to the media, but remained mostly unaffected in *copt5-2*, or with even lower, but non-significant, values than in WT leaves (5 μM Cd; **Fig. 6A**). Remarkably, the lipid peroxidation levels showed a completely reversed pattern of change in roots as the MDA content considerably increased in the *copt5-2* mutant at the lower Cd concentration tested (5 μM), and remained higher than in the WT roots at higher Cd levels (**Fig. 6B**). Accordingly, the *COPT5*^{OE} line showed the opposite effect with increased MDA content in leaves and decreased content in roots when compared with the *copt5-2* mutant (**Fig. 6A**, **B**). The location of the oxidative damage caused by Cd in roots was studied. For this purpose, histological sections were performed from the roots of those plants grown in hydroponic Hoagland (0.1×) solution with or without 10 μM Cd (**Fig. 6C**), and they were stained with toluidine blue dye. Lipid peroxidation in the tissues surrounding the vascular bundles, detected by the appearance of a reddish-blue color (**Fig. 6C**), was higher and more widely extended in the vascular bundles of the *copt5-2* roots at 10 μM Cd. Thus Cd differentially affects *copt5-2* roots.

Effect of cadmium on ethylene release according to copper levels

The possibility of the observed responses to Cd treatments being mediated by changes in ethylene biosynthesis in Cd-treated plants was studied in hydroponically grown WT and *copt5-2* adult plants subjected to four test conditions: modified Hoagland 0.1× without Cu; the same medium with Cu (0.1 μM) or Cd (5 μM); and with both Cu (0.1 μM) and Cd (5 μM). The most remarkable result obtained from these experiments was under the 0.1 μM Cu conditions, where ethylene release was significantly stronger than in the absence of Cu because, when Cu was added, the values almost doubled in the WT, and they quadrupled in the mutant, if compared with those in the modified Hoagland 0.1× without Cu medium (**Fig. 7**). Thus, surprisingly, a deficit in Cu content seemed to limit ethylene biosynthesis. The presence of Cd further reduced the biosynthesis of the hormone in both the WT and *copt5-2* plants under both Cu conditions (**Fig. 7**). In an independent experiment, the WT and *copt5-3* lines exhibited the same trends (**Supplementary Fig. S7**), which further indicates decreased ethylene production under Cu deficiency, which became more exacerbated under Cd stress.

Cadmium content in the WT and *copt5* plants with different copper statuses

The Cd concentration was separately measured in the roots and leaves of the WT, *copt5-2*, *copt5-3* and *COPT5*^{OE} hydroponically grown plants in Hoagland without Cu, and with 0.1 μM Cu, to ascertain whether Cd content or distribution is altered in the *copt5* mutants (**Fig. 8**). Cu content was also determined in the Cd-treated plants, and was significantly higher in the *copt5-2* and *copt5-3* leaves and roots than in the WT (**Supplementary Fig. S8A**, **B**). It is noteworthy that the Cd content in WT shoots increased significantly when grown in the medium supplemented with 0.1 μM Cu (**Fig. 8A**), which indicates that the Cu status in the plant influences Cd translocation. This effect did not reach levels of statistical significance in roots (**Fig. 8B**). It is remarkable that Cd translocation to upper plant parts was significantly impaired in the *copt5-2* and *copt5-3* mutants (**Fig. 8A**) since Cd content was around 15% lower in the *copt5-2* and *copt5-3* leaves than in the WT despite being similar in roots, at least in *copt5-2* (**Fig. 8**). This suggests that the

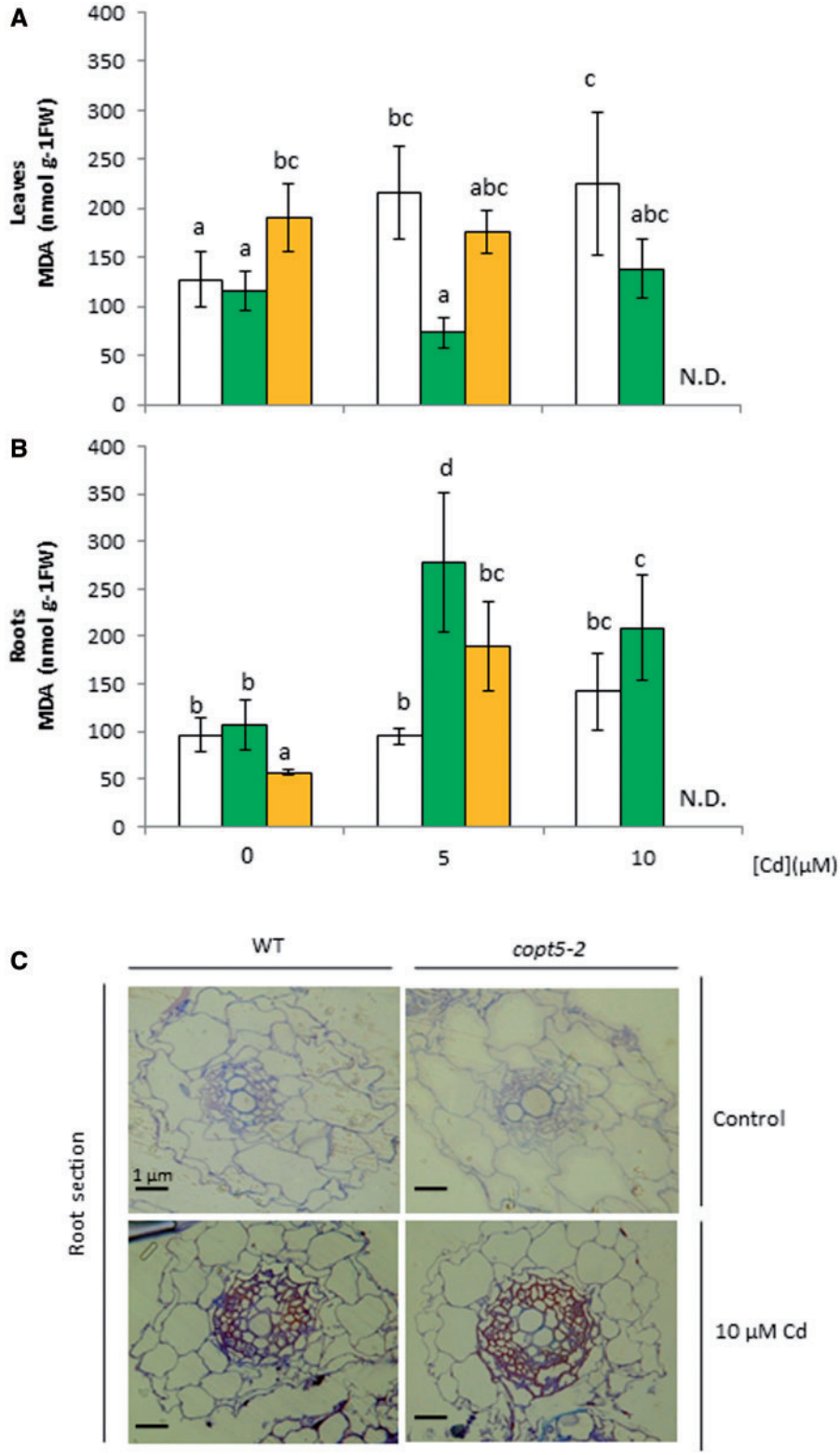

Fig. 6 Lipid peroxidation in Cd-stressed adult plants. MDA contents in the leaves (A) and roots (B) of the 47-day-old WT (white bars), *copt5-2* (green bars) and *COPT5*^{OE} (yellow bars) plants grown during the last 16 d of culture in Hoagland's hydroponic medium containing different Cd concentrations. Mean values ± SD are shown ($n = 3–6$). Different letters indicate significant differences ($P \leq 0.05$). (C) Cross-sections of the WT and *copt5-2* root tissue from the Cd-stressed and control plants stained with toluidine blue. Photographs were taken with an optical microscope (the black bar represents 1 μm). The sites of plasma membrane damage are stained in red.

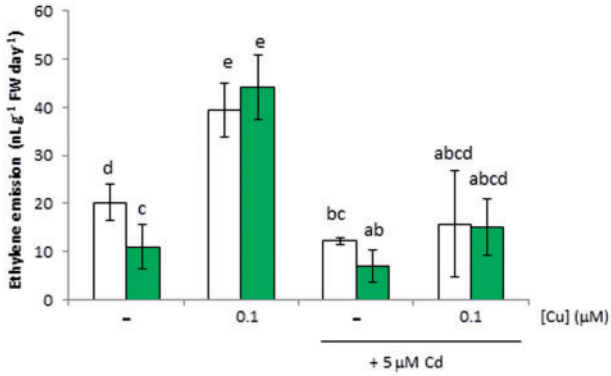

Fig. 7 Effect of Cd stress on ethylene biosynthesis at different Cu statuses. Ethylene emission in the 47-day-old WT (white bars) and *copt5-2* (green bars) plants grown in modified Hoagland's hydroponic medium without Cu (−) and treated during the last 16 d of culture with 0.1 μM Cu, containing or not Cd (5 μM). Mean values ± SD are shown ($n = 7–9$). Different letters indicate significant differences ($P \leq 0.05$).

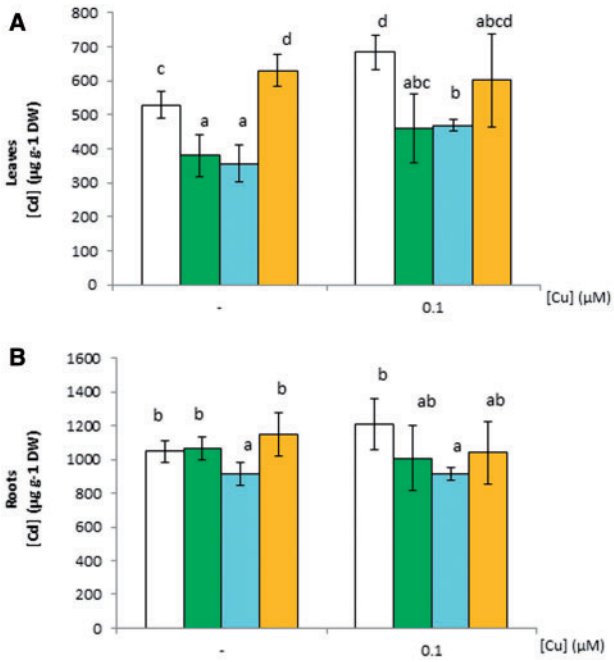

Fig. 8 Cd accumulation in different plant organs. Cd content in the leaves (A) and roots (B) of the 47-day-old WT (white bars), *copt5-2* (green bars), *copt5-3* (blue bars) and *COPT5*^{OE} (yellow bars) plants exposed to 5 μM Cd added to the Hoagland's hydroponic medium supplemented, or not, with 0.1 μM Cu during the last 16 d of culture. Mean values ± SD are shown ($n = 5–9$). Different letters indicate significant differences ($P \leq 0.05$).

COPT5 function participates in Cd translocation to plant shoots.

Discussion

Extensive studies on the underlying mechanisms of Cd toxicity in plants under metal stress or in plant hyperaccumulators have been reported (reviewed in Cuypers et al. 2010, Kramer 2010, Clemens et al. 2013), but the understanding of how plants acclimate to high Cd levels under metal deficiencies is poor. In this work, we explored the role of the Cu transporter COPT5 in Cd tolerance by using the *copt5-2* and *copt5-3* mutants, which displayed increased sensitivity to Cd toxicity, to better understand the interaction between Cu homeostasis and Cd root to shoot translocation in higher plants.

A role for COPT5 in Cd sensitivity has been previously suggested by the expression of the *COPT5* homolog gene in the low Cd-accumulating plant *S. torvum*, which was induced with mild Cd exposure (Yamaguchi et al. 2010). However, under the experimental conditions used herein (30 μM Cd), Arabidopsis *COPT5* expression remained unaffected (**Fig. 4**). Nevertheless, the obtained data indicate that Arabidopsis COPT5 plays a role in root to shoot Cd translocation and in Cd tolerance. These data are based on the characterization of the phenotypes shown by transgenic plants with altered levels in COPT5 grown in the presence of 30 μM Cd under mild Cu deficiency (1/2 MS) conditions if compared with Cu-sufficient medium (1/2 MS plus 1 μM Cu). COPT5 has been previously shown to be expressed mostly in vascular bundles in roots and to localize at the pre-vacuolar/vacuolar compartment where it functions in transporting Cu^+ towards the cytosol from the lumen of these storage or recycling compartments (Garcia-Molina et al. 2011, Klaumann et al. 2011). Mutants lacking COPT5 display impaired Cu distribution at the cellular level, where Cu accumulates at the vacuoles, and in different organs, where Cu increases in lower parts (roots and rosette leaves), whereas reproductive organs (siliques and seeds) show a decreased Cu content if compared with controls (Klaumann et al. 2011). Cu^+ efflux from root cells is mediated by the heavy metal P-type ATPase HMA5 (Andrés-Colás et al. 2006) (**Fig. 9**). A putative direct effect of COPT5 on the de/compartmentalization of Cd has been ruled out since COPT transporters have been shown to be Cu^+ specific (Sancenón et al. 2003), and Cd^{2+} is probably not a substrate for Cu^+ HMA5 ATPase. Instead Cd^{2+} is transported through divalent cation transporters, such as HMA4 (reviewed in Lin and Aarts 2012).

Our data indicate that Cd effects are indeed due to the COPT5 function on Cu transport since addition of Cu, but not other metals, reverts the growth inhibition of the *copt5* mutants caused by Cd (**Fig. 1**). Based on the presented results, an indirect effect should be considered to explain the enhanced Cd sensitivity of the *copt5* mutant. To pursue the COPT5 function in Cd tolerance further, we followed three processes where evidence for a putative interaction between Cu deficiency and Cd toxicity has already been suggested; first, the influence of ethylene biosynthesis and/or signaling on plant growth (Schellingen et al. 2014); secondly, the effect of Cd on Cu deficiency responses that affect Cd tolerance (Gayomba et al. 2013); and, finally, oxidative stress generated by both the scarcity of Cu and the presence of Cd, which could also affect root elongation (Smeets et al. 2013).

Regarding the putative role of ethylene, it has been reported that ethylene seems to play a crucial role in the Cd-induced inhibition of root growth in both barley and

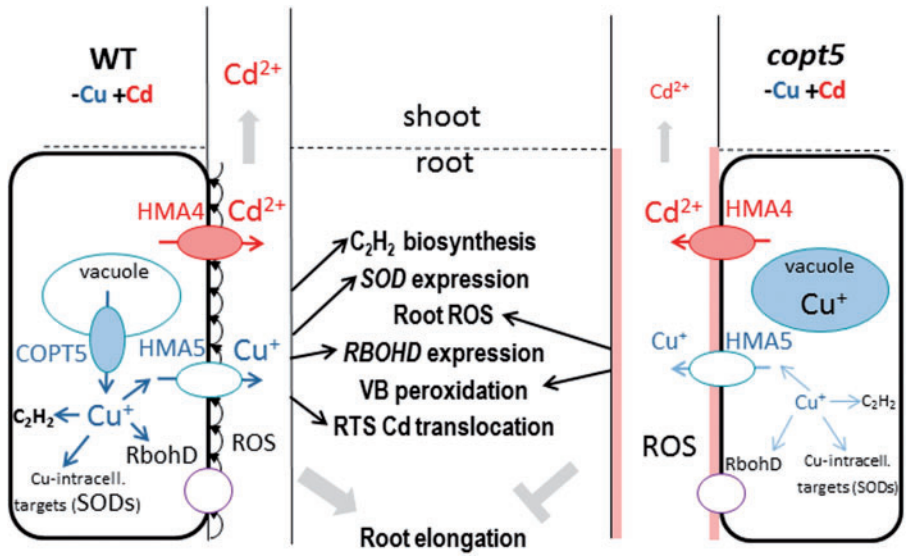

Fig. 9 Model of the COPT5 function in Cd root to shoot translocation and tolerance. The phenotypic characteristics of the WT (left panel) and *copt5* mutants (right panel) described in the text are shown under Cu deficiency and in the presence of Cd. Intracellular Cu deficiency was exacerbated in the *copt5* mutants, which led to Cu accumulation in roots, reduced ethylene emissions and defective *copt5* SOD antioxidative defenses and *RbohD* expression. As a result of the defects in both the intercellular and interorgan Cu distribution in the *copt5* mutants, Cd transport to the aerial plant parts and root elongation was impaired. RST, root to shoot; VB, vascular bundle cells. Arrows indicate the processes that were favored in the WT or the *copt5* mutants.

Arabidopsis (Valentovicova et al. 2012, Schellingen et al. 2014). Since Cd binding to the Atx1 metallochaperone provokes defects in Cu delivery to cuproproteins in yeast (Heo et al. 2012), and as ethylene receptors are cuproproteins (Rodríguez et al. 1999), a similar effect in Arabidopsis would negatively affect ethylene perception. Although according to the obtained results, the exacerbated sensitivity of the *copt5* mutants to Cd is not dependent on a poorer perception of ethylene (**Fig. 3**), since both Cd and ethylene reduce hypocotyl growth, further experiments will be needed to assess a role for ethylene perception in the *copt5* phenotype. In addition, ethylene biosynthesis can be affected by Cd. Opposite effects of Cd on ethylene biosynthesis have been reported depending on the Cd levels and the experimental system used (Arteca and Arteca 2007, Rodríguez-Serrano et al. 2009, Schellingen et al. 2014). Under the experimental conditions presented herein, long-term (16 d) Cd (5 μM) treatments inhibited ethylene biosynthesis in the WT and *copt5* plants, but not in the $COPT5^{OE}$ line, under low Cu (Hoagland 0.1× in **Fig. 7** and **Supplementary S7**). Increased ethylene levels have been shown to alleviate the Cd-induced inhibition of photosynthetic capacity in mustard (Masood et al. 2012). Along the same lines, Lu and Kirkham (1991) indicated that a correlation might exist between the genotypes that emit more ethylene and heavy metal resistance. Accordingly, the reduced ethylene production measured in low Cu plants could explain, at least partially, the increased Cd sensitivity observed in the *copt5* mutants since exacerbated Cu deficiency effects have been described in these mutants (Garcia-Molina et al. 2011). Despite the role that Cu plays in ethylene perception being well established, this work uncovers a new role in ethylene production. However, the specific effects of Cu status on ethylene biosynthesis merit further studies.

Recent data on the *copt2* mutant indicate that Cd stimulates the SPL7-dependent expression (Gayomba et al. 2013). Under the experimental conditions reported herein, the expression of plasma membrane-located COPT family members, such as *COPT1* and *COPT2*, was down-regulated by both metals, while *COPT5* expression was regulated by neither Cu nor Cd (**Fig. 4**). Moreover, we were unable to detect significant differences in Cd sensitivity in root growth of the single *copt1*, *copt2* and *copt6* mutants if compared with the controls (**Supplementary Fig. S4**). These apparent discrepancies can be attributed to the genotypic differences and diverse Cd exposure conditions. Gayomba et al. (2013) used 10-day-old seedlings which had been subjected to 50 μM Cd treatment, while in this work, 7-day-old seedlings were used which had been grown in 30 μM Cd. According to the presented results, Cd interacts with SPL7-mediated responses (**Figs. 4**, **5**), which agrees with a putative role for Cd that affects SPL7 transcriptional activation by replacing the Zn ions at the Zn fingers in the SBP DNA-binding domain (Hartwig 2001). Among the Cu deficiency SPL7 targets we find SODs, encoded by *FSD1* and *CSD1*, whose expression was reduced and increased, respectively, by Cd (**Fig. 5A**, **B**). Accordingly, *FSD1* and *MIR398* are direct SPL7 targets, and *CSD1* expression was regulated post-transcriptionally by *MIR398*, whose decreased expression led to increased *CSD1* levels (Yamasaki et al. 2007). However, both *FSD1* and *CSD1* expression was notably reduced in the *copt5* mutants, independently of Cd treatment (**Fig. 5A**, **B**). Since it has been recently suggested that Cu deficiency perception can take place in the lumen of the endoplasmic reticulum through SPL7

(Garcia-Molina et al. 2014), a complex scenario is envisaged for the Cu deficiency responses in the *copt5* mutants. Whereas Cu delivery to the target intracellular cuproproteins was impaired under Cu deficiency, surplus Cu accumulated in the lumen of another secretory pathway compartment, the vacuole, to provoke a stressful imbalance in cellular Cu redistribution in *copt5* mutants. Our results agree with the reported hypersensitivity to Cd in the *nramp3nramp4* mutant impaired in mobilization of vacuolar metals (mainly Fe and Mn) that identified chloroplasts as a target probably related to a Cd-mediated decreased availability of essential metals in these organelles (Molins et al. 2013).

Since SODs are among the main antioxidative defense enzymes, they can be involved in the deficient SOD response in the *copt5* mutants. In fact, two separate types of oxidative stress effects can be considered through the COPT5 function. First, a malfunctioning chloroplast photosynthetic electronic transfer chain has been previously described in the *copt5* mutants, when plants were subjected to severe Cu scarcity conditions, probably due to a defect in the Cu recycling from the vacuole (Garcia-Molina et al. 2011). These defects in chloroplast function are a well known source of ROS production (Yruela 2013). Secondly, the function of COPT proteins has been recently linked to Cu^{2+}-mediated production of hydroxyl radicals, which leads to the activation of cation channels (Rodrigo-Moreno et al. 2013). As a result of both the disturbed Cu localization, that exacerbates at least part of the Cu deficiency in roots, and reduced antioxidative defenses, *copt5* root cell membranes were particularly sensitive to oxidative stress, and those in the vascular bundles cells became differentially peroxidated when Cd was present (**Fig. 6C**). Xylem loading of ions is dependent on normal functioning of membrane-bound transport systems located in the parenchyma cells around the vascular bundles. Disruption of membrane integrity caused by lipid peroxidation is among the factors involved in the reduction of metals and macronutrient contents observed in leaves of Cd-treated plants (Rubio et al. 1994, Sandalio et al. 2001). Xylem loading could also be impaired by cell wall lignification. A ROS-increased lignification has been hypothesized to prevent xylem Cd entry (Sandalio et al. 2009).

The role of ROS in plant signaling and defense against biotic and abiotic stresses has begun to be deciphered (reviewed in Gilroy et al. 2014). It has also been suggested that Cd produces both oxidative damage and increased antioxidant defense (Cuypers et al. 2010). In line with this, lack of induction by Cd of the NADPH oxidase (*RBOHD*), described as a major player in Cd-induced ROS production (Smeets et al. 2009, Cuypers et al. 2010), was observed in the *copt5* mutants (**Fig. 5C**). However, *RBOHD* was not induced in an *spl7* mutant in the presence of Cu (**Supplementary Fig. S5C**), which indicates that COPT5-mediated *RBOHD* expression is dependent on SPL7. Based on the recently published data about the ROS-mediated vascular homeostatic control of root to shoot element translocation and the role of NADPH oxidases in this process (Jiang et al. 2013), a model is proposed in which lack of the COPT5 function prevents *RBOHD* induction, probably in the root stele, which leads to lower Cd concentrations in root vasculature cells and in xylem sap to reduce the delivery of damaging amounts of Cd to shoots (**Fig. 9**). Accordingly, the MDA content in leaves followed the Cd content in the WT and *copt5* mutant plants (**Figs. 6A**, **8A**), which suggests that oxidative stress damage is caused by Cd. These results agree with the recently published work on OXI1 (oxidative signal-inducible kinase 1), where *FSD1* and *MIR398* expression was regulated by the OXI1 pathway by acting downstream of NADPH oxidase activity (Smeets et al. 2013). These results demonstrate that, in addition to and independently of the SPL7-mediated responses, Cu transport plays a key role in Cd resistance, and suggest that by producing damage to membranes, oxidative stress triggers an NADPH oxidase/ROS signaling pathway, which contributes to Cd translocation and basal plant resistance (**Fig. 9**).

Taken together, the results presented herein match a model where the COPT5 function is an important component of basal Cd resistance in Arabidopsis. The inability to retrieve Cu from the vacuole in the *copt5* mutants under Cd treatment affects root growth in a complex manner, leading to defective antioxidative defenses. Under Cu deficiency and in the presence of Cd, all these defects are further aggravated, and lead to a higher and more widespread peroxidation of the membranes at the vascular bundles in the *copt5* mutants, impairing normal functioning of membrane-bound transport systems which would result in inhibition of root to shoot Cd translocation and of root elongation. The slightly impaired Cd distribution in *copt5* plants suggests a biotechnological approach to minimize entry of Cd into edible parts of crop plants to avoid Cd reaching animals and humans.

Materials and Methods

Plant growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the WT. The *copt5-2*, *copt5-3* and *spl7* knock-out mutants, the complemented *COPT5* (*pCOPT5::COPT5::GFP*) and the overexpressor $COPT5^{OE}$ (*pCaMV35S::COPT5::HA*) lines have been previously described in Yamasaki et al. (2009) and Garcia-Molina et al. (2011). Seeds were stratified for 2 d at 4°C, sown in soil pots and watered regularly. Two different culture systems were employed: seedlings in agar plates for shorter term experiments; and adult plants in hydroponic solutions for longer term experiments. For growth on plates, seeds were surface-sterilized with sequential washes in 70% ethanol (5 min), bleach (5 min) and water (2× 2 min), resuspended in 0.1% (w/v) agar and sown on plates containing 1/2 MS medium supplemented with 1% (w/v) sucrose and, unless otherwise indicated, 1 μM $CuSO_4$ and/or 30 μM $CdCl_2$. Hydroponic cultures were performed from the seedlings germinated in Eppendorf tubes filled with 0.8% agar (w/v) containing Hoagland (0.1×) solution for 1 week. Then they were transferred to black boxes containing modified Hoagland (0.1×) nutrient solution, as described in Hermans and Verbruggen (2005), but without Cu. Plants were grown in this medium for 31 d and the solution was changed every 3 d. Long-term treatments with Cu or Cd were performed over a 16 d period. To counterbalance the prolonged presence of the metal in hydroponic media, lower concentrations were tested (2.5–10 μM) to select, according to the preliminary experiments, those showing differential effects of the metal on the WT and mutant plants. The concentration of Cu, when added, was also lowered to sufficiency levels (0.1 μM). In all cases, intermediate photoperiodic conditions (12 h light, 20–23°C/12 h darkness, 16°C) were applied. Plant growth during the 16 d treatment was followed. AGRs represent the mean daily length (roots) or fresh weight (aerial part) increase during this period, and are expressed as percentages of the control WT plants.

Root length was measured by the Image J 1.42 q software (http://rsb.info.nih.gov./ij). To measure lipid peroxidation, frozen rosette leaves and roots were ground in MDA extraction buffer [15% (v/v) trichloracetic acid; 0.37% (w/v) 2-thiobarbituric acid; 0.24 N HCl; 0.0001% (w/v) butylated hydroxytoluene] and then boiled at 80°C for 1 h to generate MDA derivatives, which were determined by the formula $A_{535}-A_{600}$ ($\varepsilon = 1.56\times10^5\ M^{-1}\,cm$). Values were normalized to nmol g^{-1} FW.

Histological procedures

Plant material was fixed for 24 h in 90 ml of 70% ethanol, plus 5 ml of 40% formaldehyde and 5 ml of glacial acetic acid. Next the plant material was dehydrated in consecutive baths at increasing ethanol concentrations: 70, 96 and 100% (2× 30 min each). Finally, the plant material was incubated at 4°C for 3 h in LR-White resin in 100% ethanol (1 : 3, v/v) and the sections obtained were stained with toluidine blue.

Ethylene production and metal content determination

Plants were introduced into glass flasks containing 3 ml of nutrient solution, sealed with a rubber septum and kept for 24 h in darkness. The ethylene released to the head space was analyzed in a gas chromatograph (Shimadzu GC-14B) equipped with a packed alumina column and a flame ionization detector (FID). A 1 ml aliquot of gas from the head atmosphere of the flasks was extracted with a syringe, injected at 120°C and run isothermally at the same temperature. Detection was performed at 150°C. An external standards quantitation method was followed with the Shimadzu CLASS-VP™ program.

The fresh Arabidopsis material was washed once with 20 μM EDTA and three times with MilliQ H_2O, dried at 65°C for 2 d and digested with 65% (v/v) HNO_3 at 80–90°C. Digested samples were then diluted with Millipore H_2O (Purelab Ultra), and the Cu and Fe contents were determined by inductively coupled plasm mass spectrometry (ICP-MS) at the 'Servicios Centrales de Investigación' (Universidad de Almeria, Spain).

Gene expression analysis by real-time quantitative PCR

Total Arabidopsis RNA was extracted with Trizol Reagent (Ambion) and reverse transcription–PCR was performed with SSII (Invitrogen), as previously described (Andrés-Colás et al. 2006). RNA was quantified by UV spectrophotometry; its integrity was visually assessed on ethidium bromide-stained agarose gels and was treated with DNase I Amp Grade (Invitrogen). Real-time quantitative PCR (qPCR) was carried out with SYBR-Green qPCR Super-Mix-UDG with ROX (Invitrogen). The specific primers detailed in **Supplementary Table S1** were used in a CFX96 Touch™ Real-Time PCR Detection System (BioRad) with one cycle of 50°C for 2 min, an initial denaturation cycle at 95°C for 2 min, and a series of 40 cycles of denaturation and amplification at 60°C for 30 s. Values were normalized at the *UBQ10* mRNA levels and the WT was used as a reference under control conditions.

Statistical analyses

The statistical analysis of the relative expression was performed by comparing the relative expression of the genes (RT-PCR) based on the pair-wise fixed reallocation randomization test ($P < 0.05$) (Pfaffl et al. 2002). For the other parameters, one-way analyses of variance (ANOVAs) were performed. Significant differences between means were established after post-hoc tests (Tukey or Games–Howell, according to data homoscedasticity; $P \leq 0.05$) using the IBM SPSS Statistics software, version 19.0.0. Data are provided as the mean values ± SD of the different biological samples used in each experiment, as indicated in the figure legends.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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