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ORIGINAL ARTICLE

Copper uptake mechanism of *Arabidopsis thaliana* high-affinity COPT transporters

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Abstract

Copper (Cu) is an essential plant micronutrient. Under scarcity, Cu^{2+} is reduced to Cu^{+} and taken up through specific high-affinity transporters (COPTs). In *Arabidopsis*, the COPT family consists of six members, either located at the plasma membrane (COPT1, COPT2, and COPT6) or in internal membranes (COPT3 and COPT5). Cu uptake by COPT proteins has been mainly assessed through complementation studies in corresponding yeast mutants, but the mechanism of this transport has not been elucidated. To test whether Cu is incorporated by an electrogenic mechanism, electrophysiological changes induced by Cu addition were studied in *Arabidopsis thaliana*. Mutant (T-DNA insertion mutants, *copt2–1* and *copt5–2*) and overexpressing lines ($COPT1^{OE}$ and $COPT5^{OE}$) with altered expression of *COPT* transporters were compared to wild-type plants. No significant changes of the membrane potential (E_m) were detected, regardless of genotype or Cu concentration supplied. In contrast, membrane depolarization was detected in response to iron supply in both wild-type and in mutant or transgenic plants. Similar results were obtained for trans-plant potentials (TPP). GFP fusions of the plasma membrane COPT2 and the internal COPT5 transporters were expressed in *Xenopus laevis* oocytes to potentiate Cu uptake signals, and the cRNA-injected oocytes were tested for electrical currents upon Cu addition using two-electrode voltage clamp. Results with oocytes confirmed those obtained in plants. Cu accumulation in injected oocytes was measured by ICP-OES, and a significant increase in Cu content with respect to controls occurred in oocytes expressing *COPT2:GFP*. The possible mechanisms driving this transport are discussed in this manuscript.

Keywords *Arabidopsis thaliana* · Copper uptake · COPT transporters · Membrane (E_m) and trans-plant (TPP) potentials · Two-electrode voltage clamp (TEVC) · *Xenopus laevis* oocytes

Introduction

Copper (Cu) is an essential mineral micronutrient required for plant growth and development. It plays a key role as a redox cofactor in basic processes of cellular metabolism such as photosynthesis and respiration, and participates in hormone signaling, oxidative stress defense, and lignin biosynthesis, among other biochemical and physiological processes (Broadly et al. 2012). Both Cu scarcity and excess result in generation of reactive oxygen species (ROS) (Ravet and Pilon 2013; Rodrigo-Moreno et al. 2013) that can damage nucleic acids, proteins, and membrane lipids, thus disturbing a number of biological functions (Sharma et al. 2012). Consequently, cytoplasmic Cu levels are tightly controlled and its homeostasis depends on the balance between uptake and distribution among the different subcellular compartments and cuproproteins. The main Cu species found in aerobic conditions is the divalent form (Cu^{2+}) that may enter root cells

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through low-affinity divalent cation transporters with low specificity, such as some members of the ZIP family (Wintz et al. 2003), though this hypothesis has been questioned (Milner et al. 2013). Cu uptake through a low-affinity transport system (K_m ~580 μM), abolished in the presence of ascorbic acid, was reported in grapes (Martins et al. 2012). However, it is well established that the predominant transport mechanism, particularly under Cu scarcity, consists of its reduction by plasma membrane NADPH-dependent cupric reductases (Bernal et al. 2012) and subsequent uptake by high-affinity Cu^+ transporters of the COPT family (Sancenón et al. 2003; Puig 2014; Peñarrubia et al. 2015) expressed under the control of the Cu-responsive transcription factor SPL7 (SQUAMOSA promoter-binding protein-like7) (Yamasaki et al. 2009; Bernal et al. 2012).

The *Arabidopsis* COPT family, known as Ctr in yeast and animals, consists of six members. Three of them are located at the plasma membrane (COPT1, COPT2, and COPT6) and mediate Cu uptake from the external medium while other members (COPT3 and COPT5) are located in internal membranes (Sancenón et al. 2004; Jung et al. 2012). COPT4 is not functional in the corresponding yeast mutants (Sancenón et al. 2003). COPT5 is involved in delivering Cu from the prevacuolar compartment to the cytosol under severe Cu deficiency conditions (García-Molina et al. 2011; Klaumann et al. 2011). The integral plasma membrane COPT1 and COPT2 transporters are located at root tips, and along the rest of the root, respectively. Both are abundant in reproductive tissues and their expression is upregulated by Cu deficiency (Sancenón et al. 2003, 2004; Perea-García et al. 2013). *COPT2* is the most highly expressed among the members located at the plasma membrane (Gayomba et al. 2013; Perea-García et al. 2013). The participation of COPT transporters in Cu acquisition has been assessed through both complementation studies in the corresponding *Δctr1ctr3* yeast mutant (Kaempfenkel et al. 1995; Sancenón et al. 2003) and in transgenic plants where the expression levels of the COPT transporters have been modified (Puig 2014). However, little is known about the energetic requirements of the uptake mechanism.

Given the negative values of the membrane potential (Em) and the extremely low cytosolic concentration of free Cu ions (Rae et al. 1999), transport of this cation across membranes is expected to be thermodynamically favorable. Further, COPT/Ctr transporters do not possess obvious ATP-binding domains, suggesting that Cu^+ uptake does not use a primary active transport mechanism (Lee et al. 2002). However, this fact does not exclude the possibility of secondary active transport energized by the H^+-motive force, as reported for the uptake of other metal ions through transporters of the NRAMP, YSL, and ZIP families (Schaaf et al. 2004; Chaloupka et al. 2005; Kavitha et al. 2015). In this sense, a mammalian H^+-coupled metal ion transporter which may transport Cu^{2+} has been characterized (Gunshin et al. 1997), and Lee et al. (2002) reported a positive effect of low pH on Cu uptake through Ctr1 in yeast. Results reported by Lin and Kosman (1990) showed that Cu uptake in yeast was barely detected in glucose-starved cells, or at 4 °C; moreover, the transport presented saturable kinetics, and was inhibited by azide and dinitrophenol, which indicate an energy-dependent process. In addition, electrogenic uptake of cations can also occur by uniport mechanisms, as reported for NH_4^+ (Ludewig et al. 2002). Since electrogenic substrate transport affects the transmembrane potential, the variation of Em is a suitable tool to study uptake and effects of metals, such as Fe, Zn, Ni, Al, or Cd, in plants (Sijmons et al. 1984; Llamas et al. 2000, 2008; Sivaguru et al. 2003; Illes et al. 2006; Pavlovkin et al. 2006; Sanz et al. 2009; Bose et al. 2010; Kenderesova et al. 2012).

To our knowledge, electrophysiological studies on Cu uptake are scarce, and conflicting results have been published. A previous study on corn roots reported strong Em depolarizations induced by divalent cations, including Cu, when applied at 100 μM (Kennedy and Gonsalves 1987). Similarly, the cell resting potential of *Nitellopsis obtusa* was depolarized by 50% during a 45-min test at 110 μM Cu (Manusadzianas et al. 2002). Kennedy and Gonsalves (1987, 1989) also measured trans-root potentials (TRP) in excised corn roots and found that supplying Cu concentrations as low as 5 μM resulted in TRP depolarizations of more than 30 mV concomitantly with H^+-efflux inhibition. In contrast, Murphy et al. (1999) did not observe Em changes in *Arabidopsis* root cells upon addition of 30 μM Cu but the treatment induced K^+ leakage, suggesting an electroneutral uptake mechanism for this metal.

Electrophysiological studies of different transport proteins have also been performed in heterologous systems, such as oocytes of the African clawed frog *Xenopus laevis.* This technique has been widely used for the functional characterization of different metal transporters from diverse origin, including plants, and it has helped to elucidate uptake kinetics, substrate specificity, competition with other ions, or the need for ligands in the transport of different transition metals (Koike et al. 2004; Schaaf et al. 2004; Murata et al. 2006; Durrett et al. 2007; Zhai et al. 2014). Its use has also evidenced the mismatch that may occur between increased gene expression and actual transport activity (Kavitha et al. 2015).

In the present work, we performed an electrophysiological study in *Arabidopsis thaliana* to test whether Cu is taken up by an electrogenic mechanism through COPT transporters. To this end, plants with altered expression of *COPT* transporters located both at the plasma membrane and internal membranes (T-DNA insertion mutants, *copt2–1* and *copt5–2*, and overexpressing lines, $COPT1^{OE}$ and $COPT5^{OE}$) were compared to wild type. Further, GFP fusions with the Cu^+ transporters (*COPT2:GFP* and *COPT5:GFP*) were expressed in *X. laevis* oocytes to potentiate Cu uptake signals, and the cRNA-injected oocytes were tested for electrical currents upon Cu

addition. Additionally, Cu uptake in COPT-expressing oocytes was monitored by analyzing their metal content after incubation in a Cu-containing medium.

Materials and methods

Plant material and growth conditions

Seeds of wild-type (WT), *copt2–1* (Perea-García et al. 2013), and *copt5–2* (García-Molina et al. 2011) knockout mutant lines and transgenic plants overexpressing $COPT1^{OE}$ (Andrés-Colás et al. 2010) and $COPT5^{OE}$ (García-Molina et al. 2011) of Arabidopsis (*A. thaliana*, Col-0) plants were stratified for 2 days at 4 °C after ClO_2 sterilization (200 mL commercial bleach plus 3 mL HCl) for 5 h. Seedlings were germinated in shortened pipette tips filled with half-strength Murashige and Skoog (½ MS) medium in 0.8% agar plus 2.5 mM MES buffer, pH 5.7 and kept in boxes filled with distilled water in a growth chamber (12-h light/dark; 22 °C and 70% RH) until roots protruded a few millimeters from the cut end of the plastic tips. Subsequently, they were grown in hydroponic medium containing half-strength nutrient solution for 2 weeks and then in complete nutrient solution (3 mM KNO_3, 2 mM KCl, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 2 mM NH_4NO_3, 0.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, 50 μM NaFe-EDTA, 50 μM H_3BO_3, 10 μM $ZnSO_4$, 5 μM $MnCl_2$, 0.5 μM $CuSO_4$, and 0.01 μM Na_2MoO_4) which was renewed each week. Since expression of plasma membrane *COPT* transporters is induced under Cu deficiency (Sancenón et al. 2003), and in the case of *COPT2* also enhanced by Fe deficiency (Perea-García et al. 2013), plants were transferred either to half-strength nutrient medium, 1–3 days before Em measurements, or to fresh medium without Cu and Fe and supplemented with 50 μM of the Cu^+-chelator bathocuproine disulfonate (BCS) 3–7 days prior to TPP measurements. Plants from five different sowings were used to measure electrical responses to Cu addition.

Electrophysiological measurements

The effect of Cu on the transmembrane potential difference (Em) was measured in plants 30–40 days after sowing, as described by Llamas et al. (2000). Roots of whole plants were secured in a Plexiglass chamber that was perfused by a gravity flow system at a rate of 4–5 mL × min^{-1} with a standard solution consisting of 0.2 mM KCl, 0.2 mM $CaSO_4$, 0.4 mM $MgCl_2$, and 1 mM MES, pH 5.5. Transmembrane electrical potentials were measured with glass microelectrodes filled with 3 M KCl and reference salt bridges (3 M KCl in 2% agar), connected via Ag/AgCl electrodes with an electrometer amplifier (FD-223, WPI, Sarasota, FL). The reference electrode was kept in the perfusion chamber near the root. The micropipette was inserted with a micromanipulator. Changes in Em induced by addition of 10 or 30 μM $CuSO_4$ to the perfusion solution were followed and recorded with AxoScope (v.8.1) software. Electrical noise was attenuated with a low-pass filter (Chebyshev 8-pole, 0.01 to 20 Hz).

Whole plant electropotentials (trans-plant potentials (TPP)) were measured with two electrodes similar to the above-described reference electrode. As previously indicated, Cu was added to the perfusion solution bathing the roots; however, the probe was introduced in a small, separate, chamber containing perfusion solution, where the cut end of a leaf of the plant was also immersed. In this way, the xylem exudate closed the electrical circuit.

Heterologous expression of COPT transporters in Xenopus oocytes and two-electrode voltage clamp

The *COPT2-GFP* and *COPT5–GFP* sequences were subcloned from the p426GPD yeast vector (Sancenón et al. 2003) into the *Xenopus* expression plasmid pOO2 (Ludewig et al. 2002) using the restriction enzyme sites *HindIII* and *SalI* for *COPT2* (1.22 kb) and *BamHI* and *SalI* for *COPT5* (1.19 kb). Capped cRNA was synthesized by in vitro transcription with a mMESSAGE mMACHINE_SP6 Kit (Ambion, Inc.) according to the manufacturer's instructions.

Procedures for oocyte isolation, injection, and maintenance were as described (Osawa et al. 2006; Pike et al. 2009) with modifications: oocyte defoliculation was 2 to 4 h; 46 ng *COPT2* or *COPT5 cRNA* were injected on the following day, and the antibiotics added to the ND96 Ringer solution were 10 μg/mL streptomycin sulfate and 50 μg/mL gentamicin. Expression of *COPT-GFP* constructs was visualized with confocal microscopy (Leica SP8) 24 h after injection of cRNA. Oocyte batches from four different frogs were used. Two-electrode voltage clamp measurements were performed 2–4 days after injection of *COPT2* or *COPT5* cRNA. One to 100 μM $CuSO_4$, together with 100 μM ascorbic acid to maintain the metal in a reduced state, was added to a bath solution containing 230 mM mannitol, 0.15 mM $CaCl_2$, and 10 mM MES/Tris, pH 5.5 (Huang et al. 1999). Uninjected oocytes served as controls. A TEV-200A amplifier (Dagan, Minneapolis, MN) was used to clamp the voltage and signal was recorded with Axotape 2.0 software (Axon Instruments, Union City, CA). The effect of Cu addition was tested while the oocyte membrane voltage was clamped at − 60 mV.

Cu uptake by COPT-injected oocytes and Cu content analysis

For Cu uptake measurements, after injection with 46 ng *COPT2:GFP* or *COPT5:GFP* cRNA, oocytes were incubated in 6-well plates, using 15 to 25 oocytes in 5 mL ND96 in each well (2 experiments), or in Petri plates, with 50 oocytes in

25 mL ND96 (1 experiment). Uninjected oocytes served as controls. After 2-day maintenance in ND96 at 14 °C, the ND96 was replaced with 25 μM $CuSO_4$ plus 100 μM ascorbic acid in ND96. Subsequently, the oocytes were incubated at room temperature for 90 to 105 min with slow rotary shaking. They were rinsed four times with chilled ND96 and transferred to pre-weighed 1.5-mL Eppendorf tubes. Samples were then digested with trace metal grade HNO_3 after drying at 65 °C, 48–72 h. Cu content was analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 8000, PerkinElmer). Three batches of oocytes from two different frogs were used, with two to three replicates each. Significant differences with respect to controls were established by Student's *t* tests.

Results

Effects of Cu addition on membrane (Em) and TPP potentials

Under our experimental conditions, the transmembrane potential difference (Em) of root cells measured in intact adult WT *Arabidopsis* plants was around −60 mV. Upon addition of 10 μM Cu to the perfusion solution, no significant Em changes were observed. Increasing the Cu concentration to 30 μM produced similar results, though in around 20% of the recordings, a long-lasting small Em depolarization was registered and this trend only reverted after withdrawal of the metal (Fig. 1a). In contrast, addition of 0.5 mM Fe induced immediate transient depolarizations, which were generally followed by a spontaneous repolarization, in some cases attaining the initial potential, and always followed by a clear hyperpolarization after its withdrawal from the perfusion solution (Fig. 1a). The same patterns described for WT plants were obtained for knockout and overexpressing *Arabidopsis* lines (*copt2–1*, *copt5–2* $COPT1^{OE}$, and $COPT5^{OE}$). As an example of these results, traces obtained for $COPT1^{OE}$ plants are shown in Fig. 1b.

After placing the plants in the setup to measure TPP, voltage values oscillated for several minutes to more than 1 h. Since trans-root potentials (TRP) and hence TPP sum up electrical potential differences across cells in the external medium-xylem sap path (de Boer et al. 1983), the electric potential differences measured were smaller than those of root cell Em values. Thus, once TPP stabilized, values recorded could be positive or negative but usually around 0 mV. The oscillations observed after plant installation in the setup could also occur during the experiment. Changes of the light environment are probably involved as light/dark transitions strongly affected TPP (Fig. S1). However, the responses of TPP were similar to those of Em and addition of nutrients such as glucose, which is taken up by H^+ cotransport (Slayman and Slayman 1974), also showed typical Em transients (Fig. S1). Under our experimental

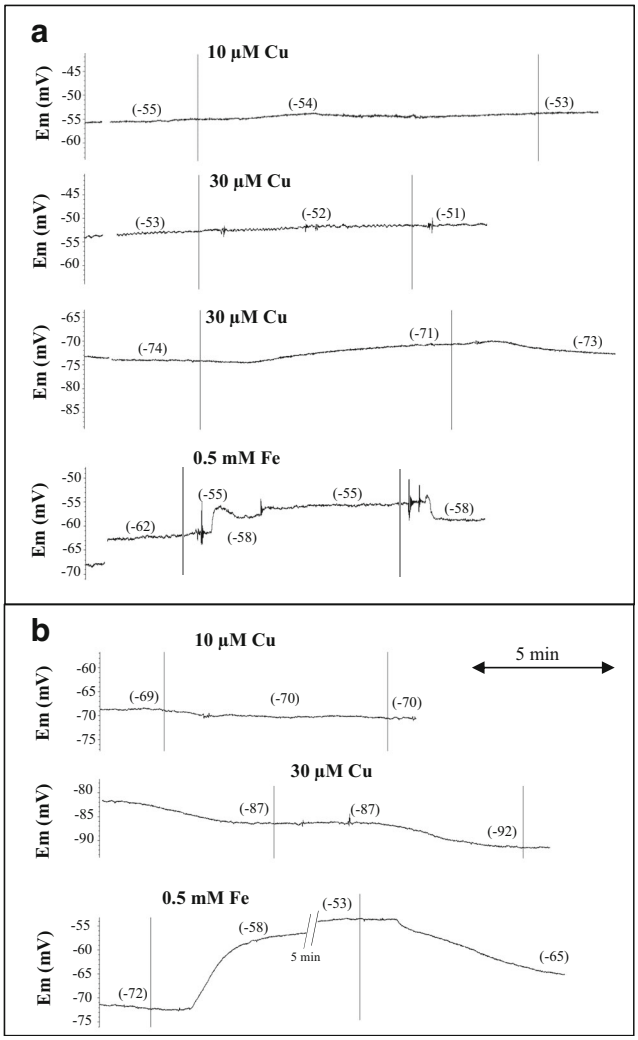

Fig. 1 Em changes in *Arabidopsis thaliana* root cells induced by metals. Ten and 30 μM $CuSO_4$ (Cu) or 0.5 mM $K_3Fe(CN)_6$ (Fe) were added to the perfusion solution bathing the roots of intact adult (30–40 days after sowing) WT (**a**) and $COPT1^{OE}$ (**b**) plants. The first and second vertical lines indicate addition and withdrawal of the metal, respectively. Temporal scale bar applies to both panels. Numbers in brackets show voltages in mV

conditions, the effects of Cu and Fe on TPP were similar to those described for Em. Representative traces registered for WT and $COPT1^{OE}$ plants are shown in Fig. 2a, b, respectively. As indicated, no depolarizations occurred upon addition of 10 μM Cu. However, 0.5 mM Fe induced a depolarization which was generally maintained while it was present in the perfusion solution and TPP only repolarized after its withdrawal. A similar pattern of TPP changes was recorded for Zn, tested in WT plants (Fig. 2a).

Overall, no electrical change (either measured as Em, or as TPP) could be detected when 10 or 30 μM Cu was supplied to the medium bathing the roots of plants from the different genotypes tested and maintained for 1–7 days under Cu-deficiency conditions (see the "Materials and methods" section).

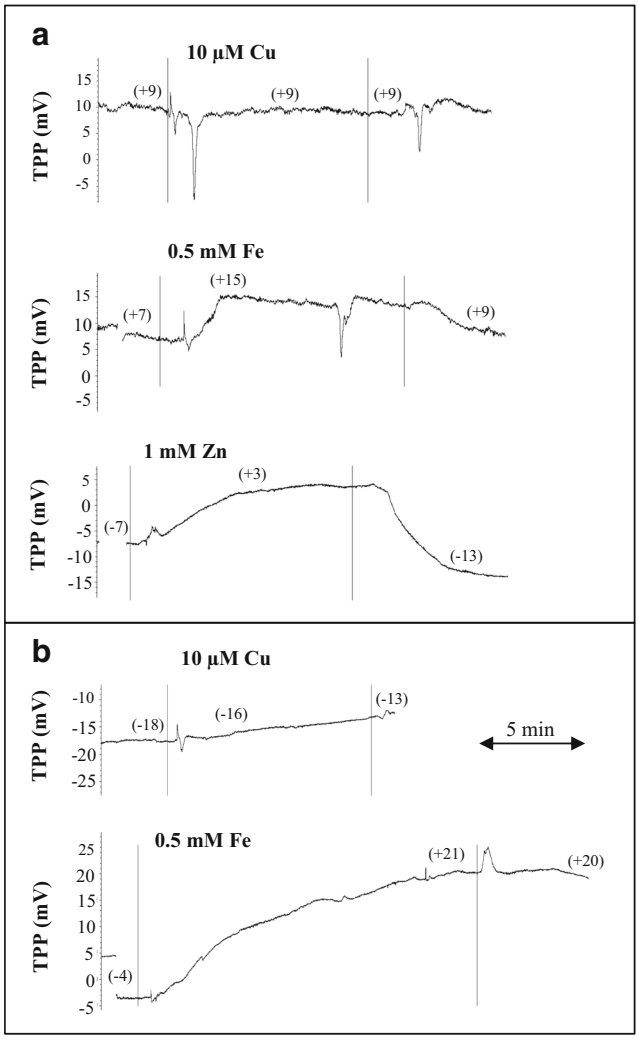

Fig. 2 TPP changes in *Arabidopsis thaliana* plants induced by metal addition to the roots. Ten µM $CuSO_4$ (Cu) or 0.5 mM $K_3Fe(CN)_6$ (Fe) and 1 mM $ZnSO_4$ (Zn) were added to the perfusion solution bathing the roots of intact adult (30–40 days after sowing) WT (**a**) and $COPT1^{OE}$ (**b**) plants. The first and second vertical lines indicate addition and withdrawal of the metal, respectively. Temporal scale bar applies to both panels. Numbers in brackets show voltages in mV. Spikes caused by electrical interferences can be seen

Effects of Cu addition on electrical currents across the membrane of COPT2- and COPT5-expressing oocytes

Because induced currents in native *Arabidopsis* tissue may be below the detection limit, we next used COPT overexpression in *X. laevis* oocytes to augment any possible COPT-mediated electrical signal. For this, we employed C-terminally GFP-tagged *COPT2* and *COPT5* constructs to enable verification of transporter localization in the oocyte plasma membrane. Importantly, both *COPT2-GFP* (Perea-García et al. 2013) and *COPT5-GFP* (García-Molina et al. 2011) were previously shown to complement the respective *Arabidopsis* mutant lines, demonstrating that the GFP tag does not interfere with transporter function. Injection of *COPT2:GFP* and *COPT5:GFP* cRNA into *X. laevis* oocytes resulted in the expression of the respective GFP-tagged transport proteins located in the plasma membrane by 24 h after injection (Fig. S2). Despite its apparent integration into the oocyte membrane, no electrical currents were recorded by two-electrode voltage clamp (TEVC) upon addition of 1–100 µM Cu to the bathing medium in the presence of 100 µM ascorbic acid. Therefore, the electrical signals were similar for uninjected (control) and *COPT*-injected oocytes (Fig. 3). Since the plasma membrane of *X. laevis* oocytes possesses endogenous transporters, including K^+ channels (Sobczak et al. 2010), oocyte membrane integrity was tested by supplying 10 mM KCl. This treatment induced similar electrical currents in injected and control oocytes (Fig. 3), indicating that the membrane integrity was not affected in the injected oocytes. Remarkably, the current/voltage relationship, measured in the absence of Cu in the bathing medium, was clearly different in *COPT2*-injected than in control and in *COPT5*-injected oocytes (Fig. 4). *COPT2*-injected oocytes showed a stronger level of transporter expression than those injected with *COPT5* (Fig. S2) and greater membrane instability, which made them less able to withstand voltage changes.

Cu uptake by oocytes injected with COPT2:GFP and COPT5:GFP cRNA

After *COPT* cRNA-injected oocytes and control uninjected oocytes were incubated with 25 µM Cu, metal ion analysis was performed by ICP-OES. As described for TEVC measurements, Cu uptake was studied in the presence of 100 µM ascorbic acid in order to maintain the metal ion in its reduced form. As shown in Fig. 5, Cu uptake by *COPT5*-expressing oocytes was only slightly higher than controls (1.2-fold increase) and not statistically significantly different from them (Fig. 5). In contrast, the Cu content in oocytes expressing the COPT2 transporter was significantly higher, accumulating over 6-fold more Cu than the control oocytes. The final Cu concentration in *COPT2*-expressing oocytes, 343 ± 34 µM, representing a 13-fold increase over the external Cu concentration, indicates that rather than passive Cu diffusion resulting from higher membrane instability and leakiness, a concentrative Cu uptake mechanism is operating.

Discussion

Given the role of Cu in physiological processes such as photosynthesis, respiration, and antioxidant defense, both deficiency and excess of this metal generate ROS, which may be deleterious for biological molecules and structures (Sharma et al. 2012). As for most plant species, *Arabidopsis*

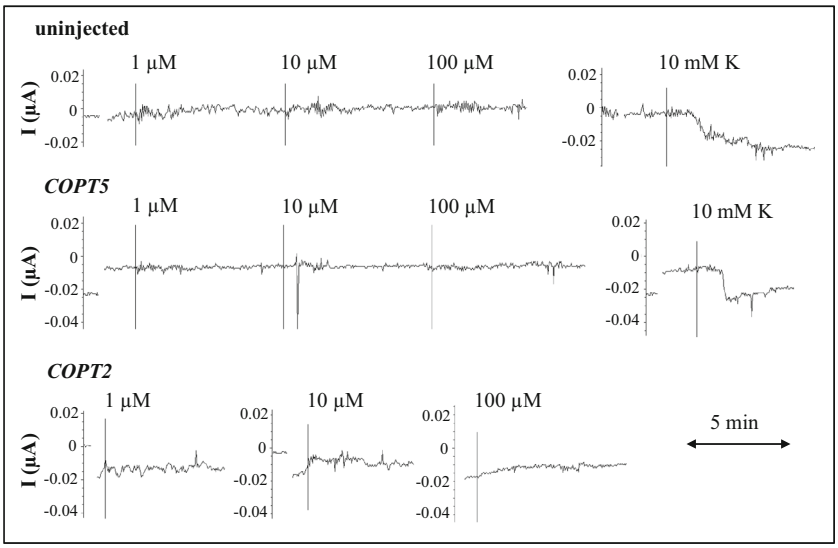

Fig. 3 Effect of adding 1–100 µM $CuSO_4$ to the bathing medium on electrical currents of control and injected oocytes at the moment indicated by the vertical bars ($n = 5$–10 oocytes from at least 3 different frogs). Ascorbic acid (100 µM) was also present in the medium to maintain Cu ions in the reduced state. Currents induced by addition of 10 mM KCl are also shown

has a narrow range of Cu concentrations for optimal growth and development; thus, deficiency responses are induced below 0.5 µM Cu (Yamasaki et al. 2007) while 50 µM is considered toxic (Lequeux et al. 2010). In this work, we added Cu concentrations in the middle and upper sufficiency range, 10 and 30 µM, to *Arabidopsis* roots to test putative changes of Em indicative of electrogenic uptake of this metal. Our results, obtained from plants maintained under Cu-deficient conditions to induce expression of *COPTs* (Sancenón et al. 2003; Perea-García et al. 2013), showed that addition of Cu to WT plants did not result in appreciable Em variations (Fig. 1a). Similar results were obtained in plants overexpressing the high-affinity Cu transporters COPT1 (Fig. 1b) or COPT5, as well as in *copt2–1* and *copt5–2* knockout mutants. The long-lasting small Em depolarization registered in some plants after supplying concentrations that were near toxicity levels (30 µM; Fig. 1a) may be related to an increased organic acid efflux associated with Cu-detoxifying mechanisms. In this sense, a rapid increase in membrane permeability, measured as K^+ efflux, together with a release of organic acids was reported by Murphy et al. (1999) in *Arabidopsis* during the first 3 h of treatment with 30 µM Cu. Transient increases in membrane permeability also occurred after addition of Cd, another toxic metal, to rice and maize roots (Llamas et al. 2000; Pavlovkin et al. 2006). Apparently, Cd uptake in rice induced detoxifying mechanisms, which eventually restored the initial Em (Sanz et al. 2009).

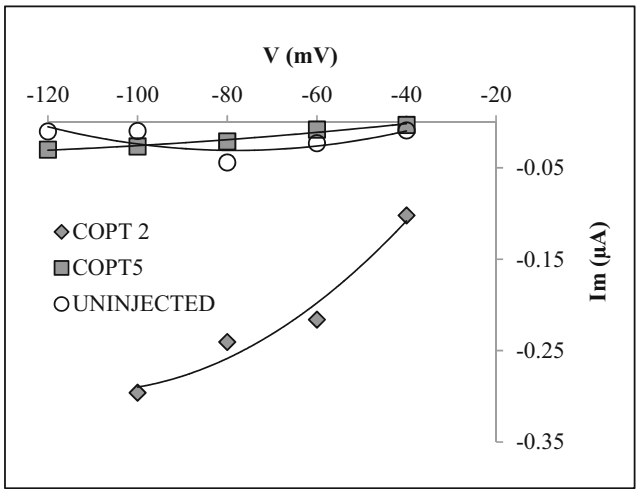

Fig. 4 Current-voltage relationship of control and *COPT2* and *COPT5* cRNA injected oocytes, in the absence of Cu in the external medium. Medians for $n = 5$–15 oocytes from 2 (COPT5) or 3 different frogs are shown

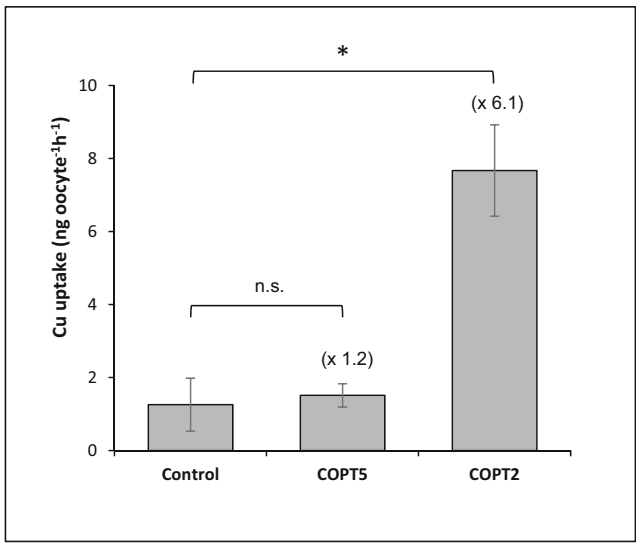

Fig. 5 Cu uptake in control and *COPT5:GFP* and *COPT2:GFP* cRNA injected *X. laevis* oocytes. Oocytes were incubated in ND96 medium containing 25 µM $CuSO_4$ plus 100 µM ascorbic acid. Significant differences with controls are shown by an asterisk ($P < 0.02$, n.s. = not significant). Standard errors ($n = 3$) are shown as vertical bars. Numbers in parentheses indicate fold concentration increases with respect to the controls

Since no Cu effect on Em was detected in WT plants, results obtained in the *copt2–1* and *copt5–2* knockout mutants were to be expected. Similarly, lack of additional response in $COPT5^{OE}$ lines as compared to WT plants was envisaged because COPT5 is a tonoplast-located transporter that delivers Cu toward the cytosol under severe deficiency conditions (García-Molina et al. 2011; Klaumann et al. 2011). In contrast, since COPT1 constitutes the major Cu acquisition system in *Arabidopsis* roots (Puig 2014), it is reasonable to anticipate a stronger response to Cu in $COPT1^{OE}$ plants; however, no electrical changes after Cu addition were detected in these plants (Figs. 1b and 2b). This undetectable effect of Cu on root cell Em is in agreement with data reported by Murphy et al. (1999) under similar experimental conditions, i.e., same species and Cu concentration (30 μM). On the contrary, Kennedy and Gonsalves (1987, 1989) reported strong depolarizations induced by Cu in root cells of *Zea mays*. Although the Cu concentration used by these authors to measure Em changes was in the toxicity range (100 μM), they showed that 10- or 20-fold lower Cu concentrations (10 and 5 μM) also depolarized TRP by more than 30 mV. Measurements of TRP have not been as broadly used as Em determinations in electrophysiological studies. TRPs sum up the electrical potential difference between the external medium and the xylem sap, and therefore, they integrate electrical changes occurring in cortical and stelar cells (de Boer et al. 1983), usually resulting in lower and more variable voltage values (Fig. 2). However, a tight electrical coupling between the cellular and tissue levels in the intact plant, leading to similar and synchronous variations of Em and TRP, has been described (Wegner et al. 1999). Since electrical contact with the xylem sap can also be established at the shoot level, this enables measurements of TPP, which respond similarly to TRP or Em during substrate uptake. Thus, addition of glucose to the medium bathing the roots elicited the typical transients also observed for Em during H^+/substrate cotransport (Fig. S2) (Slayman and Slayman 1974). Because *COPT1* expression is limited to the tip of primary and secondary roots (Sancenón et al. 2004), a lack of Em responses to Cu addition (Fig. 1) could have resulted from impalement of root cortical cells basal to the tip that do not express this transporter. However, the need for a precise insertion of the microelectrode in Em measurements can be circumvented by following TPP changes, as cells are not impaled. Using this technique, we confirmed the results obtained for Em; i.e., no electrical changes were detected upon Cu addition in either WT (Fig. 2a) or $COPT1^{OE}$ (Fig. 2b) plants.

Overall, results obtained for Em and TPP suggest an electroneutral process in Cu uptake. Alternatively, the small amount of Cu taken up by plant root cells may result in undetectable electrical signals, as pointed out by Reid (2001) for uniport or cotransport of micronutrients. In order to distinguish between these possibilities, heterologous expression of *COPTs* in *X. laevis* oocytes was carried out to potentiate Cu uptake signals. Our results showed that Cu addition did not elicit detectable currents in *COPT2*- and *COPT5*-injected oocytes (Fig. 3), though *COPT2*-injected oocytes showed an enhanced capacity for Cu uptake. Thus, a significant 6-fold increase in Cu content over the controls was measured in these oocytes after incubation in a Cu-containing medium at room temperature in the presence of ascorbic acid (Fig. 5). Uptake assays in the presence of Cu^{2+} and ascorbic acid (600 μM and 1 mM, respectively) resulted in non-viable and leaky *X. laevis* oocytes (Antala 2016) probably because Cu^+ ions damaged the oocyte membrane through lipid peroxidation reactions. In our experiments, even in the absence of added Cu, a higher membrane instability was observed in *COPT2*-injected oocytes (Fig. 4) that could have made them leaky. However, the possibility that Cu could passively diffuse into them can be discarded, since the final concentration in the oocytes was 343 μM, which represents a 13-fold increase over the external concentration. Further, the mean Em measured in *COPT2*-injected oocytes of the different batches was − 22 ± 7 mV. According to the Nernst equation [$E_N = - RT/zF \ln(ci/ce)$], passive influx after incubation at room temperature in a medium containing 25 μM Cu should result in a maximum internal Cu concentration of around 70 μM. Therefore, a Cu concentrating process is required to reach the observed 343 μM, thus pointing to an energy-dependent mechanism of Cu uptake through COPT2. Since according to the Irving–Williams series Cu has the highest capacity for binding to organic compounds, it is possible that Cu might bind to histidine or cysteine residues of proteins or other organic compounds, thus increasing passive Cu uptake. However, mature oocytes are considered "closed" systems containing all reserves needed for embryogenesis until tadpoles have hatched (Nomizu et al. 1993). The fact that (1) the functional histidine and cysteine pools measured in stage VI oocytes are in the low pmol range (Eppig and Dumont 1972); (2) 90% of total vitellogenin, a Zn protein which does not bind other transition metals, is sequestered in yolk platelets until hatch and is not accessible to cytosolic events (Montorzi et al. 1994; Falchuk et al. 1995); and (3) Cu treatments did not increase metallothionein (MT) contents in the frog oocytes (Sunderman et al. 1995) and furthermore, Cu uptake in MT-deficient strains of yeast do not differ from that in controls (Lin and Kosman 1990), argue against a passive mechanism for the increase of the internal concentration up to almost 350 μM in 90 min that we measured. In accordance with this, the existence of an energy-dependent mechanism has been reported for Cu uptake in yeast, showing strongly decreased uptake at 4 °C and in glucose-starved cells, together with saturable kinetics and inhibition by metabolic poisons such as azide or dinitrophenol (Lin and Kosman 1990).

A model proposed by Tsigelny et al. (2012), based on the structure of the human Ctr1, suggests that Cu^+ may undergo ligand exchange reactions that provide a neutral passage at the middle of the pore of the transporter endo-domain, together with negative and positive charges at the entrance and exit ecto-domains that attract and repel Cu^+ ions, respectively. According

to this model, net charge would be transferred across the membrane. However, considering the amount of Cu taken up in *COPT2*-injected oocytes during the experimental period (around 12 ng per oocyte) and using the molar mass of Cu and Avogadro's constant, about 1.13×10^{14} Cu^+ ions were transported. Taking the outdated definition of the Ampere ($1A = 6.242 \times 10^{18}$ elemental charges per second), this amount of Cu^+ ions would generate a current of 18 μA if all Cu ions were taken up in 1 s. Though uptake experiments lasted 90 min, this is not a linear process. According to data reported by Lin and Kosman (1990) in a time-course kinetics study in yeast lasting 90–120 min, more than 50% of Cu taken up through a high-affinity transport system ($K_m = 4.4$ μM) occurred in the first 20 min. Under voltage clamp, the electric driving force remains constant, whereas in uptake experiments, both the electric and chemical gradient collapse as substrate is taken up and the system moves toward equilibrium. However, even assuming the most unfavorable scenario, that is, that 50% of the uptake occurred in the first 20 min and taking into account only the electrical charges corresponding to Cu^+ ions (uniport), a current of about 8 nA should have been generated, and would be even greater in the case of a H^+/Cu^+ symport mechanism. Since a current of 8 nA is within the detection limits of our TEVC equipment and no currents were detected using a 4-fold higher Cu concentration than in uptake experiments, our results are consistent with an electroneutral process in Cu uptake through COPT2.

In summary, with the experimental approaches used in this work, a combination of different electrophysiological techniques and elemental analysis by ICP-OES, our results altogether indicate that whereas the COPT5-mediated Cu^+ remobilization does not affect Cu content or Em, Cu^+ uptake through the plasma membrane, mediated by COPT2, is an energy-dependent and electroneutral process. Further experiments are needed to establish the biophysical mechanism and source of energy for COPT-mediated Cu uptake in plants.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

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